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<table>
<thead>
<tr>
<th>CONTENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>IN MEMORIAM</td>
</tr>
<tr>
<td>----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>ORIGINAL PAPERS</td>
</tr>
<tr>
<td>Multiplex-PCR assay for identification of <em>Klebsiella pneumoniae</em> isolates carrying the <em>cps loci</em> for K1 and K2 capsule biosynthesis</td>
</tr>
<tr>
<td>GIERCZYŃSKI R., JAGIELSKI M., RASTAWICKI W., KAŁUŻEWSKI S.</td>
</tr>
<tr>
<td>----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>FAME profiles in <em>Pseudomonas vesicularis</em> during catechol and phenol degradation in the presence of glucose as an additional carbon source</td>
</tr>
<tr>
<td>MROZIK A., PIOTROWSKA-SEGET Z., LABUZEK S.</td>
</tr>
<tr>
<td>----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Reliability of the Etest in light of the correlation between an antibiotic’s critical concentration (Cc) and MIC values</td>
</tr>
<tr>
<td>BEDNAR M.</td>
</tr>
<tr>
<td>----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Antibiotic susceptibility and molecular characterisation of <em>Proteus mirabilis</em> isolates in hospitals from the west pomeranian area of Poland</td>
</tr>
<tr>
<td>MACZYŃSKA I., GIEDRYS-KALEMBA S.</td>
</tr>
<tr>
<td>----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Prevalence of antibiotic resistance profile in relation to phylogenetic background among commensal <em>Escherichia coli</em> derived from various mammals</td>
</tr>
<tr>
<td>BALDY-CHUDZIK K., STOSIK M.</td>
</tr>
<tr>
<td>----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Human neutrophil peptides in vaginitis/cervicitis of different etiology</td>
</tr>
<tr>
<td>WIECHULA B.E., FRIEDEK D.A., EKIEL A.M., ROMANIK M.K., MARTIROSIAN G.</td>
</tr>
<tr>
<td>----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Biological activity of phenylpropionic acid isolated from a terrestrial <em>Streptomyces</em></td>
</tr>
<tr>
<td>NARAYANA K.J.P., PRABHAKAR P., VIJAYALAKSHMI M., VENKATESWARLU Y., KRISHNA P.S.J.</td>
</tr>
<tr>
<td>----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Ultra-structural studies on root nodules of <em>Samanea saman</em> (Jacq.) Merr. (Leguminosae)</td>
</tr>
<tr>
<td>QADRI R., MAHMOOD A., ATHAR M.</td>
</tr>
<tr>
<td>----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Growth and antioxidant activity of <em>Desulfotomaculum acetoxidans DSM 771</em> cultivated in acetate or lactate containing media</td>
</tr>
<tr>
<td>PAWŁOWSKA-CWIĘK L., PADO R.</td>
</tr>
<tr>
<td>----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Bioremediation of aflatoxins by some reference fungal strains</td>
</tr>
<tr>
<td>EL-SHIEKH H.H., MAHDI H.M., EL-AASER M.M.</td>
</tr>
</tbody>
</table>

INSTRUCTION TO AUTHORS AVAILABLE AT www.microbiology.pl/pjm
IN MEMORIAM

Mirosław Kańtoch
(1928–2007)

On the 5th of May, 2007, passed away professor dr hab. med. Mirosław Kańtoch, full member of the Polish Academy of Sciences, eminent microbiologist, the creator of modern Polish virology, expert of the WHO for medical microbiology, initiator and main executor of scientific programs in collaboration with the Center for Disease Control in Atlanta, devoted to viral infections of social significance in Poland.

Mirosław Kańtoch was born on the 1st of January, 1928 in Sosnowiec. He studied at the Faculty of Medicine of the Medical Academy in Wrocław, where he received his medical doctor’s degree in 1951. He was a student, and then assistant of professor Ludwik Hirschfeld. He defended his doctoral thesis, the advisor of which was professor Henryk Makower, at the Medical Academy in Wrocław in 1956 and obtained the position of assistant professor at the same time at the Department of Medical Microbiology, Medical Academy and at the Institute of Immunology and Experimental Therapy of the Polish Academy of Sciences.

In 1956 he organized the Electron Microscopy laboratory which he was then the head of for close to 10 years. In 1961, at the age of 33, he presented and defended his habilitation thesis and then left on a Rockefeller Foundation stipend for Baltimore, where he worked under the guidance of professor F.B. Bang. In his recollections professor Kańtoch always mentioned the names of professors L. Hirschfeld, H. Makower and F.B. Bang as his mentors and the photos of those three professors always hung in his office.

Three years after returning from the USA, in 1965, professor M. Kańtoch was appointed by the Minister of Health as the head of the Department of Virology at the National Institute of Hygiene in Warsaw, which position he held until the year 2000.

In 1970 he became an associate professor and in 1978 full professor. In 1986 he was elected corresponding member of the Polish Academy of Sciences (PAN) and in 1994 full member of the PAN.

Prof. M. Kańtoch was a member of several Committees of the PAS, including the Committee of Microbiology, Committee of Immunology, Committee of Human Ecology Etiopathogenesis, Committee of Immunology and Human Disease Etiopathogenesis. He was honorary member of the Polish Society for Microbiology, the Society of Epidemiologists, I.I. Miecznikow Committee of Microbiologists and Infectious Disease Physicians in Russia, full member of the Warsaw Scientific Society, the Polish Society of Epidemiologists and Infectious Disease Physicians, member of the team of experts of the World Health Organization.

Prof. M. Kańtoch was on the Scientific Boards of many research institutions, such as the Ludwik Hirsfeld Institute of Immunology and Experimental Therapy of the PAS, the National Institute of Hygiene, The Military Institute of Hygiene and Epidemiology. Professor M. Kańtoch was also a member of the editorial boards of several scientific journals, e.g. “Przegląd Epidemiologiczny” “Postępy Mikrobiologii”, “Medycyna Doświadczalna i Mikrobiologia”, Postępy Higieny i Medycyny Doświadczalnej”, “Polish Journal of Microbiology” and a member of the international editorial board of “Acta Virologica”.

Professor M. Kańtoch contributed in a major way to the development of scientific research on the
etiopathogenesis, immunology and immunoprophylaxis of viral infections. Worth particular attention is his contribution in studies on the safety, effectiveness and dissemination of vaccinations against the polio virus, measles and rubella and well as in the pioneer at the time research on the teratogenic action of the rubella virus and the occurrence of congenital defects caused by the infection of mothers during pregnancy with such viruses as the cytomegaly virus, herpes simplex (cold sores) and varicella zoster (chickenpox and shingles). In continuing the work of professor Feliks Przesmycki, professor M. Kańtoch became a co-originator of Polish medical virology.

The research mentioned above was documented in over 200 original scientific papers several score congress and meeting reports, which professor Kańtoch was the author or co-author of. The Professor was also the author of several monographs and the textbook “Medical Virology” intended mainly for medical students and physicians, which had several reprints.

In recollecting professor M. Kańtoch, we cannot overlook his didactic achievements. We, the employees of the Department of Virology, National Institute of Hygiene, remember the Professor above all as a carer, doctoral thesis advisor and a person who inspired us and demanded we work on our habilitation theses. The professor was the thesis advisor of 20 doctorates, 17 of which were from the Department of Virology of the National Institute of Hygiene and three from the time of his work in Wrocław. We remember the Professor as a very demanding Person but always knew we could in every case count on his help, aid and protection.

Employees of the Department of Virology
National Institute of Hygiene
Introduction

*Klebsiella pneumoniae*, an important nosocomial pathogen, causes suppurative infection, pneumonia, urinary tract infection and septicemia in humans, especially immunosuppressed (Podschun and Ulmann, 1998) or suffering from underlying diseases like diabetes mellitus (Fung et al., 2002). Persons of low social status and alcoholics constitute the main patients population at risk, comprising up to 66% of those suffering from community acquired pneumonia, that is a very severe illness with a rapid onset and a high mortality rates (Podschun and Ulmann, 1998; Sahly and Podschun, 1997). Despite the discovery of other virulence factors such as fimbriae, siderophores and O-antigens, capsular antigens are considered to be ultimate determinants of *K. pneumoniae* pathogenicity (Podschun and Ulmann, 1998; Sahly and Podschun, 1997; Fang et al., 2004; Yu et al., 2007). Clinical isolates of this species produce capsular polysaccharide (CPS) (Fang et al., 2004; Ørskov and Ørskov, 1984). Among 77 capsular serotypes (K-types) of *K. pneumoniae* (Orskov and Ørskov, 1984), strains belonging to serotypes K1 and K2 are considered the most virulent to mice (Simoons-Smit et al., 1984) and humans (Fang et al., 2004; Yu et al., 2007). Moreover, strains of K1 and K2 are believed to escape the opsonin-independent lectin phagocytosis (Podschun and Ulmann, 1998; Kabha et al., 1995). Clinical studies on 134 patients with *K. pneumoniae* liver abscess exhibited predomination of serotypes K1 (63.4%) and K2 (14.2%) (Fung et al., 2002).

The capsular swelling (quellung) reaction and counter-current immunoelectrophoresis are the most commonly used techniques for identification of *K. pneumoniae* serotypes K1 and K2 (Janda and Abbott, 1998). The availability and costs of the antisera, which can be produced in specialised laboratories, limit the practice of serotyping. Therefore, novel molecular-serotyping tool was recently developed (Brisse et al., 2004). Although, this method is capable to identify all 77 K-types of *K. pneumoniae*, it requires time consuming long-range PCR followed by the endonuclease digestion and computer aided analysis of the electrophoretic patterns. Thus, despite its indisputable advantages, molecular-serotyping is not optimal for rapid identification of K1 and K2 strains in routine diagnostic. On the other hand, recently described PCR-based assays for differentiation of the major serovars of *Listeria monocytogenes* (Doumith et al., 2004), *Streptococcus*
pneumoniae (Kong and Gilbert, 2003), Yersinia pseudotuberculosis and Y. pestis (Bogdanovich et al., 2003) have been found a rapid and practical alternative to laborious classical serotyping. For these reasons, we aimed to develop multiplex-PCR assay for identification of K. pneumoniae strains genetically competent to produce K1 and K2 capsular polysaccharides.

**Experimental**

**Materials and Methods**

**Bacterial strains.** We examined 147 isolates (Table I) including complete set of 77 Klebsiella spp. K-antigen reference strains (Ørskov and Ørskov, 1984) and ten reference K1 and K2 strains described elsewhere. Prior to serotyping by the counter-current immunoelectrophoresis using K1 and K2 antisera (Statens Serum Institut, Denmark) all clinical isolates listed in Table I were biochemically identified by classical tube tests. Noncapsulated variants of K. pneumoniae strains K1 (n = 1) and K2 (n = 6) were designed as described previously (Kaźużycki, 1968).

**PCR procedure.** Template DNA was prepared from 0.5 ml of an overnight culture at 37°C in nutrient broth as described previously (Gierczyński et al., 2004) but the lysozyme treatment was omitted. Primers listed in Table II were used for amplification of fragments of wzc, orf10 and K. pneumoniae 16S rRNA gene in multiplex-PCR. PCRs were carried out in 20 ml reaction volumes in a thermalcycler (Mastercycler, Eppendorf, Germany), with 0.75 U of the recombinant Taq DNA Polymerase (Fermentas, Lithuania), 1×Mg-free PCR buffer with (NH₄)₂SO₄, each deoxy-nucleoside triphospate at a concentration of 0.2 mM, 3.0 mM MgCl₂, each primer at a concentration shown in Table II and 2.5 µl of the template DNA solution. A general program consisting of 35 cycles for 45 s of each denaturation at 94°C, annealing at 60°C and elongation at 72°C was used for amplification. Finally, DNA synthesis was completed at 72°C for 3 min. Prior to cycling, 5 min denaturation step at 94°C was included. The 2% gel (MP Biomedicals, Germany) in TAE (40 mM Tris-acetate pH 8.0, 1 mM EDTA) was used for the multiplex-PCR products separation. Gels were run at a constant voltage of 80 V for 2 hours, stained in 2 µg/ml ethidium bromide for 10 min and photographed under UV by Gel-Scan apparatus (Kucharczyk, Poland). Each strain was analysed in triplicate.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Number of isolates</th>
<th>Capsular type (serotyping)</th>
<th>Geno-serotype K1 (wzc)</th>
<th>K2 (orf10)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A5054 (O1:K1)²</td>
<td>1</td>
<td>K1</td>
<td>+</td>
<td>–</td>
<td>(Ørskov and Ørskov, 1984)</td>
</tr>
<tr>
<td>A5054⁴</td>
<td>1</td>
<td>NT³</td>
<td>+</td>
<td>–</td>
<td>This study</td>
</tr>
<tr>
<td>408 (SB3182)²</td>
<td>1</td>
<td>K1</td>
<td>–</td>
<td>–</td>
<td>(Brisse et al., 2004)</td>
</tr>
<tr>
<td>466 (SB3186)²</td>
<td>1</td>
<td>K1</td>
<td>–</td>
<td>–</td>
<td>(Brisse et al., 2004)</td>
</tr>
<tr>
<td>643 (SB3188)²</td>
<td>1</td>
<td>K1</td>
<td>–</td>
<td>–</td>
<td>(Brisse et al., 2004)</td>
</tr>
<tr>
<td>920 (SB3192)²</td>
<td>1</td>
<td>K1</td>
<td>–</td>
<td>–</td>
<td>(Brisse et al., 2004)</td>
</tr>
<tr>
<td>B5055 (O1:K2)⁴</td>
<td>1</td>
<td>K2</td>
<td>–</td>
<td>+</td>
<td>(Ørskov and Ørskov, 1984)</td>
</tr>
<tr>
<td>B5055⁴</td>
<td>1</td>
<td>NT</td>
<td>–</td>
<td>+</td>
<td>This study</td>
</tr>
<tr>
<td>1584 (SB3201)³ (C2b)⁴</td>
<td>1</td>
<td>K2</td>
<td>–</td>
<td>+</td>
<td>(Brisse et al., 2004)</td>
</tr>
<tr>
<td>777 (SB3202)³ (C2c)⁴</td>
<td>1</td>
<td>K2</td>
<td>–</td>
<td>+</td>
<td>(Brisse et al., 2004)</td>
</tr>
<tr>
<td>34 (SB3203)³ (C2d)⁴</td>
<td>1</td>
<td>K2</td>
<td>–</td>
<td>+</td>
<td>(Brisse et al., 2004)</td>
</tr>
<tr>
<td>778 (SB3199)³ (C2e)⁴</td>
<td>1</td>
<td>K2</td>
<td>–</td>
<td>+</td>
<td>(Brisse et al., 2004)</td>
</tr>
<tr>
<td>B4631 (O2:K2)⁴</td>
<td>1</td>
<td>K2</td>
<td>–</td>
<td>+</td>
<td>(Kauffmann, 1954)</td>
</tr>
<tr>
<td>B7380 (O2:K2)⁴</td>
<td>1</td>
<td>K2</td>
<td>–</td>
<td>+</td>
<td>(Kauffmann, 1954)</td>
</tr>
<tr>
<td>K3-K82²</td>
<td>75</td>
<td>NT</td>
<td>–</td>
<td>–</td>
<td>(Ørskov and Ørskov, 1984)</td>
</tr>
<tr>
<td>Clinical isolates K1</td>
<td>3</td>
<td>K1</td>
<td>+</td>
<td>–</td>
<td>This study</td>
</tr>
<tr>
<td>Clinical isolates K2</td>
<td>10</td>
<td>K2</td>
<td>–</td>
<td>+</td>
<td>This study</td>
</tr>
<tr>
<td>Clinical isolates K2²</td>
<td>5</td>
<td>NT</td>
<td>–</td>
<td>+</td>
<td>This study</td>
</tr>
<tr>
<td>Clinical isolates</td>
<td>40</td>
<td>NT</td>
<td>–</td>
<td>–</td>
<td>This study</td>
</tr>
<tr>
<td>Total:</td>
<td>147</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

² Klebsiella K-antigen reference strains excluding false serotypes K73 and K75-78 (Ørskov and Ørskov, 1984),
³ noncapsulated variants,
⁴ genomic DNA template (capsular type cited from the reference),
⁵ C-patterns (subgenotypes) of K. pneumoniae K2 (Brisse et al., 2004),
⁶ NT, strains not typeable by K1 and K2 antisera.
Results and Discussion

In order to select marker loci specific for serotypes K1 and K2 we performed comparative analysis of the cps gene clusters for K1 and K2 capsule biosynthesis deposited in GenBank (http://www.ncbi.nlm.nih.gov) under accession numbers: AY762939 and D21242 respectively. For multiplex-PCR assay we selected gene wzc encoding tyrosine-protein kinase and the open reading frame 10 (orf10) encoding putative inner membrane protein for K1 and K2 serotypes respectively. Fragment of the K. pneumoniae 16S rRNA gene served as a positive multiplex-PCR control. The expected size of 16S rRNA gene amplicon was 508 bp while PCR-product for wzc (K1) and orf10 (K2) was 356 bp and 663 bp respectively.

The multiplex-PCR assay result for K1 and K2 was judged as a positive when two bands were present – one specific for the 16 RNA gene and the other specific for wzc or orf10 respectively. The presence of the K. pneumoniae 16S rRNA gene served as a positive multiplex-PCR control. The expected size of 16S RNA gene amplicon was 508 bp while PCR-product for wzc (K1) and orf10 (K2) was 356 bp and 663 bp respectively.

The multiplex-PCR assay result for K1 and K2 was judged as a positive when two bands were present – one specific for the 16 RNA gene and the other specific for wzc or orf10 respectively. The presence of the 16S rRNA gene amplicon alone indicated that tested DNA sample contained neither wzc, orf10 and PCR inhibitors. In this case, the assay result was valid but negative for K. pneumoniae K1 and K2 geno-serotypes. The optimal yield of PCR products was observed for concentrations of wzc and orf10 primers ranging from 0.50 to 0.25 µM and the 16S rRNA gene primers between 0.250 and 0.125 µM.

The multiplex PCR yielded DNA fragment of about 500 bp for all tested strains, whereas additional fragments about 350 bp and 650 bp were detected for strains of capsular type K1 and K2 respectively (Fig. 1). No bands were observed for DNA-free negative control (data not shown). Specificity of the PCR products was confirmed by DNA nucleotide sequencing performed as described previously (Gierczyński et al., 2004). Notably, orf10 amplicons were obtained for strains of K. pneumoniae K2 belonging to different subgenotypes (C-patterns) (Brisse et al., 2004). This finding proved usefulness of the developed multiplex-PCR assay for identification of genetically diverse strains of capsular type K2 (Table I). Moreover, developed assay correctly identified rarely occurring strains O2:K2. The wzc and orf10 were also detected in the noncapsulated variants of strain A5054 and B5055 respectively. Consequently, orf10 was also traced in noncapsulated derivatives of clinical K2 isolates. This is in agreement to previous findings (Brisse et al., 2004), that molecular serotyping was capable to determine a potential serotype of capsule-deficient isolates. Except the 500 bp band, no PCR-products were generated for Klebsiella spp.

Table II

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Target locus</th>
<th>GenBank accession number</th>
<th>Primer sequence</th>
<th>Primer Position</th>
<th>Primer concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>wzcf wzc</td>
<td>AY762939</td>
<td>5’-GATACAGGTGTATTGTGC-3’</td>
<td>8947–8966</td>
<td>0.4 µM</td>
<td></td>
</tr>
<tr>
<td>wzcr wzc</td>
<td>AY762939</td>
<td>5’-GAGCTCTATATGTTGGATGC-3’</td>
<td>9283–9302</td>
<td>0.4 µM</td>
<td></td>
</tr>
<tr>
<td>or10f orf10</td>
<td>D21242</td>
<td>5’-CCAGAGTTAGCCCCGATTT-3’</td>
<td>14205–14225</td>
<td>0.4 µM</td>
<td></td>
</tr>
<tr>
<td>or10r orf10</td>
<td>D21242</td>
<td>5’-GAAGTCTATACCCCCGAGGCG-3’</td>
<td>14848–14867</td>
<td>0.4 µM</td>
<td></td>
</tr>
<tr>
<td>K16Sr 16S rRNA</td>
<td>AF453251</td>
<td>5’-AGGGTGCAAGCGTGTAATCGG-3’</td>
<td>493–512</td>
<td>0.2 µM</td>
<td></td>
</tr>
<tr>
<td>K16Sr 16S rRNA</td>
<td>AF453251</td>
<td>5’-GTCTTCACAGGTCGCCAAAGG-3’</td>
<td>981–1000</td>
<td>0.2 µM</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Result of the multiplex PCR for identification of K. pneumoniae capsular genosero types K1 and K2

M – DNA size ladder 100 bp step (GeneRuler 100 bp, Fermentas, Lithuania). Lines: K1 – A5054 (K1 reference strain), K2 – B5055 (K2 reference strain), 1 – B5055 (noncapsulated), 2 – F5052 (K6), 3 – 889/50 (K20), 4 – 636/52 (K58), 5 – 438 (K66), 6 – B4631 (O2:K2), 7 – Kp90 (non-K1 and non-K2 clinical isolate), 8 – Kp57 (clinical isolate K2), 9 – A5054 (noncapsulated), 10 – 408 (K1), 11 – Kp229 (clinical isolate K1), 12 – 778 (K2), 13 – 1584 (K2), 14 – 34 (K2).
tested other than A5054 or B5055, as well clinical isolates nontypeable by K1 and K2 antisera (Table I). It is noteworthy, strain K58 that was reported to cross-react with K1 in serotyping did not yield wzg specific amplicon. The lack of such cross-reactions may be an advantage of developed assay when compared with a classical serotyping.

Taken together, obtained results show that developed multiplex-PCR assay is potentially useful tool for identification of K1 and K2 serotypes of *K. pneumoniae*. Moreover, developed assay is capable to determine whether a capsule defective strain is K1 or K2 derivative. Thus, the assay detects K1 and K2 geno-serotypes of *K. pneumoniae* in fact. However, due to reported horizontal transfer of thecps cluster to strains representing other species of *Enterobacteriaceae* (Nelson and Selander, 1994; Rahn et al., 1999) the multiplex-PCR assay may not be used instead the classical biochemical tests for *K. pneumoniae* identification (Janda and Abbott, 1998). We recommend this assay as a relatively inexpensive and robust tool for screening for *K. pneumoniae* K1 and K2 genoserootypes. However, to diversify capsule producing and capsule deficient isolates classical serotyping with K1 and K2 antisera is recommended. The multiplex-PCR assay may help to reduce total cost and workload of *K. pneumoniae* K1 and K2 capsular types identification in epidemiological surveys and routine diagnostic.

Acknowledgements

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Dr. A.A. Zasada assisted in the template DNA isolation. We are thankful to Dr. S. Brisse for a support of a total DNA of selected strains of *K. pneumoniae* K1 and K2.

Literature


FAME Profiles in *Pseudomonas vesicularis* during Catechol and Phenol Degradation in the Presence of Glucose as an Additional Carbon Source

AGNIESZKA MROZIK*1, ZOFIA PIOTROWSKA-SEGET2 and SYLWIA ŁABUŻEK1

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**Abstract**

The aim of this study was to evaluate the impact of catechol and phenol added to culture media separately and with glucose as an additional, easily-degradable carbon source on fatty acid methyl ester (FAME) composition in *Pseudomonas vesicularis*. Simultaneously, the degradation rates of aromatic substrates used were investigated in single and binary substrate systems. Both catechol and phenol treatments caused changes in the distribution of tested groups of fatty acids. The most noticeable changes included an increase in degree of fatty acid saturation, the appearance of branched and disappearance of hydroxy fatty acids as compared to the control sample with glucose. Under catechol or phenol treatment sat/unsat ratio showed the values of 8.63 and 11.38, respectively, whereas in control cells it reached the value of 2.66. The high level of saturation comes from the high content of cyclopropane fatty acids in bacteria under exposure to aromatic substrates, regardless of the presence of glucose. In these treatments their content was more than 3-fold higher compared to the control. It has been demonstrated that glucose supplementation of culture media containing single aromatic substrate extended the degradation rates of catechol and phenol by *P. vesicularis*, caused an increase in number of cells but did not significantly change the fatty acid profiles in comparison with bacteria growing on catechol and phenol added to the media individually.

**Key words:** *Pseudomonas vesicularis*, catechol and phenol degradation, fatty acid composition

**Introduction**

Fatty acids are essential structural components of bacterial cell membranes that regulate their stability and fluidity. The membrane is the site of the primary contact with the environment and has an important role in maintaining the viability and functionality of bacterial cells. The main function of the membrane is to form permeability barriers regulating the passage of solutes between the cell and the external environment. This function is mainly determined by membrane lipid composition (Šajbidor, 1997; Denich *et al.*, 2003). The analysis of bacterial membrane fatty acids is also of interest for studies on toxicity of many contaminants that generate environmental stress. Many findings documented that xenobiotics, such as organic solvents and aromatic hydrocarbons influence bacterial fatty acid composition (Heipieper *et al.*, 1992; Sikkema *et al.*, 1994; Kabelitz *et al.*, 2003). Low-molecular weight aromatic hydrocarbons, such as catechol and phenol are the simplest structurally aromatic compounds and enter the environment as a consequence of human activities. For example, they widely occur during the production of dyes, pesticides, pharmaceuticals, wood processing chemicals, polymers and explosives. Since aromatic compounds exhibit toxic, mutagenic and carcinogenic properties, there is a serious concern about their elimination from environment. One of the most promising methods is the application of hydrocarbon degrading bacteria to clean-up contaminated sites. For this purpose, numerous bacteria mainly from the genera *Pseudomonas*, *Acinetobacter*, *Klebsiella* and *Bacillus* are used in the field of phenols degradation (Ahamad and Kunhi, 1996; Chang *et al.*, 1997; Ali *et al.*, 1998; Heesche-Wagner *et al.*, 1999; Beendorf *et al.*, 2001).

The rate of degradation processes of many aromatic substrates can be improved by supplementing...
of culture media with additional carbon sources or other compounds such as nitrate, phosphate as well as mineral constituents. Glucose, sodium glutamate and yeast extracts are known as conventional carbon sources that influence the biotransformation and biodegradation processes (Wang et al., 1996; Wang and Loh, 1999, 2001). For example, Yu and Ward (1994) have observed that the rate of pentachlorophenol degradation by mixed bacteria cultures significantly increased by the addition of glucose and peptone to culture medium. However, some additional carbon sources may inhibit aromatic compound degradation. In studies conducted by Ampe et al. (1998) it has been shown that Ralstonia eutropha degraded phenol less effectively in the presence of acetate as compared to the culture with phenol alone. Moreover, glucose enrichment repressed catechol degradation by Pseudomonas sp. CF600 (Mrozik et al., 2006). In turn, the addition of glucose and sodium glutamate did not affect the dynamics of phenol degradation by Pseudomonas putida ATCC49451 (Loh and Wang, 1998). Bacteria possess a regulatory mechanism that allows them to use a preferential carbon source over a mixture of several other substrates and this phenomenon is usually called catabolic repression. It has been described for various bacteria, however, the molecular mechanism of gene expression for peripheral catabolic enzymes in the presence of preferred substrate differ substantially between species (Saier, 1996; Stülke and Hillen, 1999; Petruschka et al., 2001).

In fact, many aromatic compounds partition into phospholipid bilayer and modify its fatty acids composition and membrane properties. Accumulation of these compounds in the membrane disturbs many biological processes such as respiration, growth, ions and nutrient transport and may even cause lysis of the cell (Sikkema et al., 1995; Weber and de Bont, 1996; Denich et al., 2003). As a response to phenols exposure bacteria modify their membrane lipid composition by de novo synthesis of fatty acids, isomerization of cis to trans unsaturated fatty acids, changing the proportion between iso and anteiso branched fatty acids, altering the average of chains length and protein content (Keweloh et al., 1990; Heipieper et al., 1994; Sikkema et al., 1995). These mechanisms have been related to homeoviscous adaptation and have been investigated by several authors (Shinitzky, 1984; Heipieper et al., 1992; Härtig et al., 2005).

However, there is no available information on the influence of additional carbon sources on fatty acid profiles of bacteria during the biodegradation of phenols. The objective of this work was to establish changes in cellular fatty acid patterns in Pseudomonas vesicularis during catechol and phenol degradation in culture media supplemented with glucose as an additional source of carbon and energy.

Experimental

Materials and Methods

Bacterial strain. The experiments were performed using Pseudomonas vesicularis strain isolated from mixed populations of activated sludge collected from sewage-treatment plant in Częstochowa, Poland. To select phenol-degrading bacteria the increasing doses of phenol were added to sample of sludge for 30 days. To isolate phenol-degrading bacteria 10-fold dilutions of sludge suspensions were plated onto mineral medium (Kojima et al., 1961) amended with 0.188 g/l of phenol. Among isolated strains P. vesicularis was dominant. It was identified on the basis of cellular fatty acids derivatized to methyl esters (FAMEs) and analysed by gas chromatography using the MIDI Microbial Identification System (Newark, USA).

Culture conditions. Cultures were grown in modified minimal medium containing: 3.78 g of Na₂HPO₄ x12H₂O; 0.5 g of KH₂PO₄; 5.0 g of NH₄Cl; 0.2 g of MgSO₄ x7H₂O and 0.1 g of yeast extract in 1.0 l of deionised water (Kojima et al., 1961). To study the effect of glucose on FAME profiles bacteria were cultivated in Kojima medium containing catechol or phenol, at the concentration of 0.440 g/l and 0.376 g/l, respectively and in binary mixtures containing single aromatic substrate and 1.0 g/l of glucose. The final pH of the medium was 7.2–7.3. Liquid cultures were grown in 500 ml flask on rotary shaker (125 rpm) at 30°C.

Bacterial growth. Samples of the cultures were withdrawn every two hours until 8 h of incubation, and then at 16 and 24 h of the experiments. Cell density (OD) was measured spectrophotometrically as the absorbance of the suspension at 600 nm, with reference to a standard curve calibrated by plate enumeration.

Determination of catechol and phenol concentrations. Concentrations of tested aromatic compounds were measured at the same sampling time when OD was measured. Determination of catechol concentration was based on color reaction between catechol and sodium molybdate by measuring absorbance at 480 nm (Evans, 1946). Phenol concentration was estimated using spectrophotometry method with diazotised p-nitroaniline by measuring the absorbance of color solution at 550 nm (Lurie and Rybnikova, 1968).

Determination of glucose concentration. Removal of glucose in media was calculated using tests GLUCOSE EO produced by Biochemtest, Poland. This test is based on glucose oxidation to gluconic acid by glucose oxidase with production of hydrogen peroxide in the presence of peroxidase and chromogene ABTS. Absorbance of solution was measured with spectrophotometer at 675 nm.

Enzyme activity assay. The activities of catechol dioxygenases were measured spectrophotometrically
by monitoring the formation of the first product of aromatic ring cleavage, cis, cis-muconate at 260 nm for catechol 1,2-dioxygenase and 2-hydroxymuconic semialdehyde at 375 nm for catechol 2,3-dioxygenase (Feist and Hegeman, 1969). Detailed procedure of enzymes isolation was described in the previous paper (Mrozik et al., 2006). Enzymes activities were expressed as mmol of cis, cis-muconate and 2-hydroxymuconic semialdehyde formed per mg of protein per minute for catechol 1,2-dioxygenase and catechol 2,3-dioxygenase, respectively. The protein content of cell-free extract was estimated by the method of Bradford (1976) with lysozyme as a standard.

**Fatty acid extraction and analysis.** Fatty acid composition of bacterial strain was determined at mid-exponential phase of culture growth. For analysis of cellular fatty acids cells grown in single- and binary-substrates systems were used. Bacteria were harvested by centrifugation (8000 × g) at 4°C for 30 min. The cell pellets obtained from each culture were washed with 10.0 ml of 0.85% NaCl to remove residue of culture medium. To decrease the humidity of bacterial cell, pellets were left through 2 h at room temperature. Next 10.0 ml of 0.85% NaCl was added. Then the samples were vortexed and placed in 100°C water bath for 30 min. Following this saponification step, fatty acids were converted to fatty acid methyl esters (FAMEs) by adding 2.0 ml of 6.0 M HCl:MeOH (1:0.85) to each tube and were incubated at 80°C in water bath for 10 min. FAMEs were extracted from the aqueous phase by addition of 1.15 ml of hexane:methyl tert-butyl ether (MTBE) (1:1) to each tube. Then samples were rotated end-over-end for 10 min. After removing aqueous (lower) phase, 3.0 ml of 0.3 M NaOH in H2O was added and the tubes were again rotated for 5 min (Sasser, 1990). Finally, the organic (upper) phases containing FAMEs were transferred to gas chromatography vials. Fatty acids were analysed by gas chromatography (Hewlett-Packard 6890, USA) using capillary column Ultra 2-HP (cross-linked 5% phenyl-methyl silicone 25 m, 0.22 mm ID, thickness 0.33 mm) and hydrogen as a carrier gas. FAMEs were detected by a flame ionisation detector (FID) and identified by MIS (Microbial Identification System) software, using the aerobe TSBA40 method and TSBA40 library (MIDI, USA).

**Results and Discussion**

**Cell growth and aromatic compounds degradation.** To estimate the effect of glucose on catechol and phenol degradation by *P. vesicularis* bacteria were grown in media containing only single aromatic substrate and in the same media supplemented with glucose. The strain was able to metabolize catechol completely at the concentration of 0.440 g/l and phenol at the concentration of 0.376 g/l served as a single substrate during 10 and 15 h, respectively. The highest catechol removal was observed during the first 4 h of culturing and in this time 65% of dose added to the medium was degraded. In comparison, in that time in phenol containing medium its concentration decreased about 42%. In both experiments significant differences in growth as indicated OD value of *P. vesicularis* were not found. The substrate removal profiles and growth curves are presented in Figure 1A and B.

The addition of glucose to media with aromatic substrates resulted in the increase of culture OD and altered the time of catechol and phenol degradation. The time necessary for complete removal of both aromatic substrates by tested bacteria extended to 24 h (Fig. 1A and B). In comparison with single-substrate system, OD of bacterial culture in binary mixtures was markedly higher and reached the value of 0.8 and 1.0 for catechol with glucose and phenol with glucose, respectively. In control medium with glucose served as a sole source of carbon and energy *P. vesicularis* metabolized it during 6 h of culturing (data not shown). Time of glucose utilization did not change in the medium containing phenol whereas in medium with catechol was 2 hours shorter as compared to the control sample. Interesting changes were revealed when compared the dynamics of catechol and phenol biodegradation. In mixture containing glucose and phenol both substrates started to be degraded at the same time whereas in the mixture containing catechol and glucose were not degraded simultaneously (Fig. 1A and B). Catechol biodegradation by *P. vesicularis* started when 90% of glucose added was metabolized. These results indicated that glucose was preferentially utilized by *P. vesicularis* and it might repress catechol degradation. Similar phenomenon was observed during studies on catechol biodegradation rate in the presence of glucose by strain *Pseudomonas* sp. CF600. The time of catechol degradation in binary system with glucose was longer than that when catechol served as a sole carbon source. In contrast to *P. vesicularis* it started to degrade both substrates immediately after their addition to the culture medium (Mrozik et al., 2006). The effect of glucose and sodium acetate on aromatic compounds biodegradation by bacteria from the genus *Pseudomonas* was also observed by Kao et al. (2005). They have revealed that addition of these extra carbon sources did not enhance pentachlorophenol (PCB) degradation by *Pseudomonas mendocina* NSYSU. They have explained this phenomenon by the fact that this strain isolated from PCB-contaminated soil did not receive inputs of glucose and acetate from natural sources and the cometabolism is not the...
dominant biodegradation mechanism of PCB by this bacterium. In turn, impact of glucose on phenanthrene (PHE) degradation by Sphingomonas sp. strain LB126 in chemostat cultures was studied by van Herwijnen et al. (2003). They found that PHE removal in the presence of glucose was much higher as compared to phenanthrene and fluorene grown culture without the high impact on growth cells. Besides biodegradation experiments many studies are being conducted on molecular mechanism of catabolic repression in bacteria grown on glucose or other easily-degradable carbon sources and aromatic compounds as inducers. For example, Duetz et al. (1996) described catabolic repression of the TOL pathway by succinate under different conditions of inorganic-nutrient limitation. In other studies the role of Crc regulator in the repression of several catabolic pathways for the assimilation of some sugars and aromatic compounds in Pseudomonas putida was shown (Morales et al., 2004). Such studies are necessary for better understanding the correlations among degradation ratio of various organic substrates by bacteria.

In parallel to biodegradation studies the activities of enzymes involved in aromatic ring cleavage were calculated. As shown in Table I, P. vesicularis in each experiment treatment synthesized both catechol 1,2- and 2,3-dioxygenases. In bacterial cells growing on catechol only the activity of catechol 1,2-dioxygenase...
was significantly higher as compared to activity of catechol 1,2-dioxygenase and reached the values of 2.55 and 0.39 µmol/min/mg of protein, respectively. In contrast, in bacteria cultured on phenol the activity of catechol 2,3-dioxygenase was 2.5-fold higher than catechol 1,2-dioxygenase. These results indicate that catechol and phenol degradation proceeded both via meta and ortho metabolic pathways. The addition of glucose slightly decreased the activity of measured enzymes (Table I). Similarly, Tian et al. (2003) studying the impact of glucose added on phenanthrene degradation by P. mendocina demonstrated that glucose supplementation decreased the activities of hydrocarbon dioxygenase and catechol 2,3-dioxygenase.

**FAME analysis.** To estimate the changes in fatty acid composition the profiles of whole-cell fatty acids isolated from P. vesicularis cultured in media containing catechol or phenol degradation with or without glucose were analyzed. For the detailed interpretation of results all fatty acids obtained were divided into two major groups: saturated and unsaturated. The first group of fatty acids included four sub-groups: straight-chain, branched, hydroxy- and cyclopropane fatty acids. Percentages of these fatty acid groups in each experiment treatments are presented in Table II. Both catechol and phenol treatment caused crucial changes in the distribution of the tested groups of whole cell-derived fatty acids in P. vesicularis. Bacteria cultured on aromatic substrates characterized by the higher proportion of saturated fatty acids as compared to control with glucose. The percentage of these fatty acids composed 89.41% and 91.92% of total fatty acids when bacteria were grown on catechol or phenol, respectively, whereas in control sample they represented 72.65% of total fatty acids (Table II). Similar tendency resulting in the increase of the membrane saturation in the presence of toxic aromatic compounds and aliphatic alcohols was earlier observed in studies using P. putida (Heipieper et al., 1992; Mrozik et al., 2005), Rhodococcus sp. 33 (Gutierrez et al., 1999), Ralstonia eutropha H850 (Kim et al., 2001) and Acinetobacter calcoaceticus (Kabelitz et al., 2003).

The addition of glucose to culture medium with catechol did not significant change the abundance of saturated fatty acids in P. vesicularis as compared to cells collected from medium containing catechol only. In contrast, in bacteria growing in medium containing phenol and glucose the proportion of saturated fatty acids was about 10% lower than in bacteria grown on phenol used separately. In bacterial cells, irrespective of medium content, among saturated fatty acids the dominant group was straight-chain fatty acids. This group included the following fatty acids: 10:0, 12:0, 14:0, 15:0, 16:0, 18:0 and 19:0. However, their percentages in fatty acid profiling obtained from bacteria cultured in single- and binary system was lower as compared to control (55.40%) and ranged from 48.39 to 52.58%. The increase of degree of membrane saturation is well known adaptive mechanism allowing bacteria to survive under toxic substrates stress (Sikkema et al., 1994, 1995; Weber and de Bont, 1996). Catechol and phenol exposure drastically changed the content of terminally branched and hydroxy fatty acids. Interestingly, this observed effect was independent of the presence of glucose in the culture medium. The results showed that both aromatic substrates caused the disappearance of hydroxy fatty acid 12:0 2OH, whereas in control sample with glucose it composed 5.70% of total fatty acids. In contrast, catechol and phenol in all tested systems caused the appearance of branched fatty acid 15:0 iso and additionally 15:0 anteiso was detected in cells growing on phenol as a single carbon source. However, the percentages of branched fatty acids in FAME profiles were generally low and ranged from 0.84 to 1.62% of total fatty acids for bacteria grown on catechol and/or phenol in the presence or absence of glucose (Fig. 2). Tsitko et al. (1999) studying the impact of different aromatic compounds on

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Catechol 1,2-dioxygenase µmol/min/mg of protein</th>
<th>Catechol 2,3-dioxygenase µmol/min/mg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>glucose</td>
<td>0.07 ± 0.02</td>
<td>0.11 ± 0.03</td>
</tr>
<tr>
<td>catechol</td>
<td>2.55 ± 0.07</td>
<td>0.39 ± 0.07</td>
</tr>
<tr>
<td>catechol + glucose</td>
<td>2.11 ± 0.11</td>
<td>0.19 ± 0.05</td>
</tr>
<tr>
<td>phenol</td>
<td>0.70 ± 0.05</td>
<td>1.85 ± 0.17</td>
</tr>
<tr>
<td>phenol + glucose</td>
<td>0.57 ± 0.06</td>
<td>1.12 ± 0.09</td>
</tr>
</tbody>
</table>

Number of replicates, n = 3

### Table I

Percentages of total saturated, unsaturated fatty acids and sat/unsat ratio of Pseudomonas vesicularis growing on catechol or phenol, or/and glucose in single- and binary systems

<table>
<thead>
<tr>
<th>Group of fatty acids</th>
<th>% of total fatty acids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
</tr>
<tr>
<td>Saturated</td>
<td>72.65</td>
</tr>
<tr>
<td>Straight-chain</td>
<td>55.40</td>
</tr>
<tr>
<td>Hydroxy</td>
<td>5.70</td>
</tr>
<tr>
<td>Branched</td>
<td>0.00</td>
</tr>
<tr>
<td>Cyclopropane</td>
<td>11.56</td>
</tr>
<tr>
<td>Unsaturated</td>
<td>27.35</td>
</tr>
<tr>
<td>Sat/unsat ratio</td>
<td>2.66</td>
</tr>
</tbody>
</table>

Values are the averages of three independently performed experiments (standard errors < 5%).

### Table II

Percentages of total saturated, unsaturated fatty acids and sat/unsat ratio of Pseudomonas vesicularis growing on catechol or phenol, or/and glucose in single- and binary systems.
R. opacus FAMES composition revealed that these substrates also increased content of branched fatty acids. It seems that the response of bacterial cells to membrane active substrates to a large extent depends on individual physiological and biochemical features of given bacteria. In contrast to tested strain, Pseudomonas sp. CF600 reacted to catechol and phenol exposure in an opposite way. This strain growing on catechol or phenol in single- and binary systems with glucose synthesized both hydroxy and branched fatty acids (Mrozik et al., 2006). With regards to the chromatographic profiles of saturated fatty acids, the changes in the abundance of cyclopropane fatty acids 17:0 cy and 19:0 cy ω8c were the most visible. The highest increase of these fatty acids content was detected in bacterial cells grown on phenol. Their percentage reached the value of 39.78% whereas in control sample with glucose showed the value of 11.56%. Surprisingly, in bacteria cultured in binary system containing phenol and glucose 19:0 ω8cy fatty acid was not detected that resulted in decreasing of the total amount of cyclopropane fatty acids. Such phenomenon was not observed in the experiment with catechol served as a sole carbon and energy source and in mixture with glucose (Fig. 2). The presented results as well as results obtained by other researchers indicate that content of cyclopropane fatty acids depends not only on the chemical structure and properties of hydrocarbons but also on the features of bacterial strains (Ramos et al., 1997; Kim et al., 2001; Fang et al., 2004; Mrozik et al., 2006). Cyclopropane fatty acids have been known as compounds that stabilize membrane lipids, make it more rigid and in this way improve bacteria survival under unfavorable conditions. However, the detailed role of these fatty acids in the regulation of bacterial membrane stability and fluidity in the presence of aromatic compounds is not fully understood yet and require further investigations and explanations.

It has been found that tested aromatic substrates used both in a single- and binary systems significantly decreased the amount of unsaturated fatty acids such as 16:1ω7c and 18:1ω9c. Their abundance declined about 5-fold in comparison to control. In contrast, the percentage of fatty acid 18:1ω7c/ω9t/ω12t, which is well known as typical for bacteria from the genus Pseudomonas, increased from 6.34% in control to 8.41% and 10.25% in bacterial cells growing in media supplemented with catechol or phenol and glucose, respectively. However, under exposure of catechol and phenol used individually the amount of this fatty acid slightly decreased.

The impact of various toxic compounds and their interactions with easily degradable carbon sources on bacterial MIDI-FAME profiles might be examined by analysis of saturated/unsaturated ratio (Table II). In this study it has been showed that under catechol or phenol exposure this ratio was about 3.5-fold higher than that in control and reached the value of 8.63 and 11.38 for catechol and phenol, respectively. The addition of glucose to culture medium containing the aromatic substrates changed the response of bacterial cells to these toxic compounds which resulted in decreasing of sat/unsat ratio in comparison with cultures grown in the presence of catechol or phenol separately (Table II). Additionally, the protective effect of glucose against the toxicity of aromatic substrates during their biodegradation was confirmed by marked increase of bacterial culture density (Fig. 1A and B).
The action of catechol or phenol in the presence of glucose as an additional carbon source measured as FAMEs patterns of bacterial cells was slightly different as compared to the action of these aromatic substrates added individually. Generally, the differences among the fatty acid composition in bacteria cultured in media containing catechol or phenol and in mixtures with glucose were slight but significant in comparison to pattern of FAMEs obtained for control samples. The most noticeable difference was associated with cyclopropane fatty acid abundance. Beside straight-chain fatty acids they constituted the second dominant group in FAMEs profiles. The high proportion of cyclopropane fatty acids resulted in the increase of sat/unsat ratio. The data obtained from biodegradation studies and analysis of FAME profiles of *P. vesicularis* indicated that the addition of glucose as easily-degradable carbon source to media containing aromatic substrates such as catechol and phenol stimulated the growth of bacteria while did not have distinct influence of the whole cell-derived fatty acid composition.

**Literature**


Reliability of the Etest in Light of the Correlation between an Antibiotic’s Critical Concentration (Cc) and MIC Values

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Abstract

The study relates to the theory of diffusion methods for antibiotic sensitivity testing. The aim of the study was to show the relationship between the antibiotic critical concentration (Cc) and its minimum inhibitory concentration (MIC). The results contribute to the explanation of the Etest’s reliability and support the scientific basis for MIC determination using agar diffusion methods. Susceptibility among 90 clinical isolates of 12 common aerobic bacterial species to gentamicin, erythromycin, or oxacillin was assessed using the multidisc method (for Cc), by the agar dilution method (for MIC) and by the Etest. The results of all three methods were statistically compared and found to be closely related. The regression equation for Cc values and MIC was \( \log_2(MIC) = 0.99 \times \log_2(Cc) - 0.13; r = 0.99; p < 0.05 \); the regression equation for Cc values and Etest-MIC (Et) was \( \log_2(Et) = 0.86 \times \log_2(Cc) + 0.34; r = 0.96; p < 0.05 \); the regression equation for Etest-MIC values and MIC was \( \log_2(MIC) = 1.12 \times \log_2(Et) - 0.50; r = 0.96; p < 0.05 \).

Key words: Etest, multidisc method, antibiotic critical concentration (Cc), antibiotic sensitivity testing

Introduction

Quantitative antibiotic sensitivity can be estimated by either agar/broth dilution methods, or by agar diffusion methods. The minimum inhibitory concentration (MIC) in dilution methods is not affected by the growth of standard bacterial inoculums because the antibiotic is completely active from the beginning of the incubation period. On the other hand, in diffusion methods (Etest, multidisc method) the concentration of the antibiotic at the edge of inhibition zone at the time of its formation relates to the growing bacterial population. This antibiotic concentration, the actual bacterial density and the time when the edge of inhibition zone is founded are called the critical concentration (Cc), the critical population and the critical time, respectively (Linton, 1961; Cooper, 1963; Barry, 1980; Delignette-Muller and Flandrois, 1994). The critical time generally lasts for a period of several hours after the start of incubation. In slow growing bacteria, the critical time is longer. The critical population is therefore higher than the standard inoculum and, consequently, the critical concentration can theoretically differ from MIC. The question we hope to answer is, does it really differ? If yes, then the Etest wouldn’t work properly. However, the Etest has been reported to be reliable by many authors, although it has not been well studied (Kronvall, 2000). The purpose of this paper is to uncover the theoretical explanation for Etest accuracy.

How the antibiotic critical concentration can be measured? If two or more discrete discs with different amounts of the same antibiotic are used, the critical concentration of the antibiotic can be calculated from the content of the discs and from the diameter of their respective inhibition zones. The following rules apply to the theory of inhibition zone formation in the disc method:

- The critical time for the same bacteria strain, antibiotic, and cultivation conditions is independent of the amount of antibiotic in the disc (of the disc content) (Barry, 1980).
- As soon as the edge of a zone is formed, its diameter will not change (Linton, 1961; Barry, 1980).
- The relation between the area of inhibition zone and natural logarithm of antibiotic disc content is linear (Barry, 1980; Kronvall, 1982; Delignette-Muller and Flandrois, 1994).

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The antibiotic diffuses into the agar according to Fick’s second law of diffusion (Vesterdal, 1947; Humphrey and Lightbown, 1952; Koch, 1999).

Note on rule 3): the relation between the radius of the inhibition zone and antibiotic disc content is expressed by the equation

\[ r_n^2 = a + b \ln(m_n) \]  

where \( r_n \) is the radius of inhibition zone in \( n \)-th disc and \( m_n \) is the content of the disc. A graphic image of this dependency is a straight line. Its intercept \( a \) and slope \( b \) can be calculated from the measured data by common statistical procedures. Measurement of the zone radius from the center of discs provides a better correlation with disc content and size of inhibition zone, in terms of equation \( \{1\} \), than does measurement of the zone radius starting from the edge of the disc (Kronvall, 2000).

Note on rule 4): a derived radial law for two-dimensional cylindrical diffusion applies to the disc method (Humphrey and Lightbown, 1952; Koch, 1999).

\[ Cc = \frac{m_a}{4\pi D_c d} e^{-a/b} \]  

where \( Cc (\mu g/ml) \) is the critical concentration of antibiotic, \( m_a (\mu g) \) is the antibiotic disc content at the beginning of diffusion, \( r_n \) (cm) is the radius of the respective inhibition zone, measured from the disc center \( D \) (cm\(^2\)/s) is the diffusion constant of the antibiotic, \( t_c \) (s) is the critical time, \( d \) (cm) is the agar depth, and \( e \) is the base of natural logarithms. The denominator \( 4\pi D_c d \) reflects the drop in antibiotic concentration in the disc during the critical time and the agar depth. Dimensional analysis of the formula yields \( \mu g/ml \), which confirms the mathematical validity of the formula.

It can be proven that \( b = 4Dt_c \). This is in agreement with the research done by others (Cooper, 1963; Druegon et al., 1987; Delignette-Muller and Flandrois, 1994; Koch, 1999). In formula \( \{2\} \), you can substitute slope \( b \) for \( 4Dt_c \) and we can substitute \( a + b\ln(m_n) \) for \( r_n^2 \). By consequent reduction, we arrive at resulting formula combining equations \( \{1\} \) and \( \{2\} \)

\[ Cc = \frac{1}{\pi b d} e^{-a/b} \]  

where \( a \) is the intercept, \( b \) is the slope of regression line according to equation \( \{1\} \), and \( d \) is the agar depth. Formula \( \{3\} \) calculates the critical concentration of an antibiotic without knowing its diffusion constant or critical time \( (i.e., \) without calibration). If we use at least three discs, the reliability of the result can be ascertained from the correlation of the logarithms of the discs’ content and the sizes of the respective inhibition zones.

Experimental

Materials and Methods

Susceptibility among ninety clinical isolates of common aerobic bacterial species to gentamicin, erythromycin, or oxacillin was assessed. We performed 90 concurrent sensitivity measurements using the multidisc diffusion method, standard agar dilution method and Etest. Sensitivity was measured 33 times for oxacillin, 27 times for gentamicin, and 30 times for erythromycin. These antibiotics were chosen due to their diverse mode of action. They also provide sharp, clear zones of inhibition in the diffusion method.

Bacterial strains and culture media. Forty-five strains of Staphylococcus aureus, seventeen strains of Staphylococcus epidermidis, six strains of Pseudomonas aeruginosa, six strains of Proteus mirabilis, six strains of Escherichia coli, four strains of Klebsiella spp., three strains of Acinetobacter baumannii, two strains of Enterobacter spp. and one strain of Enterococcus faecalis were used. All the strains were isolated during routine investigations of various clinical specimens in a hospital laboratory. The strains were chosen according to the qualitative sensitivity testing (NCCLS, 1993a) so as to obtain three sets of strains for measurements of the sensitivity to given antibiotic over a wide range of MIC values. Erythromycin or oxacillin sensitivity was checked in Gram-positives whereas gentamicin sensitivity in Gram-negatives. The actual experiment involved a quantitative assessment of the sensitivity to a particular antibiotic in selected strains using the multidisc method, agar dilution method, and Etest. The tests were run concurrently for each inoculum. We used Mueller-Hinton agar (Oxoid, Unipath Ltd., Basingstoke, Hampshire, England) poured to a depth of 4 mm in Petri dishes (diameter 90 mm) for all three methods. The inocula for all three methods came from colonies that were suspended in physiological saline solution to a density of 0.5 on the McFarland scale. Plates used for the multidisc method and the Etest were inoculated by swabbing.

Etest. Etest strips (Gentamicin low range; Erythromycin; Oxacillin) were used according to the manufacturer’s instructions (AB Biodisk, Solna Sweden). Agar plates were incubated at 35°C. Results were read after 24 hours according to the manufacturer’s reading guide.

Antibiotics used. Antibiotic solutions were prepared by dilution of injectable preparations: Gentamicin LEK Pharmaceuticals and Chemical Co., Slovenia (gentamicin), Erythrocin Abbott Laboratories, USA (erythromycin), and Prostaphilin Bristol-Myers Squibb S.p.A., Italy (oxacillin).

Critical concentration (Ce) determinations (multidisc diffusion method). Four 6 mm – diameter blank paper discs (Oxoid, Unipath Ltd., Basingstoke,
Hampshire, England) were placed onto the inoculated agar. An appropriate amount of antibiotic, dissolved in 20 µl distilled water, was dropped onto discs using a pipette. The amount of antibiotic on the discs was estimated on the basis of previous qualitative sensitivity testing of all strains (NCCLS, 1993a) so as to achieve a minimum of two measurable yet distinct inhibition zones when using four discs (Table I). Agar plates were incubated at 35°C for 24 hours. The diameters of inhibition zones were measured using an electronic calliper. Non-rounded zone diameters and data on antibiotic disc content were used to calculate the slope $b$ and intercept $a$ according to equation \(1\). These constants were subsequently employed for the calculation of the critical concentration of the antibiotics using formula \(3\). Where at least three inhibition zones formed, the percentage of variation in the zones explained by the disc content logarithms (coefficient of determination $R^2$ written as a percentage) was also calculated to show the reliability of the result.

### Table I

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Disc content (µg)</th>
<th>Resistants</th>
<th>Sensitives</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A     B    C     D</td>
<td>A    B     C     D</td>
<td></td>
</tr>
<tr>
<td>Erythromycin</td>
<td>10000</td>
<td>200   40.0</td>
<td>8.00 200</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>800   25.6  64.0</td>
<td>16.0 256</td>
<td>64.0 4.00 1.00</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>5000  714   102</td>
<td>14.6 102</td>
<td>14.6 2.08 0.298</td>
</tr>
</tbody>
</table>

In order that the number of discs in the multidisc method can be reduced to four, strains were initially subdivided into groups that were either sensitive or resistant using a routine qualitative disc method. Because of practical reasons, the ratio between the neighboring discs’ contents is usually (but not obligatory) regular.

**MIC determination.** The assessment of MIC using the agar dilution method was performed in accordance with NCCLS guidelines (NCCLS, 1993b). The antibiotics were the same as those used in the multidisc diffusion method. Final concentrations of antibiotics in the agar ranged from 0.012 to 512 mg/l. The dilutions were based on a geometrical order (factor 2) and were related to concentrations of 1 mg/l and 1.5 mg/l (0.012, 0.016, 0.023, 0.031, 0.047, 0.063, 0.094, 0.125, 0.19, 0.25, 0.38, 0.5, 0.75, 1.0, 1.5, 2, 3, 4, 6, 8, 12, 16, 24, 32, 48, 64, 96, 128, 192, 256, 384, 512 mg/l). The inoculum was applied to the agar surface by means of a pin replicator. Agar plates were incubated at 35°C. Results were read after 24 hours.

**Statistical evaluation of results.** The results were classified by the applied method only, not by bacterial strain or the antibiotic used. The correlations of Etest results with MIC, multidisc diffusion method results with MIC and multidisc diffusion method results with Etest results, were expressed using the Spearman rank correlation coefficient. After transforming the results into base 2 logarithms, we expressed them by means of the Pearson correlation coefficient. Statistical values were calculated by Statistica for Windows (StatSoft Inc.). Differences no greater than a twofold dilution factor between the MIC and the Etest or between the MIC and the multidisc method, were used to calculate agreement (Pfaller et al., 2000).

**Results**

Agreement of Cc with MIC was observed in 89 out of 90 concurrent critical concentration measurements. Regression straight line is shown in Figure 1.
the Etest. All disagreements occurred among resistant strains. Both the multidisc method and the Etest correlated well with the dilution agar method: both the Spearman and Pearson coefficients reached at least 0.9. The relation between the results of the multidisc method (Cc) and MIC was $\log_2(MIC) = 0.99 \times \log_2(Cc) + 0.13$; $r = 0.99$; $p < 0.05$. The relation between the Etest results (Et) and MIC was $\log_2(MIC) = 1.12 \times \log_2(Et) + 0.50$; $r = 0.96$; $p < 0.05$. The relation between Cc and Et was $\log_2(Et) = 0.86 \times \log_2(Cc) + 0.34$; $r = 0.96$; $p < 0.05$. The reliability of the multidisc method expressed as the average percentage of variations in the zones explained by the disc content logarithms was 98.85% (92.36–100.00%). The $b$ value in formula (1) was not related to the sensitivity of the strains. Thus, the critical time was independent from strain sensitivity. Neither species-dependent nor antibiotic type-dependent irregularities in Cc-MIC relationship were found.

Discussion

According to the classical theories (Cooper, 1963; Barry, 1980; Hedges, 1999), the bacterial growth rate impacts the inhibitory zone diameter. In the Kirby-Bauer qualitative disc diffusion method, this phenomenon is solved by the interpretative standards (NCCLS, 1993a). The crucial question is whether such “inaccuracy” substantially influences the Etest result. The results of our work show that it does not, because the antibiotic (critical) concentration under the edge of the forming zone practically equals MIC. So in Etest the bacterial growth rate may impact the inhibitory zone shape but not its point of intersection with the scale on the strip.

In the multidisc method, the concentration of antibiotic on each additional discs is, optimally, four to seven times lower than on the preceding disc (depending on the type of antibiotic). Because the experimental design initially divided the strains into either sensitive or resistant, we were able to reduce the number of discs used to four and still obtain at least two measurably distinct zones of inhibition for an accurate calculation of the critical concentration. Without this initial categorization, five or six discs would be required to test over the full scale of an Etest strip. Such increasing disc number brings the multidisc method closer to the Etest, which can be imagined as a chain of antibiotic discs with exponentially growing antibiotic content. The primary data in both methods are the critical concentrations of antibiotics. The Etest and multidisc methods do have similarities – the zones (including the zero zones) are always formed after the critical time passes. In the case of the Etest, the critical concentration if $r = 0$ estimates MIC using a printed scale.

The correlation between the critical concentration and MIC is not a new finding. Nevertheless, the extent of the correlation is surprising. It implies that in diffusion quantitative methods, bacterial growth up until the critical time does not influence the result. This observation contributes to an understanding of the accuracy of the Etest on a wide variety of organisms, and indicates that the results obtained with quantitative diffusion methods (Etest, multidisc method) can be expressed as MICs without any conversion.

Acknowledgement

The author would like to thank Marie Duskova and Zorka Haasova for their technical laboratory assistance and Jiri Horacek for the software production. The program for the critical concentration calculation is downloadable on http://www.lf3.cuni.cz/ustavy/mikrobiologie/download/atb_cc.zip; last accessed 7/07/07.

Literature


**Introduction**

Nosocomial infections are an important health problem worldwide and are closely related to the type of diagnostic and therapeutic procedures performed on patients. Isolating the pathogen responsible for an infection is one of the basic criteria for the identification of the type of infection, which considerably improves the chance for therapy which should be preceded by antibiogram determination. Uri-nary tract infections caused by *Proteus mirabilis* are common and often severe, leading to acute pyelonephritis, chronic inflammation, and bacteremia. The frequency of *P. mirabilis* infections in hospital patients as well as in outpatients increases the risk of endogenous infections, being infected by other patients, hospital staff, or contaminated equipment. It shows that the keeping the record of the exact characteristics of those microorganisms becomes necessary (Johnson et al., 1993).

Due to the increased antibiotic resistance, it becomes necessary to control the appearance of *P. mirabilis* strains isolated from infections in hospital environment. Wild-type strains of *P. mirabilis* are usually susceptible to β-lactams. However, a progressive increase in β-lactam resistance, mediated by the production of β-lactamases, has occurred in this species (Perilli et al., 2002).

The most recent advances in molecular biology offer promising possibilities of examining epidemiological bacteria strains in a controlled hospital environment. It is possible to determine the genetic profile of those microorganisms using pulsed field gel electrophoresis (PFGE), which can be used for analysis of chromosomal DNA restriction patterns, a gold standard in hospital epidemiology. Demonstrating evident relationship among isolated strains from different hospital wards within a few years period indicates persistence of the population of microorganisms responsible for appearance of clonal outbreaks (Fernandez-Baca et al., 2001; Hennekinne et al., 2003).

The aim of the present study was to characterise *P. mirabilis* strains, isolated during 5 years period in the West Pomeranian area of Poland, by molecular typing using PFGE procedure. The results obtained with the application of PFGE were then compared to antimicrobial resistance patterns.

**Antibiotic Susceptibility and Molecular Characterisation of *Proteus mirabilis* Isolates in Hospitals from the West Pomeranian Area of Poland**

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**Abstract**

*Proteus mirabilis* isolates (n = 177), collected between 1996 and 2000 in four hospitals in the West Pomeranian area of Poland, were characterized by antibiotype and pulsed-field gel electrophoresis (PFGE). The selected isolates were collected from different wards (intensive care unit, surgery, internal medicine, and urology). The strains were cultured from various specimen types, mostly from urine, wound samples, bronchial exudates and sputa. The identification was done by biochemical test ID 32E ATB (bioMerieux). Analysis of PFGE patterns was based on comparison of the banding patterns obtained by PFGE of chromosomal DNA digested with SfiI enzyme. Among all *P. mirabilis* isolates tested three major genotypes A (A1-A7), B (B1-B4), C (C1-C5) and 71 unique patterns were identified. The same genotypes were obtained from different patients, treated in different wards and hospitals during a 5-year period. The strains which belonged to the genotypes A and B were multiresistant and most of them produced ESBL; genotype C was more sensitive to antibiotics.

**Key words**: *Proteus mirabilis*, antibiotic susceptibility, ESBL, nosocomial infection, PFGE
Experimental

Material and Methods

Bacterial isolates and clinical data. 177 P. mirabilis clinical isolates were collected between 1996 and 2000 from different patients in 4 hospitals in the West Pomeranian area: Clinical Hospital No 2 in Szczecin (SZC; n = 152 isolates – all isolates), 3 municipal hospitals (some resistant strains): Police (POL; 6 isolates), Choszczno (CHO; 2 isolates), Gryfice (GRY; 17 isolates). The selected isolates were collected from different wards (intensive care unit, surgery, internal medicine, and urology). The strains were cultured from various specimen types, mostly from urine (124 isolates; 70%), wound samples (39 isolates; 22.1%), bronchial exudates (6 isolates; 3.4%) and sputa (8 isolates; 4.5%). Isolates were identified to the species by the ID 32E ATB test (bioMerieux).

Antimicrobial susceptibility testing. The susceptibility to antibiotics was tested by the disk diffusion method on Mueller-Hinton agar according to the criteria of the Clinical and Laboratory Standards Institute. The following amounts of antibiotics per disc were used: ampicillin – Amp (10 µg), amoxycillin/clavulanic acid – Amc (20/10 µg), piperacillin – Pip (100 µg), piperacillin/tazobactam – Tzp (100/10 µg), cephalotin – Cf (30 µg), cefuroxime – Cxm (30 µg), cefotaxime – Ctx (30 µg), ceftazidime – Caz (30 mg), imipenem – Imp (10 mg), gentamicin – Gn (10 µg), tobramycin – Tob (10 µg), netilmicin – Net (30 µg), amikacin – An (30 µg), pipemidic acid – Pi (30 µg), pefloxacin – Pef (5 µg), norfloxacain – Nor (10 µg), ciprofloxacin – Cip (5 µg) trimethoprim-sulphamethoxazole – Sxt (1.25/23.75 µg).

All isolates were recognized as ESBL producers by the double-disc test. Double-disk synergy test was performed on Mueller-Hinton agar with a central amoxycillin-clavulanic acid disk and disks of the third generation cephalosporins (ceftoxime, ceftazidime) placed 20 mm (centre to centre) from each other. The test was considered to be positive for ESBL production when the bacterial growth had a ‘champagne cork’ appearance. For each strain the test was repeated twice.

Molecular typing. Isolates were typed by determining PFGE SfiI DNA macrorestriction patterns with the GenePath Group 5 Reagent Kit (Bio-Rad Laboratories) according to the manufacturer’s recommendation. Pulsed-field gel electrophoresis (PFGE) was performed using the GenePath System (Bio-Rad). Differences detected in band patterns analysed using Molecular Analyst Fingerprinting software (Bio-Rad). The PFGE pattern was interpreted according to Tenover et al., (1995) recommendations.

Results

SfiI PFGE patterns. Analysis of PFGE patterns was based on comparison of the banding patterns obtained by PFGE of chromosomal DNA digested with the SfiI enzyme. Among all P. mirabilis isolates in the collection, three major types A: n = 48 (subtypes: A1 – 14 strains, A2 – 16, A3 – 6, A4 – 5, A5 – 4, A6 – 2, A7 – 1), B: n = 49 (subtypes: B1 – 36 strains, B2 – 9, B3 – 2, B4 – 2), C: n = 9 (subtypes: C1 – 2 strains, C2 – 3, C3 – 1, C4 – 2, C5 – 1). The remaining strains had some unrelated PFGE patterns (more than six band differences), which were designated by roman numerals and letter P: P1 – P71 (Fig. 1).

The data for the three major types A (A1-A7), B (B1-B4), C (C1-C5) from hospitals in the West Pomeranian area are presented in Table I. Molecular typing and antibiotic resistance. Genotype A (A1-A7) strains (n = 48) were resistant to ampicillin, amoxycillin/clavulanic acid, piperacillin, cephalotin, cefuroxime, gentamicin, tobramycin, netilmicin, amikacin, pipemidic acid, pefloxacin, norflo-

<table>
<thead>
<tr>
<th>Hospital (ward)</th>
<th>1996</th>
<th>1997</th>
<th>1998</th>
<th>1999</th>
<th>2000</th>
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</thead>
<tbody>
<tr>
<td>Urology SZC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>A1, A2, A3, A4, B1, C1, C2</td>
<td>A1, A2, A5, B1, C4</td>
<td>A1, A6, B1, B2, C4</td>
<td>A2, B1</td>
<td>A3, B1, B2, B4</td>
</tr>
<tr>
<td>Surgery II SZC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>A1</td>
<td></td>
<td>A1</td>
<td>A1</td>
<td>A2</td>
</tr>
<tr>
<td>Surgery III SZC&lt;sup&gt;c&lt;/sup&gt;</td>
<td>C2</td>
<td>A5, A6, B2, B3</td>
<td>A1, A2, A7</td>
<td>A1</td>
<td></td>
</tr>
<tr>
<td>Intensive Care Unit SZC&lt;sup&gt;c&lt;/sup&gt;</td>
<td>A2, A3, C1</td>
<td>A3, A4, A5, B2</td>
<td>A2, B1</td>
<td></td>
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</tr>
<tr>
<td>Internal Medicine SZC&lt;sup&gt;c&lt;/sup&gt;</td>
<td>A1, A2, A4, A5, C2, C3</td>
<td>A2, B1</td>
<td>B1, B4</td>
<td>A2, B1, C5</td>
<td>B1, B2</td>
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<tr>
<td>POL&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>CHO&lt;sup&gt;d&lt;/sup&gt;</td>
<td>A4</td>
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<td></td>
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</tr>
<tr>
<td>GRY&lt;sup&gt;d&lt;/sup&gt;</td>
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<td></td>
<td></td>
<td></td>
<td>B1</td>
</tr>
</tbody>
</table>

<sup>a</sup>SZC – Clinical Hospital No 2 in Szczecin; <sup>b</sup>POL – Municipal Hospital in Police; <sup>c</sup>CHO – Municipal Hospital in Choszczno; <sup>d</sup>GRY – Municipal Hospital in Gryfice
Antibiotic susceptibility of *P. mirabilis* hospital isolates

Only 3 isolates were sensitive to ceftotaxime, 5 to ceftazidime, all strains were sensitive to imipenem. Only 5 (10.4%) isolates were resistant to piperacillin/tazobactam.

Genotype B (B1-B4) strains (n = 49), were resistant to ampicillin, amoxycillin/clavulanic acid, piperacillin, cephalotin, cefuroxime, gentamicin, tobramycin, netilmicin, amikacin, pipemidic acid, pefloxacin, norfloxacin, ciprofloxacin, trimethoprim-sulphamethoxazole. Most of the genotype B strains were resistant to cefotaxime (96%) and ceftazidime (92%), 10 strains (20.4%) to piperacillin/tazobactam. One of the isolates representing genotype B showed very high antibiotic resistance, including imipenem. This strain was isolated from a patient’s urine in the internal medicine SZC.

All genotype C (C1-C5) strains (n = 9) were resistant to ampicillin, cephalotin and trimethoprim-sulphamethoxazole. The 3 *P. mirabilis* isolates were resistant to amoxycillin/clavulanic acid, 6 to piperacillin, 2 to cefuroxime, 1 to cefotaxime and ceftazidime, 7 to pipemidic acid, 2 to pefloxacin, norfloxacin and ciprofloxacin. All strains were susceptible to piperacillin/tazobactam, imipenem, netilmicin and amikacin.

**Identification of β-lactamases.** Thirty four isolates were found to be ESBL producers. The number of strains producing ESBL isolated in particular years showed an increasing trend. In 1996 only 2 (5.9%) strains showed the presence of ESBL, in 1997 – 4 (11.8%), in 1998 – 6 (17.6%), in 1999 – 7 (20.6%), and in 2000 – 15 (44.1%). All of the *P. mirabilis* ESBL producers belonged to multiresistant strains and to genotypes A1 (5 strains), A2 (7), A5 (2), A7 (1) and B1 (14), B2 (4), B3 (1).

The following antibiotic resistance patterns were observed:

AmpAmcPipCflCxmCtxCazGnTobNetPiNorPefCipSxt (20 isolates; 58.8%)
AmpAmcPipCflCxmCtxCazGnTobNetAnPiNorPefCipSxt (13 isolates; 38.3%)
AmpAmcPipCflCxmCtxCazGnPiNorPefCipSxt (1 isolates; 2.9%)

**Discussion**

Despite the progress of knowledge, improved preventive and scrutiny procedures, hospital infections still pose a serious clinical, therapeutic and epidemiological problem. The most common are urinary tract infections that comprise 35–45% of all hospital infections and often cause dangerous diseases such as septicemia, pyelonephritis or wound infections. The most common pathogen isolated in urinary tract infections is still *Escherichia coli*. However, other bacteria including representatives of *Proteus* genera (especially *P. mirabilis*) begin to play ever greater role in pathogenesis of hospital infections, especially in urology wards (Chippendale *et al.*, 1994; Clapham *et al.*, 1990).
P. mirabilis is the second most common cause of urinary tract infections, and it is a frequent cause of nosocomial infections as well. It was confirmed in our own research, as much as 70% isolates isolated from urine of patients suffering for urinary tract infections were classified as P. mirabilis, and only 30% from other materials. The existence of multiresistant P. mirabilis strains in hospital environment makes constant monitoring for presence of those microorganisms in specific hospital wards a necessity. It is also necessary to monitor emerging new resistance mechanisms as well as transmission of strains between patients and wards as it is commonly done in case of other bacteria (Bonnet et al., 2002; Mamberger et al., 2001).

In our research we used PFGE as a method for analysing restriction patterns of chromosomal DNA of P. mirabilis strains in order to classify them into particular genotype. It has been shown that up to 60% of P. mirabilis strains isolated from hospital infections belong to the three main genotypes A, B, C.

Type A and B showed high antibiotic resistance to all tested aminoglycosides and chinolons, and most of β-lactam antibiotics. One strain belonging to genotype B also showed resistance to imipenem. Subtypes C1-C5 differed from types A and B in regard to antibiotics susceptibility. All those strains showed higher sensitivity to antibiotics, but the diversity of antibiotic profiles was observed among subtypes.

Demonstrating clear genetic relationship between P. mirabilis strains isolated from different environments within a few years period indicates existence of outbreak clones in wards of the clinical hospital as well as the transmission of those strains to other hospitals in the region. β-lactamases producing P. mirabilis strains with extended spectrum, ESBL, pose additional therapeutic problems in treatment of infections. The selection of ESBL producers occurs most frequently in surgery, urology and neonatal wards (Goering 1993; Saladin et al., 2002). In our research seven-fold increase of ESBL producing strains was observed: from 5.9 % in 1996 to 44.1% in 2000 year.

ESBLs are functionally differentiated. Some of them efficiently hydrolyse cefotaxime and ceftriaxone but not ceftazidime, while others clearly prefer ceftazidime and aztreonam. These differences may cause problems, particularly in interpretation of sensitivity to combinations of penicillins with inhibitors. ESBL are inhibited by β-lactamase inhibitors, but some β-lactamases hydrolyse penicillin to a considerable degree and so the inhibition effect may not suffice for the strain to be sensitive to a combination of penicillin with and inhibitor. Genes encoding for ESBL are often located on plasmids which in a short time may cause a spread of resistance genes among different bacteria through conjugation and exchange of plasmids (Neuwirth et al., 2001; Palucha et al., 1999). The results concerning aminoglycosides resistance confirm that genes responsible for aminoglycoside resistance are often located on the same plasmids, where genes encoding ESBL reside.

Our research has shown constant increase ESBL producers among P. mirabilis strains, which indicates proliferation of plasmid-coded resistance mechanism in the given environment, a disconcerting tendency. The phenomenon of proliferation of plasmid located resistance genes does not seem to be related to other genetic traits of the tested strains. ESBL producers belonging to multiresistant genotypes A and B, not to more sensitive to antibiotics genotype C. No unique pattern among ESBL producing strains has not been found.

Appearance of P. mirabilis strains with the same restriction patterns in different wards of the same hospital as well as in different hospitals in the West Pomeranian area proves the spread of the strains in hospital environment. In addition to the strains classified as particular genotypes, in our research 71 unique strains characterized by a singular restriction pattern were found. It indicates a relatively great variety among P. mirabilis strains present in our environment. The unique strains usually showed high sensitivity to antibiotics. They were probably “patients own strains” that caused endogenous infection. Appearance and remaining of the same clones of strains in different wards, their transmission between wards and their appearance in other hospitals may certainly cause the problems. It is also an irrefutable proof for circulation of hospital strains in the environment and poses danger to potential patients. Hospital strains are characterized by much higher resistance to antibiotics than patients’ indigenous strains and frequently require treatment with expensive antibiotics of wide spectrum of antibacterial activity. It results in the prolongation of the time of hospitalisation and in increasing the cost of treatment. Scarcie information appearing in the literature concerning increasing number of clonal strains among P. mirabilis is probably due to the fact that until recently these bacteria have not been considered to be particularly dangerous in hospital environment, contrary to MRSA or Klebsiella pneumoniae strains which are commonly known to be alert-pathogens (Fiet et al., 2000; Traub et al., 1996). Our results indicate that also P. mirabilis may turn out be a dangerous pathogen in hospital environment causing dangerous clinical infections. This fact should make us aware that greater attention must be paid to the situation. Periodic surveillance tests for P. mirabilis presence should be regularly performed as it is done with other bacteria in a properly managed, modern hospital. It would certainly aid determining the frequency and type of hospital infections in the relation to a given ward’s specific function and sanitary conditions.
Moreover, more intensive efforts of research teams working on hospital infections could contribute to a decrease in infection rates caused by Gram-negative rods of *Proteus* genus, especially hospitals isolates.

**Literature**


Introduction

Commensal bacteria inhabiting human and animal intestine, *E. coli* among others, are subjected to contact with various antibiotics applied at various concentrations and with varied frequency. Antimicrobial agent resistance genes are situated in mobile genetic elements such as plasmids, transposons and integrons (Carattoli, 2001; Rowe-Magnus and Mazel, 1999). Once acquired resistance genes can be transferred between bacteria. The host organism’s selective pressure selects the resistant bacteria that have specific patterns of resistance (Sayah *et al*., 2005). The observations of the development of resistant bacteria have resulted in hypothesis that commensal bacteria serve as a reservoir of resistance genes (Wray and Gnanou, 2000). The research on the prevalence of the resistance within the four main phylogenetic groups is raised in the aspect of the diversified genetic structure of *E. coli*. It seems that mammals, which are kept in zoological gardens and safari parks may serve as good model objects for

Prevalence of Antibiotic Resistance Profile in Relation to Phylogenetic Background among Commensal *Escherichia coli* Derived from Various Mammals

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Abstract

The paper describes the prevalence of resistant strains within the genetic structure of *E. coli* (phylogenetic group A, B1, B2 and D). A total of 200 commensal *E. coli* strains have been derived from 10 species of healthy animals residing on ZOO Safari Park area, in Świerkocin, Poland. The phylogenetic structure of *E. coli* has been analysed with the use of a PCR-based method. The strains were tested in terms of their susceptibility to eight classes of antibiotics: aminoglycosides, penicillins, cephalosporins, tetracyclines, nitrofurans, sulphonamides, phinicsols, and quinolones. The genetic structure of *E. coli* revealed a not uniform distribution of strains among the four phylogenetic groups with significantly numerous representation of groups A and B1. Resistant *E. coli* were found within each of the phylogenetic groups. Strains resistant to one class of antibiotics occurred significantly more frequently in phylogenetic groups B2 and D (potential pathogens), whereas strains resistant to more than one class of antibiotics belonged to phylogenetic groups A and B1 (typical commensals) in a prevailing number of cases.

Keywords: commensal *E. coli*, phylogenetic groups of *E. coli*, resistance to antibiotics

1) common occurrence of *E. coli* as an element of intestine microflora in mammals; 2) the occurrence, within *E. coli* species, of pathogens causing both intestinal and extraintestinal diseases in humans and animals; 3) the genetic structure of *E. coli*, which is of a clonal character and is composed of 4 main phylogenetic groups A, B1, B2 and D. This is evidence that pathogenic *E. coli* originate from commensal strains revealing diversified preference to the acquisition of certain virulence factors. *E. coli* of phylogenetic group B2 accumulate extraintestinal virulence factors. Enteropathogenic *E. coli* are assigned to group D in prevailing number of cases. Groups A and B1 are determined as typical commensals (Duriez *et al*., 2001; Reid *et al*., 2000). Thus, the question of the prevalence of the resistance within the four main phylogenetic groups is raised in the aspect of the diversified genetic structure of *E. coli*. It seems that mammals, which are kept in zoological gardens and safari parks may serve as good model objects for

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such research, as they stay in a limited area for a long time and are under continuous control.

Our research involved the analysis of commensal *E. coli* derived from carnivorous and herbivorous mammals staying in the grounds of ZOO Safari Park in Świerkocin. The aim of the research was to analyse the prevalence of resistant strains within the four main phylogenetic groups of *E. coli*.

**Experimental**

**Materials and Methods**

**The source of strains.** The material was derived from adult, healthy individuals. The source organisms were five species of herbivorous animals from the taxonomic order Artiodactyla: water buck (*Kobus ellipsiprymnus*), eland (*Taurotragus oryx*), yak (*Bos mutus graniens*), aurochs (*Bos primigenius*), buffalo (*Bubalus bubalis*) and five species from the taxonomic order Carnivora: lion (*Panthera leo*), lynx (*Felinis lynx*), wildcat (*Felinis silvertis*), racoon (*Procyon lotor*), dingo (*Canis familiaris dingo*). The research included both herbivorous and carnivorous species because of the different diets and the different layout of the walks in the Zoo’s area. The buffaloes and the yaks walks were separated with a walk common for aurochs, eland and water buck. The walks of the carnivorous animals were isolated from both other carnivorous as well as herbivorous animals.

**Identification of *E. coli*.** The samples were drawn once. Each of the 10 tested animal species was represented by samples drawn from three individuals. Thus, a total of 30 faecal samples were obtained. *E. coli* were isolated from each individual faecal sample. Inoculation was performed onto m-FC agar with rosolic acid (Merck). After 24 h of incubation at 44.5°C blue colonies were passaged onto MacConkey agar plates (Difco). Lacto-positive isolates were verified with a series of tests IMV and C (indole, methyl red, Voges-Proskauer, citrate) just as described earlier (Baldy-Chudzik and Stosik, 2003). All *E. coli* isolates were stored at –70°C in Luria-Bertani (LB) broth containing 25% glycerol. For the experiments, the strains were cultured in LB broth for 18 h at 37°C.

**DNA template preparation.** A single bacterial colony was suspended in 25 µl sterile water then heated to 99°C, 10 min, then cooled and centrifuged. The obtained supernatant was the source of DNA template for PCR reaction.

**BOX-PCR DNA fingerprinting.** All the isolates identified in IMV and C tests as *E. coli* were used to DNA rep-PCR fingerprinting with BOX A1R primer (BOX-PCR). The primer sequence BOX A1R and the amplification conditions were used according to the ones described earlier (Baldy-Chudzik and Stosik, 2005). BOX-PCR products were analysed electrophoretically in 0.8% (w/v) agarose in 1xTBE buffer and stained with ethidium bromide. Gels were documented as TIFF files and analysed with Fingerprinting II informatix software (BioRad). BOX-PCR gel lanes were normalized using 1 kb DNA Ladder (Fermentas), as external reference standards. The similarity matrices were calculated based on Pearson’s similarity coefficient with 1.5% tolerance for the position of a band. Cluster analysis of similarity matrix was performed by the unweighted pair group method using arithmetic averages (UPGMA). The correlations were expressed as the percentage of similarity. The comparative analysis of BOX-PCR patterns generated by repeated analyses of strain *E. coli* K12 (CIP, Paris, France) (n = 50), revealed similarity >90%. On this basis, the similarity of BOX-PCR patterns of order 85% was established as a cut-off value for determination of unique strains. The isolates, which were derived from hosts of the same species and which revealed the similarity of BOX-PCR pattern >85% were regarded to be the same and were eliminated from subsequent analyses. Based on the similarity analysis of genomic patterns, 200 unique *E. coli* strains were selected for the subsequent studies (Fig. 1, Table I).

**Determination of phylogenetic groups.** The method used three pairs of primers of PCR reaction of sequences homologic to genetic markers specific for phylogenetic groups of *E. coli*: gene *chuA*, gene *yjaA*, and an anonymous DNA sequence TspE4C2. The PCR primers, the amplification steps and the electrophoretic analysis were all used according to those given by Clermont et al. (2000). On the basis of a specific profile of PCR products, the determination of the phylogenetic group was carried out in the following way: *chuA*+, *yjaA*+, group B2; *chuA*–, *yjaA*–, group D; *chuA*–, TspE4.C2+, group B1; *chuA*–, TspE4.C2–, group A.

**Antimicrobial agents susceptibility.** Antibiograms and their interpretation were made using the disk diffusion method following the CLSI (formerly NCCLS) standards (National Committee for Clinical Laboratory Standards).

Fig. 1. Dendrogram of the similarity relation of BOX-PCR fingerprinting patterns of the 200 unique *E. coli* strains derived from ten source species. Each of the isolates is defined by: taxonomic species of the host / I, II or III refers to an individual of a given species, an Arabic numeral refers to a number of an isolate identified in an individual.
Antibiotic resistance in commensal E. coli strains

Buffalo III. 1
Aurochs III. 2
Buffalo I. 4
Buffalo II. 3
Buffalo III. 4
Buffalo I. 6
Buffalo II. 5
Buffalo II. 5
Buffalo III. 3
Buffalo I. 1
Buffalo III. 2
Buffalo II. 4
Buffalo II. 2
Lion I. 5
Lion II. 6
Lion II. 3
Lion III. 8
Lion II. 5
Lion I. 7
Lion II. 1
Lion II. 7
Lion III. 6
Lion I. 8
Lion III. 7
Lion I. 2
Lion I. 9
Lion I. 4
Lion I. 1
Lion III. 4
Lion III. 1
Lion III. 3
Lion II. 8
Yak I. 2
Yak II. 3
Yak III. 2
Yak I. 5
Yak II. 6
Yak III. 1
Yak I. 1
Yak I. 6
Yak II. 5
Yak I. 4
Yak II. 1
Yak III. 3
Yak I. 4
Aurochs I. 5
Aurochs I. 2
Aurochs I. 3
Aurochs II. 2
Aurochs II. 5
Aurochs III. 4
Aurochs III. 6
Aurochs III. 1
Aurochs II. 4
Aurochs I. 8
Aurochs III. 5
Aurochs I. 1
Aurochs I. 4
Aurochs II. 6
Aurochs III. 2
Aurochs III. 3
Aurochs II. 1
Waterbuck II. 2
Waterbuck II. 3
Waterbuck III. 5
Waterbuck II. 7
Waterbuck III. 3
Waterbuck II. 6
Waterbuck III. 2
Waterbuck II. 7
Waterbuck III. 8
Waterbuck III. 4
Waterbuck III. 6
Waterbuck II. 4
Waterbuck I. 6
Waterbuck III. 7
Waterbuck II. 8
Waterbuck I. 5
Waterbuck II. 1
Raccoon I. 1
Raccoon III. 3
Raccoon III. 9
Raccoon I. 1
Raccoon III. 2
Raccoon I. 2
Raccoon II. 4
Raccoon III. 1
Raccoon I. 6
Raccoon I. 3
Raccoon III. 5
Raccoon I. 4
Raccoon II. 2
Raccoon III. 4
Raccoon I. 5
Raccoon II. 5
Standards, 2003) for 14 antimicrobial agents (Table II). These antimicrobial agents were selected on the basis of their importance in treating human or animal bacterial infections and their use as feed additives to feed efficiency and/or disease prophylaxis in animals and on the basis of their ability to provide diversity for representation of different antimicrobial agent classes. Each of \textit{E. coli} isolate was grown overnight in LB broth at 37°C and diluted with LB broth to an absorbance at 600 nm of \(\sim 0.1\). The diluted \textit{E. coli} inoculum was swabbed onto a Mueller-Hinton agar (Difco) plate. Fourteen commercially prepared antimicrobial agent disks (Becton Dickinson) were placed on the inoculated plates. The plates were incubated at 35°C for 18 to 20 h. The diameters (in millimeters) of the clear zones of growth inhibition around the antimicrobial agent disks, including the 6-mm disk diameter, were measured by using precision callipers. The breakpoints used to categorize isolates as resistant or not resistant to each antimicrobial agent were those recommended by manufacturer (BBL Sensi-Disc Antimicrobial Susceptibility Test Discs, Becton Dickinson) for \textit{E. coli} (Table II). Intermediate zones of inhibition were counted as sensitive for purposes of this study. \textit{E. coli} ATCC 25922 was used for quality control strain.

### Results

A total number of 200 \textit{E. coli} strains were identified, among which 100 were derived from carnivorous and 100 from herbivorous animals. The analysis of the genetic structure of \textit{E. coli} isolates showed significant differences between strains derived from carnivorous and herbivorous animals (Table I). Strains derived from carnivorous animals occurred in groups A and D significantly more frequently than the ones derived from herbivorous animals (\(p<0.001\), \(p<0.01\) respectively). \textit{E. coli} from herbivorous species were significantly more frequently classified to group B1 (\(p<0.001\)). The observed diversity of the genetic structures of \textit{E. coli} between carnivorous and her-
bivorous species may be explained with different diet requirements (resulting from their taxonomic position). Diet has been reported to be the key factor determining the relative abundance of *E. coli* phylogenetic groups in mammals (Gordon and Crowling, 2003). The revealed higher homogeneity in the genetic structure of *E. coli* in herbivorous species has resulted from the fact that the examined species were all ruminants rather than from the incidences of transmission of *E. coli* between them. The conclusion is supported by the results of BOX-PCR fingerprints, where the genomic similarity (>80%) proving the transmission of strains, has been revealed in individual *E. coli* derived from: lynx and eland; wildcat and buffalo; yak, waterbuck, and eland as well as buffalo and aurochs (Fig. 1).

Among the 200 examined strains, 74 were susceptible to all the antimicrobial agents tested. Resistances to cephalosporins, tetracyclines, and sulphonamides were generally most frequent (Table III). Between *E. coli* from herbivorous animals, 65% of strains from yak were susceptible to all the antibiotics tested, whereas 38, 35, 29, and 17% of *E. coli* from eland, buffalo, waterbuck, and aurochs showed no resistance at all, respectively. In carnivorous animals, 47% of *E. coli* from dingo, 37% from wildcat, 36% from lion and lynx, and 35% from raccoon were susceptible to all the antibiotics tested. Strains from carnivorous animals were more frequently resistant to aminoglycosides (p<0.001), penicillins (p<0.001), as well as nitrofurans (p<0.001) and quinolones (p<0.001) in comparison to *E. coli* from herbivorous animals. *E. coli* multi-resistant to antibiotics belonging both to the same class as well as various classes were found in carnivorous animals more frequently than in herbivorous ones. One strain from wildcat presented resistance to 12 antimicrobial agents (N, GM, S, AM, AMC, CF, CFP, TE, FT, SXT, C, and NA). Among strains of all the herbivorous species, the most frequently identified multi-resistance pattern comprised the following antibiotics: CF, TE, and SXT. The same resistance pattern was identified also in three strains derived from lynx, what proves the transmission of resistance factors between animals living in a neighborhood. Among multi-resistant *E. coli*, simultaneous resistance to cephalosporins (CF or/and CFP) and AM, characteristic for penicillins, was found in 26 strains among which 17 were derived from carnivorous. Simultaneous resistance to cephalosporin and AM was shown in five *E. coli* whose multi-resistance patterns comprised five classes of the applied antibiotics. Resistance to TE, D or SXT exclusively, occurred with a comparable frequency in *E. coli* from herbivorous and carnivorous animals. The resistance pattern comprising SXT and D or SXT and TE occurred in both herbivorous and carnivorous, whereas resistance to SXT and CF was a characteristic feature for herbivorous animals exclusively.

Strains from carnivorous animals differed from *E. coli* of herbivorous both in the genetic structure and the resistance patterns. The observed differences constituted the base for the following generalizations:

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**Table III**

Occurrence of resistant *E. coli* in the examined animal species

<table>
<thead>
<tr>
<th>The class of agent (antibiotic)</th>
<th>Heribvorous:</th>
<th>Carnivorous:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total n = 200</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lion n = 25</td>
<td>Lynx n = 22</td>
</tr>
<tr>
<td></td>
<td>Racoon n = 17</td>
<td>Dingo n = 17</td>
</tr>
<tr>
<td></td>
<td>Wildcat n = 19</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Buffalo n = 17</td>
<td>Waterbuck n = 24</td>
</tr>
<tr>
<td></td>
<td>Aurochs n = 18</td>
<td>Eland n = 24</td>
</tr>
<tr>
<td></td>
<td>Yak n = 17</td>
<td></td>
</tr>
<tr>
<td>Aminoglycosides: neomycin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gentamycin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Streptomycin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amikacin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Penicillins: Ampicillin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amoxicillin/clavulanic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cephalosporins: cephalothin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cefoperazone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tetracyclines: Tetracycline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Doxycycline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrofurans: Nitrofurantoin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulphonamides: Risthemoprim</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulphamethoxazole</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenicolos: Chloramphenicol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quinolones: Nalidixic acid</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---
1) strains derived from carnivorous and herbivorous species were treated as two separate sets, without specifying the source species. 2) the resistance was later analyzed with regard to a class not to a particular antibiotic, since strains multi-resistant to antibiotics of the same class occurred more frequently. What is more, because of a low percentage of *E. coli* resistant to chloramphenicol (phincolics), the class was neglected in subsequent analyses. The analysis of the genetic structure of resistant *E. coli* showed that a prevailing percentage of strains resistant to any class of the applied antibiotics belonged to phylogenetic groups A and B1 (typical commensals), derived from both carnivorous and herbivorous animals (Fig. 2). Not numerous resistant *E. coli* from groups B2 and/or D usually co-occurred with the resistant strains from groups A and B1.

The susceptible strains constituted 36% of the set from herbivorous animals and 38% of set of *E. coli* derived from carnivorous animals (Fig. 3). A high percentage of multi-resistant strains were revealed in each of the sets. Among *E. coli* from herbivorous animals 24% strains revealed resistance to three classes of antibiotics, whereas 38% strains revealed resistance to more than three classes of antibiotics in the set from carnivorous animals (from 4 up to 7 classes) (Fig. 3). The genetic structure of multi-resistant strains revealed distinct features common for both analyzed sets of *E. coli*: 1) strains resistant to one class of antibiotics were represented by groups B2 and D (potential pathogens) whereas the representation of strains from group A and B1 did not occur (typical commensals); 2) strains resistant to two and three classes of antibiotics were represented by all the four phylogenetic groups; 3) strains resistant to more than three classes of antibiotics in vast majority belonged to group A and B1 and the representation of group B2 was insignificant or did not occur, and group D did not occur.

### Discussion

The highest levels of resistance were observed for sulfonamides, tetracyclines, and cephalosporins in strains derived from all animal species. Resistance to
Antibiotic resistance in commensal *E. coli* strains was shown in each of the phylogenetic groups of *E. coli*, but strains resistant to a single antibacterial agent (sulphonamide or tetracycline) belonged exclusively to phylogenetic groups B2 and D (potential pathogens). The prevalence of resistance to these classes of antibiotics could be the result of widespread and lengthy use of these antimicrobial agents in non-food producing animals (Klein and Bulte, 2003). Resistance to tetracycline is plasmid mediated. The large numbers of genetic determinants for tetracycline resistance make it more possible for a susceptible bacterium to acquire resistance factors than if only a few determinants were available (McEwen and Fedorka-Cray, 2002). Resistance to sulphonamides is plasmid mediated, but chromosomal mutations for sulphonamide resistance take place very seldom. Resistance to sulphonamides is widespread in the environment and cross-resistance between sulphonamides is complete (Sköld, 2001).

A high level of resistance to cephalosporins was observed in both carnivorous and herbivorous animals, whereas resistance to penicillins occurred considerably more frequently in carnivorous animals. The resistance to these classes of antibiotics as well as to the remaining examined classes co-occurred, forming patterns of multi-resistance in strains from phylogenetic groups A and B1. Cefoperazone (the third generation of cephalosporins) is used in veterinary medicine for various species of animals. The first-generation cephalosporin’s – cephalothin are heavily used for the treatment of bacterial infections and particularly of urinary tract infections in cats and dogs but rarely or never in ruminants (all herbivorous animals in this study are ruminant) (Lanz et al., 2003;
Donaldson et al., 2006). In herbivorous animals, the resistance against the third-generation of cephalosporins can develop resistance to cephalothin. The most common mechanism of resistance to β-lactam antibiotics (including the tested penicillins, and cephalosporins) is the production of various β-lactamases, which hydrolyze β-lactam ring. The E. coli resistance may be caused by both mutations in ampC gene (conditioning constitutive β-lactamase) located on chromosomes, and a broad spectrum of β-lactamase genes located on plasmids. AmpC β-lactamases are not inhibited by inhibitors such as clavulanic acid. The plasmid resistance is the effect of a stable mutation and is easy to maintain by bacteria even at the absence of the selective pressure of antibiotics. β-lactamases located on a plasmid are sensitive to inhibitors such as clavulanic acid (Livermore, 1995; Siu et al., 2003). The family of β-lactamases is numerous, and growing. Among strains of a complex resistance patterns including: penicillins, penicillins with clavulanic acid and cephalosporins, a univocal identification of types of developed resistance may be achieved by specific gene identification. It is the consequence of the fact that a simultaneous occurrence of both chromosomal and plasmid genes conditioning β-lactamases are found more and more frequently in multi-resistant strains (Tenover et al., 2003). The differences in resistance patterns between carnivorous and herbivorous animals may be caused by exposure to different agents because of differences in the husbandry of these species or other factors that may have increased or decreased the likelihood of the development and conservation of resistant bacteria in the animal species. For example, resistance to quinolones occurred considerably more frequently in carnivorous than in herbivorous. The resistance to this class of antibiotics is the result of chromosomal mutations, and not the result of acquiring genes from other bacteria of the same or other species, and the occurrence of resistance is conditioned by the frequency of applying the medicine (Chen et al., 2001).

The research comprised 10 species of healthy mammals with the aim to analyze the genetic structure of antibiotic-resistant commensal E. coli. Such direct comparative analyses of E. coli in various host species are rare in the literature and are usually concerned with clinical strains derived from a single host species (Hill et al., 2003; Selander et al., 1986). The obtained results are essential for the better recognition of population biology of E. coli, because they indicate the fact that within a genetic structure of E. coli of various species, phylogenetic groups A and B1, i.e. typical commensal strains, compose a basic reservoir of multi-resistant strains. They also suggest that E. coli from groups A and B1 occupy similar niches in the organisms of the examined animals. In such niches, gradual acquiring of resistance factors may result in increased surviving. It has been reported that multi-resistant E. coli survive better beyond the host organism than the susceptible strains (Abu-Ghazaleh, 2001). The contamination of the habitat of animals with multi-resistant E. coli increases the real hazard of the prevalence of both such strains as well as the resistance factors. The prevalence of multi-resistant E. coli from groups A and B1 in the environment may be essential for the recently reported failures in the treatment of extraintestinal infections of E. coli in humans (particularly reoccurring infections of urinary tract in females) (Johnson et al., 2005; Moreno et al., 2006). The problems in the treatment resulted from the occurrence of opportunistic, multi-resistant E. coli from groups A and B1 rather than uropathogenic E. coli from group B2, while in most cases uropathogenic E. coli from group B2 constituted a bacterial subpopulation sensitive to antibiotics.

Literature


Klein G. and M. Bulte. 2003. Antibiotic susceptibility pattern of Escherichia coli strains with verocytotoxigenic E. coli associated
virulence factors from food and animal species. *Food Microbiol.* 20: 27–33.
Baldy-Chudzik K. and Stosik M.
Introduction

The female birth canal is rich in microorganisms. Infection starts when a pathogenic microorganism enters into the birth canal and dominates the physiologic microflora, breaking protective barriers and starting an inflammatory process. Vaginal environment (stable temperature, humidity, presence of nutrients, and low level of oxygen) is appropriate for the growth of microorganisms. Development of infection depends on many factors, such as virulence of microbes, immune system activity, production of different factors inhibiting growth of microorganisms and others.

In Poland about 35% of infections of female genito-urinary tract are caused by yeasts (Gołębiewska and Kurnatowska, 2001), especially *Candida albicans* (80–95%), urogenital mycoplasmas; *Ureaplasma urealyticum* – 20% of cases of NGU (Denys, 2006), and *Chlamydia trachomatis* – 20–40% (Choroszy-Król et al., 2000; Zbroch et al., 2004).

Although group B streptococci (GBS) is a part of the physiologic microflora of vagina (colonization – 34%) the frequency of infection by this bacterium increases during inflammatory processes in the genito-urinary tract (Dyba et al., 2005).

Taking into account the possibility of relapses or severe complications as infertility, and possibility of transmission of infectious microorganisms to newborns, it is very important to appropriate diagnose and treat such infections. Because of recently observed increase of microbial resistance to antibiotics, researchers are looking for alternatives. In our study we evaluated and compared the concentration of human neutrophil peptides (HNP 1–3) in cervico-vaginal lavages (CVL), obtained from women with vaginitis/cervicitis. Swabs from the posterior vaginal fornix and from the endocervical canal as well as CVL samples were obtained from 32 patients with vaginitis/cervicitis and 29 healthy women (control group). Supernatants of CVL were used for determination of concentration of HNP by ELISA. The difference between concentrations of HNP 1–3 in studied and control groups was statistically significant (p = 0.018). The maximal concentration was determined in patients with mixed infections (28.41 ng/ml), and Group B Streptococci, GBS, (28.06 ng/ml), the minimal concentrations in cases of *C. trachomatis* (mean concentrations did not differ from those in the control group: 16.93 ng/ml and 16.39 ng/ml, respectively). Maximal correlation was determined for control-studied group with isolation of GBS (r = 0.79), and very high negative correlation for group of GBS – *C. trachomatis* (r = –0.98).

**Key words:** CVL, genito-urinary infections, HNP 1–3
localization and elimination of pathogenic microorganisms mainly by nonspecific mechanisms. Human neutrophil peptides, HNPs, are α-defensins produced in female birth canal against infectious agents. HNPs are localized in the azurophilic granules of neutrophils as the main proteins, participating in oxygen – independent phagocytosis of microorganisms (Ganz et al., 1985). α-Defensins are part of host’s natural antimicrobial immunity, responsible for the first line of defense against pathogenic microorganisms. Additionally they play an important role in acquired antimicrobial immunity through the production of specific antigens and promote maturation of dendritic cells (Yang et al., 2002).

Defensins are part of host’s natural antimicrobial immunity, responsible for the first line of defense against pathogenic microorganisms. Additionally they play an important role in acquired antimicrobial immunity through the production of specific antigens and promote maturation of dendritic cells (Yang et al., 2002).

In vitro HNPs demonstrate a cytolytic effect against bacterial strains, yeasts and viruses. This causes increasing permeability of microbial membrane, pore formation and outflow of ions and bigger molecules. HNPs can also competitively substitute divalent cations, which form bridges between lipopolysaccharide molecules (Hancock, 1997; Zasloff, 2002). It is possible that development of different mechanisms of co-influence with bacterial cell wall plays an important role among the properties of α-defensins against different microorganisms. Mechanisms of α-defensin actions are similar, but effects are different, depending on specific construction of target cell wall (Lynn et al., 2004).

The aim of this study was to evaluate and compare the concentration of HNP 1–3 in cervico-vaginal lavages, obtained from women with vaginitis and cervicitis, caused by different etiological agents.

**Experimental**

**Materials and Methods**

**Samples source.** Samples taken from 61 non-pregnant women aged 19–40 (mean age 28.5) attending the Department of Medical Microbiology at the Medical University of Silesia, Katowice for diagnostic purposes were studied. The study group includes 33 patients (mean age 28.6) with symptoms of vaginitis/cervicitis (redness of vaginal and cervical epithelium and/or mucopurulent endocervical discharge and/or pain and contact bleeding) before antibiotic treatment. The control group includes 29 healthy women (mean age 28.4). All patients gave informed consent for this study.

**Sampling procedure.** Swabs from the posterior vaginal fornix and from the endocervical canal and also cervico-vaginal lavage samples were obtained from each patient for this study (Fig. 1). Gram stained microscopic slides were studied for bacterial vagi-
nosis (BV) using Amsel and Nugent criteria and patients with BV were excluded from this study (Zbroch et al., 2004).

**Microorganisms culturing and identification.** Vaginal swabs were used for Gram staining and microorganisms culturing. Culturing was performed according to routine microbiological practice. Typing of β-hemolytic streptococci was performed by Microscreen Strep (Microgen Bioproduct Ltd., UK). Yeasts and other fungi were identified by ID 32C test (bioMérieux, France). Identification of mycoplasmas was performed by Mycoplasma IST 2 (bioMérieux, France), and cervical Dacron swabs were used for Chlamydia Direct IF (bioMérieux, France) determining antigens of Chlamydia trachomatis according to the manufacturer’s instructions.

**HNP assay.** Cervico-vaginal lavage samples were obtained by introducing 5 ml of PBS by sterile syringe followed by aspiration. Lavage-samples were centrifuged for 10 min at 1000 × g at 4°C. Supernatants were used for determination of concentration of HNP 1–3 by ELISA test kit (Cell Sciences, Inc., USA) according to the manufacturer’s instructions by using spectrophotometer mQuant (Biotek Instruments Inc., USA) at λ 450 nm.

### Results

No statistically significant difference was observed between mean ages of women in the studied and control groups (28.6 and 28.4 respectively), also socio-economic conditions in both groups were comparable.

The studied group was divided into subgroups according to detected etiological agents: GBS (n = 5), C. albicans (n = 7), C. trachomatis (n = 6), U. urealyticum (n = 7) and mixed infections (n = 7). Etiologic agents of mixed infection are presented in Table I. In control group only microorganisms of physiological microflora were detected. In the studied group the maximal concentration of HNP 1–3 was found in patients with mixed infections (28.41 ng/ml) and in cases of GBS, mean concentrations did not differ from those in the control group (16.93 ng/ml and 16.39 ng/ml, respectively) (Table II). The difference between concentrations of HNP 1–3 in studied and control groups was statistically significant (p = 0.018).

### Discussion

In the beginning phase of infection neutrophils adhere to the surface of epithelial cells and their proteins determine the first defense against infection.
Infection process triggers morphological changes in epithelial cells (mainly in nucleus and cytoplasma). These changes usually depend on the type of infectious agent.

Our study demonstrated differences in expression of HNPs depending on isolated etiological agent, although further genetic studies are required for confirmation of these results. In the medical literature we did not find any data regarding the expression of HNP 1–3 in GBS infection, as well as mixed-infection. In cases of mixed infection concentrations of HNP 1–3 determined in our study evidenced increasing immune response, only in one case the level of HNP 1–3 was low – 6.45 ng/ml (Table 1). Such high immune response to GBS infection may be connected with the fact, that GBS is also a part of vaginal physiological microflora. It was found that saprophytic microorganisms can induce defensin expression, but they also demonstrate relative tolerance against them. On the other hand, pathogenic microorganisms do not induce defensin expression and also can evade innate immune mechanisms and cause disease (Yeaman and Yount, 2003). Low concentration of defensins in women infected with C. trachomatis, observed in our study, confirms the results described by Wiesenfeld et al. (2002) and Porter et al. (2005), who described a lower concentration of HNP 1–3 in women with chlamydiosis compared with women infected by Trichomonas vaginalis or Neisseria gonorrhoeae. Studies of Yasin et al. (1996) demonstrated minimal role of α-defensins against C. trachomatis. It is also a well known fact that HNP 1–3 with other antimicrobial peptides, like LL-37, and HBD-1, acts as a synergistic barrier and kills pathogenic microorganisms (Tollin et al., 2003). This mean that even low expression of HNP 1–3 by epithelial cells (lower than active concentration) also promotes development of immune mechanisms. A very active response was detected during C. albicans infection. Lehrer et al. (1988) and Raj et al. (2000) demonstrated that HNP-1 has a high antifungal activity, but HNP-3 – very low. Structures of HNP-1 and HNP-3 differ by one amino-acid in N-terminus of peptide, which determines defensin properties (Oren and Shai, 1997). This was confirmed also in for other yeasts; Cryptococcus neoformans is inhibited by HNP-1 and HNP-2 (Ganz et al., 1985), but intracellular growth of Histoplasma capsulatum in murine macrophages is inhibited by xenogenic expression of HNP-1 (Couto et al., 1994; Salzman et al., 2003).

There are no reports on the effect of HNP 1–3 on urogenital mycoplasmas. In our study we demonstrated similar results for U. urealyticum as in the case of yeasts. We didn’t observe correlation between defensins level and age of woman. Similar results were obtained by Wiesenfeld et al. (2002). They demonstrated lack of correlation between level of HNP and hormonal anticonception, phase of menstrual cycle and use of condoms. Increasing of NHP 1–3 concentration is a response to inflammation by neutrophil and epithelial cells. Valore et al. (2002) demonstrated that because antimicrobial effect of vaginal proteins depends on concentrations, increasing of neutrophil defensins concentration contributes to host defense. It is also very important that these peptides can act synergistically with other factors, such as LL-37 (Nagaoka et al., 2000). On the other hand many microorganisms developed mechanisms to evade bacteriocidal antimicrobial molecules (Ganz, 2001). In the opinion of Lynn et al. (2004) probability, that these mechanisms stimulate adaptive evolution of α-defensins is very high. For example, pathogens Pseudomonas aeruginosa, Enterococcus faecalis and Streptococcus pyogenes produce sulfur compounds, which bind and neutralize HNP-1 (Schmidtchen et al., 2001), but protein SIC (streptococcal inhibitor of complement) inhibits antibacterial activity of several antimicrobial peptides, like lizozyme, SLPI, LL-37, HNP-1 and β-defensins 1, 2 and 3 (Fernie-King et al., 2006).

We have shown that the ability of interaction between AMPs and microorganisms changes host response to infection depending mainly on structure and properties of etiological agent. Further in vivo and in vitro studies of interactions of AMPs with different microorganisms are required to shed light on the possibility of using them against antibiotic-resistant microorganisms.

Acknowledgements

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Literature


**Biological Activity of Phenylpropionic Acid Isolated from a Terrestrial Streptomyces**

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**Abstract**

The strain ANU 6277 was isolated from laterite soil and identified as *Streptomyces* sp. closely related to *Streptomyces albidoflavus* cluster by 16S rRNA analysis. The cultural, morphological and physiological characters of the strain were recorded. The strain exhibited resistance to chloramphenicol, penicillin and streptomycin. It had the ability to produce enzymes such as amylase and chitinase. A bioactive compound was isolated from the strain at stationary phase of culture and identified as 3-phenylpropionic acid (3-PPA) by FT-IR, EI-MS, ¹H NMR and ¹³C NMR spectral studies. It exhibited antimicrobial activity against different bacteria like *Bacillus cereus*, *B. subtilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *P. fluorescens*, *Staphylococcus aureus* and some fungi including *Aspergillus flavus*, *A. niger*, *Candida albicans*, *Fusarium oxysporum*, *F. udum* and *Penicillium citrinum*. The antifungal activity of 3-PPA of the strain was evaluated in *in vivo* and *in vitro* conditions against *Fusarium udum* causing wilt disease in pigeon pea. The compound 3-PPA is an effective antifungal agent when compared to tricyclozole (fungicide) to control wilt caused by *F. udum*, but it exhibited less antifungal activity than carbendazim.

**Key words:** *Streptomyces* strain ANU 6277, taxonomic studies, 3-phenylpropionic acid, biological activity of 3-PPA

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**Introduction**

Actinomycetes are Gram-positive bacteria that are wide spread in nature and play a significant role in the production of bioactive metabolites mainly antimicrobial compounds (Sanglier et al., 1993). At least 90% actinomycetes isolated from soil have been reported to be *Streptomyces* spp. (Anderson and Wellington, 2001). Most of these are potentially useful as pharmacologically and agriculturally active agents (Berdy, 2005). Pathogens found to be drug resistant revealed the importance of novel bioactive compounds (Bonjan et al., 2004). Microbial metabolites cause increasing attention as potential plant protection agents because they are expected to overcome the pollution problems caused by the synthetic chemical pesticides. Several novel metabolites of actinomycetes were widely useful for the control of plant diseases, insects and weeds (Li et al., 2003). Phenylpropionic acid is a member of the phenylpropanoid family, comprising a wide variety of C₆-C₃ compounds synthesized by plants from phenylalanine and important in plant physiology and defense mechanisms for the synthesis of flavonoids, insect repellents, UV protectants and signal molecules (Hahlbrock and Scheel, 1989). Phenylpropionic acid is rarely encountered as microbial metabolite. Cremin et al. (1994) reported that 3-phenylpropionic acid (3-PPA) is found in ruminal fluid as product of chemical reduction of dietary phenolic monomers by ruminal microorganisms. During the screening of actinomycetes for bioactive compounds, an actinomycete strain was found to be predominant in the random sampling of laterite soils present in different locations of Acharya Nagarjuna University (ANU) campus. The isolate was identified as *Streptomyces* and designated as strain ANU 6277. The strain was deposited at Microbial Type Culture Collection Centre (MTCC), IMTECH, Chandigarh (India) with accession number 6277. Very little is known about the biological activity of 3-PPA and no

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reports were found on its production by actinomycetes. Hence an attempt was made to evaluate biological properties of the bioactive compound from the strain. The present paper define the taxonomy position of the new isolated strain, presents the procedure of the extraction and elucidation of the biological activity of a compound obtained from the strain.

Experimental

Materials and Methods

Strain used. *Streptomyces* strain ANU 6277 was isolated from laterite soil sample collected at Acharya Nagarjuna University (ANU) campus by dilution plate technique using asparagine-glycerol-salts agar medium supplemented with streptomycin sulphate (10 µg/ml) and amphotericin-B (50 µg/ml). The strain was maintained on yeast extract-malt extract-dextrose (YMD) agar medium at 4°C (Williams and Cross, 1971).

Phenotypic studies. Morphological, cultural and physiological characteristics of the strain were performed according to the methods described by Shirling and Gottlieb, (1966). The strain was cultivated on different media including those recommended by International Streptomyces Project (ISP) media and non-ISP media. The media such as tryptone-yeast extract agar (ISP-1), YMD agar (ISP-2), oat meal agar (ISP-3), starch-casein-salts agar (ISP-4), glycerol-asparagine-salts agar (ISP-5), peptone-yeast extract-iron agar (ISP-6), tyrosine agar (ISP-7), nutrient agar and Czapek-Dox agar media were employed for the study of growth characteristics of the strain (Dietz and Thayer, 1980). Utilization of different carbon sources was studied in minimal medium containing those at 1% concentration. The strain grown at 37°C for 5 days on ISP medium 2 was used to study the micro-morphology with scanning electron microscope (SEM) (Yassin et al., 1997). The culture was fixed with glutaraldehyde, and dehydrated with ethanol. The dehydrated samples were dried, mounted on aluminium stubs, and sputter coated with gold-palladium. Finally, they were observed with digital SEM (model JEOL JSM-5600).

Phylogenetic analysis. The chromosomal DNA of the strain ANU 6277 was isolated according to the procedure described by Rainey et al. (1996). The 16S rRNA gene was amplified with primers 8-27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1500r (5'-GAAAGGTTGATCCAGGC-3'). The amplified DNA fragment was separated on 1% agarose gel, eluted from the gel and purified using Qiaquick gel extraction kit (Qiagen, Germany). The purified PCR product was sequenced with four forward and three reverse primers namely 8-27f (5'-AGAGTTTGATCCTGGCTCAG-3'), 357f (5'-CTCTACCGGGAGGCACGACG-3'), 704f (5'-TACGGGTTAATGCTGTAAGTCA-3'), 1114f (5'-CAGGAGCAGCGCAACC-3'), 685r (5'-TCTACCGATTCCAGCTAC-3'), 1110r (5'-GGGTTGACGTCTGTC-3') and 1500r (5'-GAAAGGTTGATCCAGGC-3'), respectively (Escherichia coli numbering system). The rDNA sequence was determined by the dideoxy chain-termination method using the Big-Dye terminator kit using ABI 310 Genetic Analyzer (Applied Biosystems, USA).

The 16S rDNA sequence of the strain ANU 6277 generated in this reaction (1478 bases) was aligned with the 16S rDNA sequence of other closely related *Streptomyces* species retrieved from the GenBank database. A sequence similarity search was done using GenBank BLASTN (Altschul et al., 1997). Sequences of closely related taxa were retrieved, aligned using Cluster X programme (Thompson et al., 1997) and the alignment was manually corrected. For the neighbour-joining analysis (Saitou and Nei, 1987), the distances between the sequences were calculated using Kimura’s two-parameter model (Kimura, 1980). Bootstrap analysis was performed to assess the confidence limits of the branching (Felsenstein, 1985).

Cultivation of the strain for secondary metabolites production. Actively growing pure culture of the strain was inoculated into 250 ml Erlenmeyer flasks, each containing 50 ml of seed medium consisting of 0.4% dextrose, 0.4% yeast extract, 1% malt extract and 0.2% calcium carbonate (pH 7.2). The culture was incubated on a rotary shaker (250 rpm) at 28°C for two days. The seed culture (10%) of the strain was transferred into a culture medium (4% dextrose, 0.9% proteose peptone, 0.1% yeast extract, 0.6% calcium carbonate, 0.1% K2HPO4, 0.1% MgSO4×7H2O, 0.01% MnSO4×H2O, 0.005% FeSO4×7H2O (pH 7.2) and incubated at 28°C for 5 days.

Extraction, purification and identification of active metabolite. The culture filtrate was collected at the end of five day incubation period and extracted twice with equal volume of ethyl acetate. The solvent extract was evaporated in vacuo to dryness. The dark brown residue was obtained and partially purified on silica gel column chromatography (22×5 cm, Silica gel 60, Merck) and eluted with gradient solvent system consisting of ethylacetate: hexane. Active fraction was collected and concentrated. Further purification was carried out in HPLC preparative column (10 mm ×250 mm, 5 µ using hexane: 2-propanol (8:2 v/v)). Structure elucidation of pure bioactive compound from the strain was carried out by FT-IR, EI-MS, 1H NMR and 13C NMR spectral studies.

Biological activity testing. Minimum inhibitory concentrations (MIC) of 3-PPA obtained from the strain against different microorganisms including bacteria and fungi were determined by conventional agar
Phenylpropionic acid from terrestrial Streptomyces

3

dilution method (Cappuccino and Sherman, 1999) using nutrient agar for bacteria and Sabourud’s agar medium for fungi. Different concentrations of 3-PPA (0 to 1000 µg/ml) were prepared in dimethyl sulfoxide (DMSO) and assayed against tested organisms. The organisms used in this assay are Bacillus cereus MTCC 430, B. subtilis MTCC 441, Escherichia coli MTCC 40, Klebsiella pneumonia MTCC 109, Proteus vulgaris MTCC 742, Pseudomonas aeruginosa MTCC 424, P. fluorescens MTCC 103, Staphylococcus aureus MTCC 96, Aspergillus flavus, A. niger, Candida albicans MTCC 183, Fusarium oxysporum, F. udum MTCC 2204 and Penicillium citrinum. The antifungal activity was observed after 24–48 h incubation at 37°C for bacteria and 48–72 h incubation at 28°C for fungi. Each experiment was performed in triplicates and proper controls were done. Lowest concentration of compound that showed antifungal activity against test organisms was recorded as MIC value (Hwang et al., 2001).

The cytotoxic activity of 3-PPA was tested on U-937 (Human leukemic monocyte lymphoma cell line) cells using MTT assay (Plumb et al., 1989). U-937 cells were obtained from National Centre for Cell Science, Pune (India) and were cultured at 37°C with 5% CO₂ using RPMI-1640 (Himedia®, India) media containing fetal bovine serum. U-937 (2×10⁴ cells per well) were seeded in a 96-well plate containing 100 µl of RPMI medium and incubated for 24 h. The cells were then treated with different concentrations of 3-PPA (0–150 µg/ml). After 48 h incubation, 100 µl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5,-diphenyltetrazolium bromide) reagent (Sigma Chemicals, USA) was added to each well, and the plates were incubated in a CO₂ incubator at 37°C for 4 h. Thereafter, the supernatant was removed from each well. Then 100 µl DMSO was added to dissolve the colored formazan crystals produced by the MTT. Subsequently, the optical density was measured at 570 nm using an ELISA reader (Molecular Devices Corp., USA).

In vitro and in vivo antifungal activity of 3-PPA from the strain was studied against Fusarium udum MTCC 2204, the causal agent of Fusarium wilt in Cajanus cajan L. The antifungal efficacy of 3-PPA was also compared with the activity of commercial fungicides such as carbenzadim and tricyclozole. Conidial suspension of F. udum was prepared using the culture grown on potato dextrose agar for 10 days at 30°C (Hwang et al., 2001). The conidial suspensions were mixed with 3-PPA, carbenzadim and tricyclozole to give the concentration of 0, 1, 10, 50, 100, 500 and 1000 µg/ml. After incubation for 4 h at 28°C, conidial germination was microscopically examined in three replicates.

In vivo antifungal activity of 3-PPA was evaluated for its ability to suppress Fusarium wilt on red gram plants in a growth chamber. Antifungal substances including 3-PPA, carbenzadim and tricyclozole dissolved in water + methanol (95:5) were diluted to give different concentration of 0, 10, 100, 500 and 1000 µg/ml. Seeds of red gram (Cajanus cajan L.) were sown in glass beaker (18–14 cm) containing steam sterilized soil drenched with antifungal solution (30 ml). The soil was drenched with conidial suspension (10⁵ spores/ml) when the seedlings were three day old (Hwang et al., 2001). Disease severity on plants was rated 15 days after inoculation based on a scale from 0 to 5 as follows: 0 for no visible disease symptoms, 1 for slightly wilted leaves, 2 for 30 to 50% of the entire plant diseased, 3 for 50 to 70% of the entire plant diseased, 4 for 70 to 90% of the entire plant diseased and 5 for a dead plant. Data are the mean of 10 plants per treatment and result of two trials.

**Results and Discussion**

Cultural and physiological characteristics of the strain are presented in Table I. The strain showed good growth on ISP-1, ISP-2, ISP-4 and ISP-5 media. Moderate growth was observed on ISP-3, ISP-6, ISP-7 and nutrient agar media. Pigment production by the strain varied with the culture media employed. Dark brown pigment was produced by the strain when grown on ISP-1,2 and 3, while yellowish brown to yellow pigments were found with ISP-4 and 5 and nutrient agar media. Diffused melanoid pigments were observed when grown on ISP-6 and ISP-7. Micromorphology of the strain was examined by SEM (Fig. 1). The culture showed extensively branched aerial mycelium and bear short chains of spores. As the sporogenous hyphae (sporophores) were straight to flexuous in nature bearing the spores with smooth surface, the strain may be placed in the rectus-flexibilis group of Streptomyces species (Pridham et al., 1958).

Fig. 1. Scanning electron microscopic photograph of strain ANU 6277 (magnification x 10,000, Bar 1 µm →)
The strain had the ability to hydrolyze casein, esculin, gelatin, starch, tyrosine and xanthine, but not hypoxanthine. The culture could tolerate NaCl levels up to 7%. It produced enzymes such as amylase and chitinase. It exhibited resistance to chloramphenicol, penicillin-G and streptomycin and showed sensitivity to ampicillin, rifampicin and tetracycline. The utilization of various carbon sources by the strain indicated its wide pattern of carbon assimilation potential. D-arabinose, D-fructose, D-glucose, D-galactose, glycerol, lactose, maltose, mannitol, mannose, raffinose, rhamnose, trehalose and xylose supported growth of the strain, whereas cellulose, dextrin and sucrose did not support its growth.

Phylogenetic study of the strain was performed by 16S rRNA analysis. An almost complete 16S rDNA gene nucleotide sequence (1478 bp) of the strain was identified by BLASTN programme and submitted to Genbank with accession number EF 142856. The strain showed high homology (96% identity) with Streptomyces albidosflavus. Theses findings are in conformity with reports of Williams et al. (1989), Gurtler et al. (1994) and Augustine et al. (2004).

The structure of white crystalline compound obtained from the crude extract after purification was elucidated by FT-IR, EI-MS, 1H NMR and 13C NMR studies. In the FT-IR spectrum, \( \nu_{\text{max}} \) was obtained at 697.96, 931.96/cm (aromatic, C-H), 1218.19/cm (aromatic, C = C), 1301.93/cm (C-O), 1699.90 (C = O), 2928.47, 3030.43/cm (CH3-C-H) and 3390.13 (OH-group broad peak). The compound gave molecular ions in positive mode at \( m/z \) are 150(50), 104(95), 91(100), 78(40) and 51(35) suggested a molecular weight of 150 from EI-MS analysis. NMR data indicated a hydrogen count of 10 and a carbon count of 9 in CD3OD at 300MH Z. 1H NMR showed protons at 2.70 \( ^{\text{t}} \) (t, 2H), 2.90 \( ^{\text{t}} \) (t, 2H), 7.10 to 7.20 \( ^{\text{dd}} \) (aromatic-protons) and 11.0 \( ^{\text{b}} \) (broad, s, O-H,). The \( 13C \) NMR spectrum of bioactive compound exhibited peaks at 30.0 (s, C-2), 36.0 (s, C-3), 126.0,128,129 and 140 (aromatic carbons) and 180.0 (s, C-1). Based on above data, the bioactive compound was characterized as 3-phenylpropionic acid with molecular formula \( C_{9}H_{10}O_{2} \) (Fig. 3).

The bioactive compound, 3-phenylpropionic acid (3-PPA) from strain showed antimicrobial activity against fungi and bacteria.

### Table I
**Characteristics of strain ANU6277**

<table>
<thead>
<tr>
<th>Hydrolysis of</th>
<th>Pigment production in</th>
</tr>
</thead>
<tbody>
<tr>
<td>casein</td>
<td>ISP-1 +, DB</td>
</tr>
<tr>
<td>esculin</td>
<td>ISP-2 +, DB</td>
</tr>
<tr>
<td>gelatin</td>
<td>ISP-3 +, DB</td>
</tr>
<tr>
<td>hypoxanthine</td>
<td>ISP-4 +, YB</td>
</tr>
<tr>
<td>starch</td>
<td>ISP-5 +, YB</td>
</tr>
<tr>
<td>tyrosine</td>
<td>ISP-6 +, M</td>
</tr>
<tr>
<td>xanthine</td>
<td>ISP-7 +, M</td>
</tr>
<tr>
<td>Nitroblue (0.05%)</td>
<td>Nutrient agar medium</td>
</tr>
<tr>
<td>Lysozyme (0.05%)</td>
<td>+, Y</td>
</tr>
<tr>
<td>NaCl (7%)</td>
<td>Czapek-Dox –</td>
</tr>
<tr>
<td>Phenol (0.1%)</td>
<td></td>
</tr>
<tr>
<td><strong>Growth at 45°C</strong></td>
<td></td>
</tr>
<tr>
<td>Melanoid pigments</td>
<td>D-arabinose +</td>
</tr>
<tr>
<td>H2S</td>
<td>cellulose –</td>
</tr>
<tr>
<td>Amylase</td>
<td>D-glucose +</td>
</tr>
<tr>
<td>Chitinase</td>
<td>D-fructose +</td>
</tr>
<tr>
<td><strong>Production of</strong></td>
<td>D-galactose +</td>
</tr>
<tr>
<td>Melanoid pigments</td>
<td>Glycerol +</td>
</tr>
<tr>
<td>H2S</td>
<td>Lactose +</td>
</tr>
<tr>
<td>Amylase</td>
<td>Mannitol +</td>
</tr>
<tr>
<td>Chitinase</td>
<td>D-mannose +</td>
</tr>
<tr>
<td><strong>Resistance to antibiotics (µg/disc)</strong></td>
<td>Raffinose +</td>
</tr>
<tr>
<td>Ampicillin (50)</td>
<td>Rhamnose +</td>
</tr>
<tr>
<td>Chloramphenicol (50)</td>
<td>Sucrose –</td>
</tr>
<tr>
<td>Penicillin-G (50)</td>
<td>Trehalose +</td>
</tr>
<tr>
<td>Rifampicin (50)</td>
<td>Xylose +</td>
</tr>
<tr>
<td>Streptomycin (100)</td>
<td></td>
</tr>
<tr>
<td>Tetracycline (100)</td>
<td></td>
</tr>
</tbody>
</table>

+, Positive result; –, Negative result; DB, Dark Brown; YB, Yellowish Brown; M, Melanin; Y, Yellow

The strain had the ability to hydrolyze casein, esculin, gelatin, starch, tyrosine and xanthine, but not hypoxanthine. The culture could tolerate NaCl levels up to 7%. It produced enzymes such as amylase and chitinase. It exhibited resistance to chloramphenicol, penicillin-G and streptomycin and showed sensitivity to ampicillin, rifampicin and tetracycline. The utilization of various carbon sources by the strain indicated its wide pattern of carbon assimilation potential. D-arabinose, D-fructose, D-glucose, D-galactose, glycerol, lactose, maltose, mannitol, mannose, raffinose, rhamnose, trehalose and xylose supported growth of the strain, whereas cellulose, dextrin and sucrose did not support its growth.
Phenylpropionic acid from terrestrial *Streptomyces* 3

*Streptomyces* *aeruginosa* and *P. fluorescens* are highly susceptible to 3-PPA followed by *B. subtilis*, *Escherichia coli* and *Proteus vulgaris*. Among fungi, *F. udum* exhibited high sensitivity followed by *Aspergillus flavus*, *Penicillium citrinum* and *A. niger*. The bioactive compound (3-PPA) from *S. albidoflavus* strain ANU 6277 did not exhibit significant cytotoxicity on U-937 cells up to the concentration of 100 µg/ml. The compound 3-PPA showed inhibitory activity (IC50) on U-937 cell growth at 128.20 µg/ml, while the widely used anti-cancer drug, Etoposide (positive control) exhibited cytotoxicity activity (IC50) on U-937 cells at 10.26 µg/ml (Table III).

![Molecular structure of 3-phenylpropionic acid](image)

**Fig. 3.** Molecular structure of 3-phenylpropionic acid against different test microorganisms including bacteria and fungi. The minimum inhibitory concentration (MIC) of 3-PPA ranged between 10 and 100 µg/ml (Table II). Among the test bacteria, *Pseudomonas aeruginosa* and *P. fluorescens* are highly susceptible to 3-PPA followed by *B. subtilis*, *Escherichia coli* and *Proteus vulgaris*. Among fungi, *F. udum* exhibited high sensitivity followed by *Aspergillus flavus*, *Penicillium citrinum* and *A. niger*. The bioactive compound (3-PPA) from *S. albidoflavus* strain ANU 6277 did not exhibit significant cytotoxicity on U-937 cells up to the concentration of 100 µg/ml. The compound 3-PPA showed inhibitory activity (IC50) on U-937 cell growth at 128.20 µg/ml, while the widely used anti-cancer drug, Etoposide (positive control) exhibited cytotoxicity activity (IC50) on U-937 cells at 10.26 µg/ml (Table III).

**Table II**

<table>
<thead>
<tr>
<th>Tested microorganism</th>
<th>MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacteria:</strong></td>
<td></td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
<td>75</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>50</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>50</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>100</td>
</tr>
<tr>
<td><em>Proteus vulgaris</em></td>
<td>50</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>10</td>
</tr>
<tr>
<td><em>P. fluorescens</em></td>
<td>10</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>100</td>
</tr>
<tr>
<td><strong>Fungi:</strong></td>
<td></td>
</tr>
<tr>
<td><em>Aspergillus flavus</em></td>
<td>25</td>
</tr>
<tr>
<td><em>A. niger</em></td>
<td>50</td>
</tr>
<tr>
<td><em>Fusarium oxysporum</em></td>
<td>50</td>
</tr>
<tr>
<td><em>F. udum</em></td>
<td>10</td>
</tr>
<tr>
<td><em>Penicillium citrinum</em></td>
<td>25</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>100</td>
</tr>
</tbody>
</table>

**Table III**

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-PPA</td>
<td>128.20</td>
</tr>
<tr>
<td><em>Etoposide</em></td>
<td>10.26</td>
</tr>
</tbody>
</table>

* Positive control

In *in vitro* conditions, conidial germination of *F. udum* was totally inhibited with carbendazim at 50 µg/ml, with 3-PPA at 100 µg/ml and tricyclozole at 500 µg/ml (Fig. 4). *In vivo* efficacy of 3-PPA, carbendazim and tricyclozole for the control of *Fusarium* wilt was evaluated (Fig. 5). The symptoms of wilt began to appear on red gram plants one week after inoculation. Initial symptoms of the disease consist of
wilting of individual branches. The foliage symptoms are characterized by drooping of the leaves followed by upland curling. Treatment with the antifungal substances, 3-PPA and carbendazim greatly inhibited the wilt in red gram plants. The suppressive effect of 3-PPA against *Fusarium* was observed at 500 µg/ml. In contrast, the commercial fungicide carbendazim completely inhibited the development of *Fusarium* wilt at the concentration of 100 µg/ml, while the treatment with tricyclozole showed maximum antifungal activity at 1000 µg/ml. The efficacy of 3-PPA against *Fusarium* wilt was better than tricyclozole but less effective than carbendazim.

The bioactive compound, 3-PPA from the strain exhibited less cytotoxicity, while carbendazim the synthetic fungicide showed high toxicity to humans, animals and plants (Mantovani *et al*., 1998). Soil bacteria such as *Achromobacter, Nocardia* and some *Pseudomonas* species could degrade 3-PPA (Fu and Oriel, 1999) indicating its susceptibility to the microbial degradation in soil what results in the lack its accumulatin in nature like synthetic fungicides. Hence 3-PPA can be preferred over carbendazim to control *Fusarium* wilt as an eco-friendly compound.

Among *Streptomyces* spp., *S. albidoflavus* is one of the potential species that elaborate number of industrially and agriculturally important metabolites. Enzymes like chitinase and serine proteinases are reported from *S. albidoflavus* (Broadway *et al*., 1995; Bressollier *et al*., 1999). An odoriferous actinomycete, *S. albidoflavus* strain DSM 5415 was reported to produce a new sesquiterpene, albaflavenone (Gurtler and Pedersen, 1994). Antimicrobial properties of a non-polyene antibiotic (poly-hydroxy-poly ether compound) have been reported from *S. albidoflavus* strain PU 23 (Augustine *et al*., 2005). The bioactive compound dibutyl phthalate was reported from *S. albidoflavus* strain 321.2 (Roy *et al*., 2006). In the present study, the strain ANU 6277 was found to elaborate a bioactive compound, 3-phenylpropionic acid (3-PPA). This is the first report of 3-PPA from actinomycetes especially *Streptomyces* spp.

Phenyl acids like phenylacetic acid from *Streptomyces humidus* are known to possess antimicrobial activity against several bacteria and fungi (Hwang *et al*., 2001). Phenylpropionic acid was reported to be detected in culture filtrates from media after inoculation with isolated rumen bacteria or rumen fluid in the absence of added phenolic acids (Chesson *et al*., 1982). *Clostridium bifermentans* strain TYR-6 reported from oil mill waste waters could convert cinnamic acid to 3-phenylpropionic acid (Chamkha *et al*., 2001). Phenylpropionic acid derivatives are pharmaceutically important agents. Anti-inflammatory and analgesic drugs like isoprofen, ketoprofen, naproxen *etc* are phenylpropionic acid based drugs (Saisho and Ishibashi, 1998). Nagano *et al*. (2001) reported pyloricidin, a novel anti-*Helicobacter pylori* antibiotic produced by *Bacillus* sp. Phenylpropionic acid moiety of pyloricidin is essential for anti-*H. pylori* activity. The present paper investigated the extraction, physico-chemical properties and biological activities of 3-PPA from *Streptomyces* strain ANU 6277. The bioactive compound, 3-PPA from strain ANU 6277 is a promising compound as it exhibited antimicrobial activity against gram-positive as well as gram-negative bacteria and fungi. It can also be useful as biocontrol agent against *Fusarium* wilt.

Acknowledgement

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Literature


**Introduction**

_Samanea saman_ is a fast growing woody mimosoid legume that is cultivated in many parts of Pakistan in farmlands and along roadsides. It provides shade and fuel wood. Its wood is also used for making bowls, trays, furniture _etc_. The tree forms nitrogen-fixing nodules with rhizobia (Allen and Allen, 1981). The indigenous woody legumes and their root nodule bacteria play an important role in the overall nitrogen increment of Pakistani soils (Mahmood, 1999). A diverse group of Gram-negative nodule forming bacteria namely _Rhizobium_, _Bradyrhizobium_, _Allorhizobium_, _Azorhizobium_, _Mesorhizobium_, _Sinorhizobium_ and _Methyllobacterium_ have been recognized. They are the members of the α and β subgroup of the phylum _Proteobacteria_ (Chen _et al_. 2003), collectively known as rhizobia (Amarger, 2001; Vessey _et al_., 2004). The fixed nitrogen is used by _S. saman_ for its growth and enrichment of the rhizosphere. The process of nodule formation is closely related to the infection of roots by appropriate rhizobia. Rhizobia enter the root via root hairs in majority of legumes (Iqbal and Mahmood, 1992; Qadri and Mahmood 2003, 2004, 2005).

**Experimental**

**Materials and Methods**

**Material collection and preparation for microscopy.** Nodules of _S. saman_ were collected from roots of trees growing in the garden of the Department of Botany, University of Karachi. For light microscopy, the nodules and roots were fixed in F.A.A. (formaline-acetic acid-ethyl alcohol) in the ratio of 5:5:90 for
18 hours. Pieces of nodules (1–2 mm) were dehydrated in ethanol series and infiltrated with L.R. (London resin) white at room temperature and polymerized at 60°C for 24 hours. Serial sections (0.5–2 mm) were cut with a glass knife using a Sorrasl J.B.-4 ultra microtome and transferred to glass slides in a large drop of water. The sections were dried on a hot plate at 40°C, stained with aqueous toluidine blue (in 1.0% borax, pH 4.4) and mounted in Canada balsam (Faria et al., 1986). They were than examined under a Zeiss student microscope.

**Transmission electron microscopy assay.** For transmission electron microscopy (TEM), small pieces of nodules (1–2 mm) were fixed in 2% gluteraldehyde in 0.1 M phosphate buffer (pH 7) for 4 hours, washed with three changes of buffer solution during three hours and transferred to 1% aqueous osmium tetroxide for 2–4 hours at room temperature. The fixed material was processed for transmission electron microscopy as described by Qadri and Mahmood (2003).

**Scanning microscopy assay.** Complete nodules and free hand sections of nodules were fixed for scanning electron microscopy as for TEM. They were dehydrated in 100% ethanol followed by an ethanol/acetone mixtures up to 100% acetone (Faria et al., 1986). The specimens were then dried using a Polaron critical point drier (BIO-RAD), coated with gold and free hand sections of nodules were fixed for scanning microscopy as for TEM. They were then examined under a Zeiss student microscope.

**Results and Discussion**

Nodules of *S. saman* were distributed on the main as well as lateral roots and occurred singly and in clusters (Fig. 1A). Although root hair curling was observed, infection threads could not be seen (Fig. 1B). Faria et al. (1987a, b) have surveyed the occurrence of infection threads in the three sub-families of legumes namely Caesalpinoideae, Mimosoideae and Papilionoideae. According to their survey, infection in members of Mimosoideae occurs by the movement of rhizobia intercellularly rather than by infection threads. Similar observations have been reported by Dart (1977), Chandler et al. (1982) and Calvert et al. (1984). The bacteria entered the ruptured epidermis of the root, from where they spread intercellularly into the cortical region (Fig. 1C). Continuous proliferation of rhizobia in host cells resulted in the formation of well organized indeterminate nodules (Fig. 1D).

The general structure of the nodules of *S. saman* shared similarities with the majority of leguminous plants in having a nodule meristem (M), nodule cortex (NC), bacteroid region (B) and vascular supply (VS) (Fig. 1D). The nodule meristem was comprised of numerous small compact cells. This is the region of active nuclear division. Normally these cells contain neither infection threads nor rhizobia. The meristematic region persisted throughout nodule development. The nodule cortex was comprised of 4–10 layers of non-infected parenchyma, isodiametric in shape. Cortical cells are derived by division of cells of the meristematic zone. Tannins were found scattered throughout the cortical region as idioblasts (Fig. 1D). Bacteroid region occupied the central part of the nodule. The bacteroid tissues of the nodules showed both infected (IN) and uninfected (UN) cells mixed together (Fig. 1E). Similar observations have been made for *Sesbania grandiflora* (Harris et al., 1949), *Cajanus indicus* (Arora, 1956), *Cyamopsis tetragonoloba* (Narayana, 1963), *Glycine max* (Bergersen and Goodchild, 1973), *Trifolium alexandrium* (Naz and Mahmood, 1976), *Albizia spp.* (Dart, 1977), *Sesbania sesban* (Mahmood and Jamal, 1977), *Parasponia andersonii* (Trinick, 1979), *Phaseolus vulgaris* (Baird and Webster, 1982), *Leucaena leucocephala* (Iqbal and Mahmood, 1992), *Dalbergia sissoo* (Qadri and Mahmood, 2002, 2004), *Albizia lebbeck* (Qadri and Mahmood, 2005) and *Pithecellobium dulce* (unpublished). A group of bacteria were enclosed in a common peribacteroid membrane (Fig. 2B). The bacteria contained prominent granules of polyhydroxybutyrate (PHB) (Fig. 2B). Both oval and rod shaped bacteria were observed (Fig. 2C). Enclosure of a group of bacteria in a common peribacteroid membrane is a distinctive feature of infected cells in leguminous root nodules as reported by a number of investigators (Newcomb, 1976; Lawrie, 1983; Chalifour and Benhamou, 1988; Qadri et al., 2006). The peribacteroid membrane is derived initially from the host plasma membrane and is a plant product. The peribacteroid membrane become lost at certain points and bacteria are released into the cytoplasm of the cell from these sites (Fig. 2C). The liberated or free bacteria are always surrounded by a peribacteroid membrane which is derived from the bulges of the plasma membrane surrounding a group of bacteria (Fig. 2C) as described by Newcomb (1976).

The vascular differentiation of the nodule is discernible within a week after nodule initiation. Literature on the subject has been reviewed by Bond (1948), Naz and Mahmood (1976) and Baird et al. (1985). The first indication of formation of conducting tissue becomes evident in the form of a few cortical cells that start dividing parallel to the radius of the root forming the procambial strands. Very soon these strands get connected with the protoxylem points of the vascular cylinder of the parent root. The vascular supply may consist of one to four vascular strands (Bond, 1948). In *S. saman* two vascular strands were seen making connection with the vascular supply of the main root (Fig. 1D). Two vascular strands have been
Fig. 1. A: Distribution of nodules on roots of *S. saman*.
B: Light photomicrograph of *S. saman* root, showing curled root hair (RH) (magnification × 400).
C: Infection taking place through ruptured epidermis (E) Rhizobia move intercellularly. Patches of bacterial mass (BM) can be seen in the root cortex (magnification × 200).
D: Light photo micrograph of longitudinal section of *S. saman* nodule showing deeply situated meristem (M) central bacteriod region, B, and nodule cortex (NC). There is a heavy deposition of tannins (T) in the nodule tissue. Vascular tissue of the parent root VS(R), making connection with the vascular supply of nodule VS (N) (magnification × 283).
E: A scanning electron micrograph of root nodule of *S. saman* showing infected (IN), and uninfected (UN) cells, and vascular bundles (VB) in the cortex (magnification × 68).
Fig. 2. A: An enlarged view of inversely collateral vascular bundle of *S. saman* showing xylem (X), phloem (P) and endodermis (EN) (magnification × 3000).

B: Transmission electron micrograph of a portion of bacteroid region (B) showing rhizobia enclosed in a common peribacteroid membrane (PM). They show a high content of polyhydroxybutyrate (PHB) granules (magnification × 6364).

C: Transmission electron micrograph of a portion of bacteroid region (B) of a root nodule cell. Note that rhizobia (R) are enclosed in a common peribacteroid membrane (PM) and some of them are coming out of the membrane at certain points. Both elongated (E) and oval (OV) forms are present (magnification × 15 600).

D: Transmission electron micrograph of a single bacteroid (B) cell of a root nodule showing rhizobia (R) and vesicular-arbuscular mycorrhizae – VAM (VA) (magnification × 2160).

E: Transmission electron micrograph of an enlarged portion of bacteroid cell in Fig. 2-D showing a single hypha (H) along with rhizobia (R) (magnification × 15000).
reported in *Vicia faba* (Bieberdorf, 1938), *Sesbania grandiflora* (Harris et al., 1949), *Pisum sativum* (Bond, 1948), *Melilotus alba*, *Trifolium alexandrinum* (Naz and Mahmood, 1976) and *Pithecellobium dulce*. Once formed, the strands branched repeatedly encircling the central bacteroid region (Fig. 1E). The vascular strands never come in direct contact with the bacteroid tissue. A few layers of parenchyma always separate the vascular tissue from the bacteroid tissue (Fig. 1D). Vascular bundles were inversely collateral (Fig. 2A). The xylem elements faced away from while phloem elements faced towards the center. Inversely collateral bundles have been reported in pea nodules by Bond (1948). The vascular bundles were enclosed by an endodermis (Fig. 2A).

The infected cells of *S. saman* along with rhizobia also contained mycorrhizal hyphae (Figs. 2D and 2E). Vascular-arbuscular mycorrhizae (VAM) have been reported in nodules of some leguminous trees such as *Sesbania grandflora* (Habte and Aziz, 1985), *Acacia mangium*, *Albizia falcata*, (Delia Cruz et al., 1988) and *Leucaena leucocephala* (Young, 1990). The presence of mycorrhizae is known to enhance nodulation and nitrogen fixation by legumes (Amora-Lazecano et al., 1998; Johansson et al., 2004). Mycorrhizal fungi and nitrogen fixing bacteria often act synergistically on infection rate, mineral nutrition and plant growth (Rabie and Almadini, 2005). The beneficial effects of nitrogen-fixing bacteria in combination with mycorrhizal fungus on plant growth have been discussed by Patreze and Cordeiro (2004) and Domenech et al. (2004).

In conclusion it may be said that most of the mycorrhizal research with nitrogen fixing trees has revolved around only a few selected tree species (Aziz and Sylvia, 1992). Studies on VAM interactions with nitrogen-fixing tree species should be conducted on a large scale. Mahmood (1999) has analyzed the nitrogen-fixing potential of indigenous woody legumes and discussed their role in the improvement of denuded and derelict lands of Pakistan. *S. saman* with dual rhizobial and mycorrhizal infection is a potential tree for plantation in Pakistani soils in future afforestation schemes.

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**Literature**


Growth and Antioxidant Activity of *Desulfotomaculum acetoxidans* DSM 771 Cultivated in Acetate or Lactate Containing Media

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**Abstract**

Three independent 28 or 32-day stationary cultures of *Desulfotomaculum acetoxidans* DSM 771 strain were carried out under anoxic conditions in acetate or lactate-containing media. The acids were the sole carbon and energy sources in these media. During cultivation the turbidity (for calculation of cell division index) and hydrogen sulfide contents were determined in culture broth and reduced glutathione and protein concentrations were assayed in culture broth supernatant. In these three successive cultures, the bacterium initially grew much faster on lactate than on acetate. However, after two weeks of culture this difference disappeared and in fact the growth rate was higher on acetate than on lactate. The level of H$_2$S formed (product of the dissimilatory pathway of sulfate reduction) demonstrated that this pathway was more effective when lactate was a carbon source and the average H$_2$S concentration was from over 3-fold to about 9-fold greater in lactate than in acetate cultures. Also GSH (glutathione, product of the assimilatory sulfate reduction pathway) average level was about 2-fold higher in lactate-grown cultures. The high negative values of the correlation coefficients between GSH and O$_2$ levels, especially during the first 4 days of cultivation, indicate that GSH is a very important antioxidizing extracellular agent of *D. acetoxidans*. The rapid increase in GSH level, preceding the release of H$_2$S, indicates the metabolic priority of the assimilation pathway of sulfate reduction. For both carbon sources the highest coefficient of correlation was found between protein and H$_2$S levels. These results suggest that hydrogen sulfide is bound by proteins (which contain cysteiny1 residues) secreted by *D. acetoxidans* cells. Indicated way of H$_2$S binding could result in its accumulation. This coefficient of correlation increased gradually in the successive cultures. The ratio of H$_2$S concentration to protein concentration increased gradually in the successive cultures, too.

**Key words:** growth of *D. acetoxidans* on acetate and on lactate, antioxidant activity

**Introduction**

The sulfate-reducing bacteria (SRB – group 7) are capable not only of assimilative sulfate reduction but also of dissimilatory reduction of sulfate or sulfur. The dissimilatory pathway is the source of energy for SRB. The reductive character of metabolism, especially of the dissimilatory pathway requires strictly anaerobic conditions for SRB growth. These pathways are coupled to the utilization of hydrocarbon derivatives; lactate is a very good substrate for most SRB. However, Widdel and Pfennig (1981) postulated that the Gram-positive strains of *Desulfotomaculum acetoxidans* never utilized lactate as an electron donor and sporulated only when acetate was the organic substrate. Consequently, Campbell and Singleton (in Bergey’s Manual of Systematic Bacteriology) described this species as growing on media with acetate, but not with lactate (Campbell and Singleton, 1986).

In contrast, we found that *D. acetoxidans* DSM 771 consumed lactate, too (Pado and Pawłowska-Ćwięk, 2004). Because the ability of this species to grow on medium with lactate remains controversial (Holt et al., 1994), we have attempted to thoroughly investigate the growth of *D. acetoxidans* DSM 771 on acetate and on lactate. We also determined the effects of these carbon sources on the antioxidative activity of this bacterium.

**Experimental**

**Material and Methods**

**Material.** *Desulfotomaculum acetoxidans* strain DSM 771 was grown at room temperature (19–23°C). The primary inoculum was 1 ml active culture from Deutsche Sammlung von Mikroorganismen. Half volume of the inoculum was used immediately
to inoculate 50 ml of medium with 42 mM acetate and the other half to inoculate 50 ml of medium with 42 mM lactate. After 3 weeks both cultures were supplemented with 50 ml of the respective fresh medium and further kept in the dark at room temperature. After the next 3 weeks these cultures broths were used as the inocula (45 ml) for the first 32-day culture (culture I). Each inoculum was supplemented with the respective fresh medium 3 weeks before the next culture (culture II – strictly on completion of the first cultures and culture III – six months after the first culture was completed). All the cultures were conducted in parallel for acetate or lactate-supplemented media in 500 ml Erlenmeyer flasks each containing 450 ml of the culture medium. The lactate concentration was determined on the basis of the results of earlier cultures (Pado and Pawłowska-Ćwięk, 2004). In the first series of cultures, also the culture in lactate-supplemented medium but inoculated with the acetate-containing inoculum was executed. After fixing oxygen detectors and inoculation, the media were immediately covered with a liquid paraffin layer (about 5 mm thick), which was maintained throughout the culture. This paraffin layer made easier monitoring, particularly of oxygen level in the culture, without the risk of the culture being exposed to air.

Other medium components were as follows: 21.12 mM Na₂SO₄, 1.15 mM KH₂PO₄, 4.02 mM KCl, 5.61 mM NH₄Cl, 1.97 mM MgCl₂, 85.55 mM NaCl and trace elements (according to DSM-bank instruction) (Pado and Pawłowska-Ćwięk, 2004, Pawłowska-Ćwięk and Pado, 2005).

Growth. The classic Monod’s method (Monod, 1949) of graphical representation of bacterial growth in continuous cultures (bacterial growth curve) consists in plotting the number of living cells in 1 ml of culture broth as a function of cultivation time but this assay is very time-consuming. Therefore it is frequently replaced by a simpler method, based on culture turbidity measurements (nephelometry) used for cell number evaluation (Gottschal, 1992). On the basis of the results of our earlier study the high correlation coefficient (0.6295) between the Monod’s and nephelometry methods was found. The culture turbidity measurements facilitated much faster determination of the cell division index (CDI). The turbidity of culture broth was measured throughout the cultures (as shown in Table I) at 580 nm using a Specol 11 colorimeter with a TK attachment (Carl Zeiss Jena). Each result in the table is an arithmetic mean from five measurements with standard deviation ranging from 4 to 11%. Prior to sampling the flasks were gently manually agitated for 10 min. On the basis of turbidity values the cell division index (CDI) was calculated:

CDI = \frac{\tau_x}{\tau_0}

where: \( \tau_0 \) – turbidity at \( \tau_0 \) (CDI on the day of inoculation is 1.00), \( \tau_x \) – turbidity in successive days of the culture (\( \tau_x \)).

Chemical analysis. Proteins and reduced glutathione contents were assayed (without using any cell membrane disrupting agents) in culture broth supernatant after centrifugation at 6000×g for 15 min. The amount of proteins was estimated by the Lowry’s method (Lowry et al., 1951). Reduced glutathione concentration was determined by the method described by Akerboom and Sies (1981). The concentration of hydrogen sulfide was measured in culture broth by the methylene blue method of Fago and Popowsky (1949) but the samples with reagents were left overnight.

The colorimetric analyses and spectrum scanning were performed using the CECIL 8020 spectrophotometer. The standard curves obtained for known concent-

### Table I

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ª – inoculation day; ª – standard deviation [%]
Antioxidant activity of *D. acetooxidans* DSM7713
tations of respective standards. All the reagents (including standards: albumin for protein, Na₂S for hydrogen sulfide and GSH) were of analytical grade from Merck or Fluka. The results of triplicate assays are presented as arithmetic mean ± the standard deviation (the latter was similar for protein, GSH and H₂S) (Table I).

The oxygen level in culture broth was measured before mixing the cultures to avoid an error caused by air diffusion (the differences of oxygen concentration before and after mixing were 2.6–4.3 µM O₂). Because between the measurements, the cultures were kept without mixing, these measurements were made very gently for five different positions of the culture CTN-980 R oxygen detector (ELSENT Poland) coupled to a CX-315 microcomputer pH/oxygenmeter (ELMETRON Poland).

**Results and Discussion**

**Cell division index.** The obtained values of CDI revealed that the bacterium grew faster on lactate (about 2-fold higher turbidity) than on acetate within the first two weeks of the culture (Fig. 1). After this time, in the first and the third cultures, the CDI was slightly higher on acetate. After 24 days CDI was again higher (about 3-fold) for the lactate containing culture medium (in culture III). However, the culture on lactate medium but inoculated with acetate inoculum showed increase of neither cell division index nor hydrogen sulfide level. On the other hand, the lack of an increase in CDI values when the lactate medium was inoculated with acetate inoculum indicates that the adaptation process requires a relatively long time for the changeover of metabolic pathways, essential for switching on lactate catabolism. The necessity of a changeover of metabolic pathways was confirmed by an earlier observation regarding the synthesis of different redox proteins in cultures of three *Desulfovibrio* strains grown on hydrogen or lactate (Steger* et al.*, 2002).

The cell division is related to biosynthesis processes, particularly the production of proteins. According to some authors (Hancock and Poxton, 1988; Russel, 1988) “free” wall-associated proteins will continue to be synthesized and will be released directly into the culture supernatant. The obtained average content of determined protein in supernatant was at least 2-fold higher in lactate cultures and in culture I (when the cultures were inoculated with the youngest inoculum) were even 3-fold higher than in acetate cultures (Table I and II). Interestingly, as the inoculum grew older the average protein level was progressively reduced in both cultures, but more in the cultures on lactate. Thus, the above-mentioned decrease in the protein content in cultures inoculated with aged

![Fig. 1. Cell division index during cultivation of cultures on acetate (thin lines) or lactate (thick lines): culture I – solid lines; culture III (six months after the first series was completed) – dashed lines; culture on a medium with lactate but inoculated with an acetate inoculum – pointed line. For more clear illustration culture II (after the first series was completed) is not presented, since it was similar to series I. Student’s t-test values for lactate to acetate culture in successive cultures: I – 1.967 (statistically insignificance where p>0.05); II – 3.969 (0.01 significance level); III – 2.588 (0.05 significance level).](image)
inoculum suggests that the age of the inoculum compromises the capability of cells to synthesis and secretion of proteins. The highest coefficient of correlation between CDI and protein level was found for culture I (Table III). However, this correlation coefficient in culture III (with 7-month-old inoculum) was much higher for the lactate than acetate culture.

Reports on *D. acetoxidans* are rather scarce. This species has not been grown earlier on lactate (Stackebrandt et al., 1997; Hristova et al., 2000; Scholten and Stams, 2000; Boscher et al., 2001; Londry and Des Marais, 2003; Londry et al., 2004), because it was commonly believed to be unable to grow on media containing lactate as a sole carbon source (Widdel and Pfenning, 1981; Campbell and Singleton, 1986; Holt et al., 1994). Our experience showed that *D. acetoxidans* DSM 771 was also capable of catabolic utilization of lactate (Pado and Pawłowska-Ćwik, 2004; Pado and Pawłowska-Ćwik, 2005; Pawłowska-Ćwik and Pado, 2005). However, in agreement with earlier findings (Widdel and Pfenning, 1981; Campbell and Singleton, 1986), we did not observe sporulation, even after 80 days of culturing in the presence of lactate (Pado and Pawłowska-Ćwik, 2004).

The obtained relationship between the secreted protein level and CDI is reflected in the correlation coefficient but only in the first series, especially in the acetate culture (Table III). These results are in accordance with the data of Londry and Des Marais (2003) who used $^{13}$C acetate. Those authors proved that *D. acetoxidans* (unlike three other species of SRB) effectively incorporated acetate into biomass via acetyl-CoA. Moreover, they observed that this species was capable of lithotrophic growth using carbonate and gaseous CO$_2$. This lithotrophic growth capability could explain the better growth of *D. acetoxidans* in acetate culture but only after two weeks of the cultivation (in the first and second cultures), when carbonate (including dissolved CO$_2$) accumulated as a consequence of acetate catabolism (Fig. 1).

**Reduced glutathione.** Also the GSH level was higher in cultures with lactate than in those with acetate (Fig. 2–4). As the cultures with lactate produced slightly higher levels of both GSH and H$_2$S it suggests that lactate is more advantageous for the assimilatory and dissimilatory sulfate reduction pathways too. It is known that lactate contains more hydrogen atoms than acetate and this is very important in sulfate reduction processes. Since cell membranes were not disrupted prior to GSH determination, the measured GSH was extracellular. The results obtained in all three cultures (designated I, II and III) indicate that GSH biosynthesis and secretion began immediately after inoculation and in early cultures the GSH level increased more rapidly than that of H$_2$S (Fig. 2–4). The early GSH domination over H$_2$S suggests priority of the assimilatory over the dissimilatory pathway.

The initial sulfate concentration in the media was 21 mM. On the basis of the highest GSH levels (always during the first four days of the cultures), sulfur incorporation from sulfate into GSH was counted: it ranged from 0.35 to 2.55‰ in acetate cultures and from 0.68 to 3.74‰ in lactate cultures. Contrary to

![Fig. 2. Hydrogen sulfide (solid lines) and glutathione (dotted lines) levels within culture I: acetate cultures (thin lines) or lactate cultures (thick lines).](image-url)
Antioxidant activity of *D. acetooxidans* DSM7713

As it is well known, GSH is the major antioxidant agent (both extra- and intracellular) in all live organisms (Poot *et al*., 1995; Deneke 2000; Hand and Honek, 2005). The obtained results (the ratio of GSH and protein concentration) showed that lactate stimulated the production of GSH, so thus increasing the antioxidant activity of the examined strain. These levels of GSH were lower (Table II) as compared to Fareleira *et al*. (2003) (1.8±0.6 nmol GSH per mg protein of *Desulfovibrio gigas* cells). However, we determined the extracellular GSH, while those authors determined the total GSH. In this work, a rapid increase in GSH level was observed at the beginning of our expectation we did not find any correlation between GSH and H₂S levels (Table III). The absence of such correlation can explain different metabolic requirements of bacterial cells during of the cultivation. The observed regular, significant and negative values of correlation coefficients between GSH and oxygen (the more GSH the less oxygen) prove that the reduced glutathione performs the role of an antioxidant, as expected (Table III, compare Fig. 2–4 and 5). The antioxidant role of GSH was especially clear at the beginning of cultivation (the first 4 days), because within this period the greatest decreased in oxygen level was observed (by about 100 µM).

![Fig. 3. Hydrogen sulfide (solid lines) and glutathione (dotted lines) levels within culture II: acetate cultures (thin lines) or lactate cultures (thick lines).](image1)

![Fig. 4. Hydrogen sulfide (solid lines) and glutathione (dotted lines) levels within culture III: acetate cultures (thin lines) or lactate cultures (thick lines).](image2)
the culture (Fig. 2–4). However, Fareleira et al. (2003) did not observe any significant differences when oxygen concentration in the medium or the duration of the oxic period in *D. gigas* cells were increased. This phenomenon may be explained by GSH secretion initiated by O₂ and/or reactive oxygen species present in the fresh medium and taxonomic differences between these strains. Extracellular oxygen-utilizing processes are well known in eucaryotic *Deuteromycotina* (Odière and Artaud, 1992; Leonowicz et al., 2001). This strategy should also apply to anaerobic bacteria because it protects the cells against the penetration of toxic radicals into the cytosol.

**Hydrogen sulfide.** The amounts of H₂S (or sulfide) were much greater in cultures grown on lactate than on acetate (Table II): in the first culture about 10-fold higher (during the first week of cultivation – Fig. 2); in the second culture about 30-fold higher (during the first week – Fig. 3); and in the third culture over 45-fold higher (during the first week of cultivation – Fig. 4).

The absorption spectra of 2-days samples (in the first culture) after addition of methylene blue method’s reagents showed the presence of peaks at 411.6 and 665 nm (which is characteristic for product formed in this assay) only for the lactate culture (Fig 5A). This absorption maximum was characteristic for complex ferrous ions and 4-hydroxy-3-sulfobenzoate and this ligand was product of 4-hydroxybenzoate sulfonation. The 4-hydroxy-3-sulfobenzoate as extracellular metabolite was requisite for sulfate transport processes in this strain (Pawłowska-Ćwik and Pado, 2005). The obtained results suggest that lactate was more efficiently for sulfate transport processes than acetate in *D. acetoxidans*. The more efficiency of lactate requires further research. The incorporated sulfur index was counted: in lactate cultures it ranged from 4.66 to 6.07‰, while in acetate ones from 0.83 to 1.45‰ (Table II). Kaplan and Rittenberg (1964) observed sulfur isotope fractionation for *D. desulfuricans* increasing in the order lactate, acetate and ethanol. A correlation between sulfate reduction rates and fractionation was also confirmed by continuous culture experiments (Chambers et al., 1975). Detmers et al. (2001) examined 32 species of SRB and found that all incomplete-lactate-oxidizing sulfate reducers fractionated 2.0–17‰ of an isotope of sulfur (34S), whereas all examined acetate-oxidizing species fractionated 18.0–22.0‰.

The present results show that *D. acetoxidans* is capable not only of complete acetate oxidation, but it can also utilize lactate. The incomplete oxidation of lactate to acetate by sulfate yields 3-fold more energy than the complete oxidation of acetate to CO₂ (Londry and Des Marais, 2003). So probably the examined strain in the first stage metabolizes lactate to acetate but produces less hydrogen sulfide, and in the next stage it oxidizes acetate generating more H₂S (Fig. 2–4), as follows:

\[
SO_4^{2-} + 2 \text{CH}_3\text{CHOH} + 2 \text{HCO}_3^- \rightarrow 2 \text{acetylCoA} + 2 \text{H}_2\text{S} + 4 \text{CO}_2
\]

**Table II**

<table>
<thead>
<tr>
<th>Series</th>
<th>Protein Lactate</th>
<th>Protein Acetate</th>
<th>A</th>
<th>H₂S Lactate</th>
<th>H₂S Acetate</th>
<th>A</th>
<th>GSH Lactate</th>
<th>GSH Acetate</th>
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<td>94.73</td>
<td>3.08</td>
<td>4.480</td>
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<td>9.13</td>
<td>27.738</td>
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<td>1.92</td>
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<td>58.97</td>
<td>2.26</td>
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<td>3.61</td>
<td>7.719</td>
<td>13.249</td>
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<td>0.146/b</td>
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<td>0.387/b</td>
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<td>0.296/a</td>
<td>0.225/a</td>
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<td>(0.02)</td>
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<td>10.040</td>
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<tr>
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<td>0.236/b</td>
<td>0.837/b</td>
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<td>0.836/b</td>
<td>2.050</td>
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<td>0.586/a</td>
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<td>(0.001)</td>
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<td>(0.01)</td>
<td>(0.01)</td>
<td></td>
<td>(0.15)</td>
<td>(0.02)</td>
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</tr>
</tbody>
</table>

A – the ratio of protein or H₂S or GSH average concentration in lactate culture to in acetate culture; bold values – Student’s t-test for lactate to acetate culture; ( ) – significance level; nss – statistically insignificance (p>0.05); /a – decrease relative to previous series (%); /b – the ratio of H₂S and protein concentration (nmol/mg protein); /c – sulfur incorporation from sulfate (initial concentration 21 mM) into hydrogen sulfide (on final cultivation day) (%); /d – the ratio of GSH and protein concentration [nmol/mg protein]; /e – sulfur incorporation from sulfate (21 mM) into glutathione (in early exponential phase) (%).
Although the average H$_2$S level decreased in consecutive cultures, this decrease was smaller in the cultures with lactate than acetate (Table II). The calculated correlation coefficients show that H$_2$S amounts correspond to levels of determined proteins (Table III). Despite the levels of both proteins and hydrogen sulfide were reduced in the next culture, but their correlation coefficients increased in the successive cultures, both with acetate and lactate. In lactate culture, in the third culture, was found higher H$_2$S level in samples from immediately inoculated culture than on the next day (in contrast to other culture—compare Fig. 2, 3 and 4). The higher H$_2$S amount in samples from freshly inoculated lactate culture proves extracellular accumulation of hydrogen sulfide. Moreover, the average amount of H$_2$S per mg of protein increased in subsequent series, but only in lactate cultures (Table II). These results suggest that extracellular

Fig. 5. Absorption spectra of culture samples: 1 – acetate culture; 2 – lactate culture after 2 days of cultivation (A) and 17 days (lactate culture) or 32 days (acetate culture) of cultivation (B) with methylene blue method’s reagents.

<table>
<thead>
<tr>
<th>Series Culture</th>
<th>CDI vs. Protein</th>
<th>CDI vs. H$_2$S</th>
<th>GSH vs. CDI</th>
<th>GSH vs. Protein</th>
<th>GSH vs. O$_2$</th>
<th>GSH vs. H$_2$S</th>
<th>H$_2$S vs. Protein</th>
<th>H$_2$S vs. O$_2$</th>
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</thead>
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<td>I acetate</td>
<td>0.6772$^{a}$</td>
<td>0.2647</td>
<td>-0.2760</td>
<td>-0.5801</td>
<td>-0.4386$^{b}$</td>
<td>-0.6049</td>
<td>0.1223$^{b}$</td>
<td>-0.1394$^{b}$</td>
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<td></td>
<td>0.7105$^{b}$</td>
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<td></td>
<td></td>
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<td></td>
<td>0.1278$^{b}$</td>
<td>0.5968$^{b}$</td>
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<td>I lactate</td>
<td>0.2033$^{a}$</td>
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<td>0.8451</td>
<td>0.3093</td>
<td>-0.5916$^{b}$</td>
<td>0.0382</td>
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<td></td>
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<td>0.1295$^{b}$</td>
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<tr>
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<td>0.7549</td>
<td>-0.1341$^{b}$</td>
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<td></td>
<td>-0.7690$^{b}$</td>
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<td>-0.5587$^{a}$</td>
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<td>0.4016$^{b}$</td>
<td>0.8278$^{a}$</td>
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<tr>
<td>II lactate</td>
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<td>-0.6095</td>
<td>-0.6221</td>
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<td>-0.2531$^{b}$</td>
<td>0.7020</td>
<td>0.5886$^{a}$</td>
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<td>-0.7415$^{b}$</td>
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<td>-0.6826$^{b}$</td>
<td></td>
<td>0.6046$^{a}$</td>
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<td>III acetate</td>
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<td>0.7875$^{a}$</td>
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<tr>
<td>III lactate</td>
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<td>0.9357$^{a}$</td>
<td>0.2519$^{a}$</td>
</tr>
</tbody>
</table>

CDI – cell division index; $^{a}$ – whole period of cultivation; $^{b}$ – from the fourth day to the end; $^c$ – for the first 4 days of cultivation; $^d$ – from inoculation day to 21-th day; $^e$ – from the second day to the end of cultivation.
proteins bound dissimilated H$_2$S. Our earlier research showed H$_2$S accumulation by proteins linked to the cell wall this strain, because hydrogen sulfide levels were much higher in the lysozyme-treated samples than in the untreated samples (Pado and Pawłowska-Ćwičk, 2004). Hydrogen sulfide can be bound by cysteinyl residues of proteins forming disulfides, which release the so-called labile sulfur in acidic environment (Ogasawara et al., 1994). However, the results obtained in this work show a decrease of the amount of extracellular proteins with the age of used inoculum, but may be, the copies number of protein containing of Cys residues increased (e.g. proteins including in the transport processes). According to Russell (1988), these proteins could be associated with the cell wall, but not covalently linked.

Surprising was the fact that, contrary to expectation, O$_2$ did not decrease H$_2$S level; in acetate cultures, in the first and the second series oxygen presence was even advantageous, especially from the second day until the end of cultivation (compare Fig. 2–4 and 6). Also the high values of correlation coefficients for these cultures (Table III) show that oxygen could even be a positive factor for the dissimilatory sulfate reduction pathway (e.g. through the influence on sulfate transport processes) (Pawłowska-Ćwičk and Pado, 2005). Also Johnson et al. (1997) found a positive influence of oxygen (48 μM) on the growth of *Desulfovibrio vulgaris* if 250 μM hydrogen sulfide was added to the medium.

**Conclusion.** Although growth of *D. acetoxidans* DSM 771 on lactate requires at least two successive passages on this medium the species grows better on lactate than on acetate, which is contrary to earlier observations of other researches. *D. acetoxidans* lactate cultures produced higher levels of both GSH and H$_2$S than cultures with acetate, so lactate is a better substrate for metabolic processes, especially the sulfate reduction pathways. The higher level of reduced glutathione in lactate cultures results in the increase of *D. acetoxidans* antioxidant activity, which could be very important for the survival in natural environment.

**Literature**


Chambers L.A., P.A. Trudinger, J.W. Smith and M.S. Burns. 1975. Fractionation of sulfur isotopes by continuous culture of...
Introduction

The aflatoxins are a biologically active polyketide-derived secondary metabolites (Bhatnagar et al., 1992). The aflatoxins are a group of closely related highly oxygenated bisfuranocoumarin heterocyclic compounds (Buchi and Rae, 1969; Ellis et al., 1991). Aflatoxins are produced by some strains of Aspergillus flavus and most, if not all, Aspergillus parasiticus Speare (Smith and Moss, 1985) as well as the closely related species Aspergillus nomius Kurtzman (Kurtzman et al., 1987; Cotty et al., 1994). These aflatoxin-producing species differ in their ability to produce aflatoxins and some are entirely non-toxicogenic (Smith, 1997).

Chemically, aflatoxins are defined as a series of 18 known bisfuranocoumarin compounds that fluoresce strongly in ultraviolet light (Park et al., 2001). There are four naturally occurring aflatoxins B₁, B₂, G₁, and G₂, together with other aflatoxins which occur endogenously as metabolic products of microbial, animal, or human metabolic systems (Smith, 1997).

Aflatoxins can be acutely toxic, carcinogenic, mutagenic, teratogenic, and immunosuppressive to most mammalian species. The rank order of toxicity, carcinogenicity, etc., is AFB₁ > AFG₁ > AFB₂ > AFG₂, implying that the unsaturated terminal furan of AFB₁ is critical for determining the level of biological activity of the aflatoxins (Eaton and Gallagher, 1995; Smith, 1997).

The biological detoxification or the biotransformation or degradation of aflatoxin by microbial systems to a metabolite(s) that is either nontoxic when ingested by animals or less toxic than the original toxin and readily excreted from the body is being studied in several laboratories (Smith and Bol, 1989). As yet, such methods do not constitute a realistic practical approach (Smith, 1997) to the problem. Boller and Schroeder (1973, 1974) reported that A. cheralieri and A. candidus that dominated the mycoflora in rice also showed marked inhibition in aflatoxin production by A. parasiticus Speare, Aspergillus oryzae and Rhizopus nigricans (formerly Rhizopus stolonifer) and have also been reported to inhibit A. parasiticus and aflatoxin production (Christensen et al., 1973; Weckbach and Marth, 1977).

The capabilities of several fungal strains to resist aflatoxins as well as to biotransform and/or biode-
grade these compounds into several other metabolites that are either non- or less toxic than the original have been reported in this paper.

**Experimental**

**Materials and Methods**

**Organisms used.** Several strains of *Aspergillus flavus* group were examined for aflatoxin production capabilities. These cultures were provided by the Regional Center for Mycology and Biotechnology (RCMB) culture collection unit. *A. flavus* RCMB 002002 (strains designated as 1, 2, 3, 4, 5, and 6), *A. flavus* var. *columnaris* RCMB 002003, *A. parasiticus* CMB 002001 (strains: 1 and 2), *A. tamarii* RCMB 002004(2), *A. oryzae* RCMB 002015(1). Several other fungal (provided by the same culture collection) were also examined for their capabilities to grow in the presence of aflatoxins. These strains were: *P. griseofulvum* RCMB 001007(2), *P. urticae* RCMB 001017, *P. italicum* RCMB 001018(1), *P. roquefortii* RCMB 001009(6), *P. nigricans* RCMB 001013, *P. citrinum* RCMB 001011(1), *P. notatum* RCMB 001016, *C. nectriella* sp. RCMB 013001, *Rhizopus nigricans* RCMB 014001, *Mucor rouxii* RCMB 015002, *P. variotii* RCMB 018002, *P. variotii* RCMB 018003, *C. clavata* RCMB 019002, *C. solani* RCMB 031001, *Trichoderma viride* RCMB 017002, *Paecilomyces lilacinus* RCMB 018002, *C. nectriella* sp. RCMB 013001, *Rhizopus nigricans* RCMB 014001, *Mucor rouxii* RCMB 015002, *Syncephalastrum racemosum* RCMB 016001(2), *Trichoderma viride* RCMB 017002, *Paecilomyces lilacinus* RCMB 018002, *P. variotii* RCMB 018003, *Curvularia lunata* RCMB 019002, *C. clavata* RCMB 019003, *Rhizoctonia solani* RCMB 031001, *Torulomyces lagenoides* RCMB 030001, *Acremonium rutihum* RCMB 020002, *Saccharomyces cerevisiae* RCMB 006001 and *Candida utilis* RCMB 005002.

**Preliminary detection of aflatoxin-producing fungi by fluorescent agar technique.** Several strains of *A. flavus* group were screened for their ability to produce aflatoxin(s) on Sabouraud-dextrose yeast extract agar plates, using the fluorescent agar technique of Harra et al. (1974).

**Application of agar plug method.** Seven-day old cultures of *Aspergillus* spp. grown on MEA were examined for aflatoxins production using an agar plug technique (Paterson and Bridge, 1994). An agar plug was cut out with a flamed cork borer (inner diameter ~0.4 cm) from the center of the colony. The plug was removed with a sterile dissecting needle, wetted the mycelial side of the plug with a drop of chloroform/methanol (2:1, v/v) for a few seconds and touched the agar side to a thin layer chromatography (TLC) plate. The agar plug was placed at the origin of the TLC plate (20×20 cm Merck aluminum sheet, silica gel 60, layer thickness 0.2 mm). The diameter of the application spot should not be more than 0.6 cm. After drying the spots, the TLC plate was developed in TEF eluent (toluene/ethyl acetate/90% formic acid, 5:4:1, v/v/v) in a solvent saturated atmosphere using saturation pads. Griseofulvine was used as an external standard. The dry developed TLC plates were viewed in normal white light, under long wave (366 nm), and short wave (254 nm) UV light; then compared with the standards and published data on colors and Rf values according to Paterson and Bridge (1994).

**Production of aflatoxins.** For the production of aflatoxins yeast-extract sucrose (YES) liquid medium was used. For enhancement of aflatoxin production, 1 ml trace element solution was added to 1 liter of YES medium. The trace element solution is prepared by dissolving 0.5 g magnesium sulphate, 0.5 g cupric sulphate and 0.5 g zinc sulphate in 100 ml of distilled water.

**Preparation of spore suspension.** Mould inoculum was prepared by growing *A. parasiticus* on MEA slants for 7–10 days at 25°C until sporulation. The spores were harvested by adding 10 ml of sterile distilled water to the cultures on the surface of the agar slants and gently dislodging spores from conidio- phores with an inoculation loop. The spore suspension was filtered through four layers of sterile cheese-cloth followed by filtration through Whatman No 1 filter paper to remove mycelial debris. Spores were counted using an Improved Neubauer bright line hemocytometer. Appropriate dilutions were made from the stock spore suspension in 0.1% peptone water as the diluent to obtain the desired inoculum’s density of 4×10^2 cells/ml (Ellis et al., 1991).

**Extraction of aflatoxins.** The broth filtrates were mixed with an equal volume of chloroform in a separating funnel. The residue was re-extracted twice for complete extraction. The chloroform extract was defatted with hexane, concentrated in a rotary evaporator and purified using silica gel column chromatography. The column was washed with 3 ml each of hexane, ethyl ether and methylene chloride. The aflatoxins were then eluted with 6 ml chloroform: acetone (9:1, v/v) mixture. The solvent was removed by evaporation on a rotary evaporator. The residues were reconstituted in 1 ml methanol for further chromatographic analyses.

**Analysis of aflatoxins derivative formation.** 50 µl of trifluoroacetic acid (TFA) were added to 200 ml of the methanol solution of toxin extract. The mixture was allowed to react at room temperature for 15 min and then was evaporated to dryness. The residue was dissolved in 2 ml of water:acetoni-tire (75:25, v/v) for HPLC (Frisvad and Thrane, 1993).

**Determination of the fungus ability to grow on aflatoxin B1.** Different fungal strains were examined for their ability to grow on mineral medium amended with three different concentrations of aflatoxin B1 (100, 250 and 500 ppb). The broth mineral medium as given by Atlas (1995) was used.
Inhibition of aflatoxin production by toxigenic strain when co-cultivated with other fungal strains. Tested fungal strains were co-cultivated with an aflatoxigenic producing *A. parasiticus* strain on MEA broth medium. Five Erlenmeyer flask of 250-ml capacity were provided with 50 ml of the medium and inoculated with a spore suspension of *A. parasiticus* as well as the tested strain. The pH of the medium was adjusted to 6.5 with 0.1 N NaOH. The flasks were incubated for two weeks at 25°C. The growth of the fungal strains as well as the aflatoxin production was estimated by HPLC.

Separation of the degradation products. The degradation products of the aflatoxin bioremediation were analyzed using SHIMADZU GC/MS-QP 5050A gas chromatograph-mass spectrometer using CLASS 5000 software. WILEY Mass Spectral Database was used in the identification of the separated peaks.

Results

Preliminary detection of aflatoxin-producing fungi. Four strains of *A. flavus*, two strains of *A. parasiticus* and one strain of *A. flavus* var. *columinaris* exhibited blue green or bluish-green fluorescence surrounding the colonies on the agar medium indicating the possible production of aflatoxins. While *A. oryzae*, *A. tamarind* and *A. flavus* RCMB 002002(6) were not showing any fluorescence.

A confirmative test for aflatoxin production by these strains was performed by TLC analysis. Four strains of *A. flavus* and the two strains of *A. parasiticus* and the strain of *A. flavus* var. *columinaris* that showed positive fluorescence were found to produce one or more spots on the TLC plate. Two strains of *A. parasiticus* produced AFB₁, AFB₂, as well as AFG₁ and AFG₂. Also, a marked increase in aflatoxin concentrations in *A. parasiticus*, strain RCMB 002001(2) culture was observed (Table I). Thus this strain was used for the further studies as aflatoxin-producing strain. The chromatographic analysis of *A. flavus* RCMB 002002(6) indicated that this strain produced no or undetectable quantities of aflatoxins. Consequently, it was considered a non-aflatoxigenic fungus.

Investigation of the ability of selected fungal strains to grow on medium emended with different concentrations of aflatoxin. Twenty three strains were investigated for their ability to grow on mineral medium containing different concentrations of aflatoxin B₁ (Table II). Results indicate that several fungal species including *P. griseofulvum*, *P. urticae*, *P. lilacinus*, *T. viride*, *C. utilis*, *S. cerevisiae* as well as a non-toxigenic strain of *A. flavus* were able to grow on a medium containing different concentrations of aflatoxin B₁; 500 ppb, 250 ppb and 100 ppb. While, *P. roquefortii*, *R. nigricans* (= *Rhizopus stolonifer*), *S. racemosum*, *P. variotii*, *C. clavata*, and *A. fumigatus* were less tolerant to aflatoxin B₁ and showed weak growth. Alternatively, two fungal species; *P. notatum* and *R. solani* were highly sensitive to the aflatoxin B₁. *A. rutilum* showed absence of growth on the media containing aflatoxin B₁. Whereas, *P. notatum*, *R. solani* showed only a weak growth on the lowest (100 ppb) concentration.

<table>
<thead>
<tr>
<th>Table I</th>
<th>Aflatoxin production by different strains of the <em>A. flavus</em> group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strains</td>
<td>Aflatoxin (ppb)</td>
</tr>
<tr>
<td>---------</td>
<td>-----------------</td>
</tr>
<tr>
<td><em>A. flavus</em> RCMB002002(1)</td>
<td>0 0 0 0</td>
</tr>
<tr>
<td><em>A. flavus</em> RCMB002002(2)</td>
<td>0 0 0 0</td>
</tr>
<tr>
<td><em>A. flavus</em> RCMB002002(6)</td>
<td>0 0 0</td>
</tr>
<tr>
<td><em>A. flavus</em> RCMB002002(5)</td>
<td>0 0 0 0</td>
</tr>
<tr>
<td><em>A. parasiticus</em> RCMB002001(1)</td>
<td>+ + + +</td>
</tr>
<tr>
<td><em>A. parasiticus</em> RCMB002001(2)</td>
<td>+ + + +</td>
</tr>
<tr>
<td><em>A. flavus</em> RCMB002003</td>
<td>+ 0 + 0</td>
</tr>
</tbody>
</table>

0 = not detected under the experimental conditions.
+ = detectable growth, ++ = good growth.

<table>
<thead>
<tr>
<th>Table II</th>
<th>Investigation of the ability of fungal strains to grow on aflatoxin B₁ containing media</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organism name</td>
<td>Aflatoxin B₁ concentration (ppb)</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------</td>
</tr>
<tr>
<td><em>P. griseofulvum</em> RCMB001007(2)</td>
<td>++ ++ ++ ++</td>
</tr>
<tr>
<td><em>P. roquefortii</em> RCMB001009(6)</td>
<td>++ ++ ++</td>
</tr>
<tr>
<td><em>P. urticae</em> RCMB001017</td>
<td>++ ++ ++</td>
</tr>
<tr>
<td><em>P. nigricans</em> RCMB001013</td>
<td>++ ++ ++</td>
</tr>
<tr>
<td><em>P. notatum</em> RCMB001016</td>
<td>++ ++ +</td>
</tr>
<tr>
<td><em>P. italicum</em> RCMB001018</td>
<td>++ ++ ++</td>
</tr>
<tr>
<td><em>P. citrinum</em> RCMB001011</td>
<td>++ ++ +</td>
</tr>
<tr>
<td><em>R. nigricans</em> RCMB001014</td>
<td>++ ++ +</td>
</tr>
<tr>
<td><em>Circinella sp.</em> RCMB013001</td>
<td>++ ++ ++ +</td>
</tr>
<tr>
<td><em>S. racemosum</em> RCMB016001</td>
<td>++ ++ +</td>
</tr>
<tr>
<td><em>M. rouxii</em> RCMB015002</td>
<td>++ ++ ++ +</td>
</tr>
<tr>
<td><em>A. rutilum</em> RCMB020002</td>
<td>++ ++ 0 0</td>
</tr>
<tr>
<td><em>P. variotii</em> RCMB018003</td>
<td>++ ++ ++</td>
</tr>
<tr>
<td><em>P. lilacinus</em> RCMB018002</td>
<td>++ ++ ++ ++</td>
</tr>
<tr>
<td><em>C. lunata</em> RCMB019002</td>
<td>++ ++ ++</td>
</tr>
<tr>
<td><em>C. clavata</em> RCMB019003</td>
<td>++ ++ +</td>
</tr>
<tr>
<td><em>T. viride</em> RCMB017002</td>
<td>++ ++ +</td>
</tr>
<tr>
<td><em>T. lagena</em> RCMB030001</td>
<td>++ ++ +</td>
</tr>
<tr>
<td><em>C. utilis</em> RCMB005002</td>
<td>++ ++ ++ ++</td>
</tr>
<tr>
<td><em>S. cerevisiae</em> RCMB006001</td>
<td>++ ++ ++</td>
</tr>
<tr>
<td><em>R. solani</em> RCMB031001</td>
<td>++ + 0</td>
</tr>
<tr>
<td><em>A. fumigatus</em> RCMB020008(2)</td>
<td>++ + 0</td>
</tr>
<tr>
<td><em>A. flavus</em> (atoxicin strain) RCMB020002(6)</td>
<td>++ ++ ++</td>
</tr>
</tbody>
</table>

0 = not detected under the experimental conditions.
+ = detectable growth, ++ = good growth.
Influence of antagonistic activities of *A. parasiticus* against some other fungal strains. Cultivation of *A. parasiticus* in mixed cultures with other fungal strains demonstrated inhibition of the aflatoxins production. The growth of *A. parasiticus* was inhibited by the presence of these fungal strains. *T. viride* was found to be capable of inhibiting the growth of *A. parasiticus*. At the highest level, this was followed by *P. chrysogenum, P. lilacinus* and *P. urticae*. The aflatoxin concentrations were decreased with all strains examined (Table III) and their aflatoxin inhibition percentages are given hereafter in between parenthesis.

The concentration of aflatoxin B₁ was found to decrease reaching a minimum value of 5.32 ppb (75.2%) by *T. viride* compared with 21.5 ppb of the control. This is followed by 7.8 ppb (63.7%), 7.81 ppb (63.6%), 8.1 ppb (62.3%), 8.2 ppb (61.8%), 9.83 ppb (54.3%) and 10 ppb (53.5%) for *S. cerevisiae, P. griseofulvum, C. utilis, P. lilacinus, M. rouxii* and *P. urticae*, respectively.

The concentration of aflatoxin B₂ was found to be decreased and reaching a minimum value of 3.1 ppb (56.2%) by *T. viride* compared with 9.6 ppb of the control. This is followed by 4.7 ppb (51.0%), 4.8 ppb (50.0%), 5.2 ppb (45.8%), 5.9 ppb (38.5%) and 6.1 ppb (36.4%), for *S. cerevisiae, C. utilis, P. griseofulvum, P. lilacinus and P. urticae* respectively.

The concentration of aflatoxin G₁ was also decreased reaching a minimum value of 3.1 ppb (65.1%) by *T. viride* compared with 8.9 ppb of the control. This is followed by 4.1 ppb (53.9%), 4.9 ppb (44.9%), 5.1 ppb (42.6%), 5.4 ppb (39.3%), 5.8 ppb (34.8%), and 6.2 ppb (30.3%) for *P. lilacinus, S. cerevisiae, C. utilis, P. griseofulvum, P. urticae, and R. nigricans*, respectively.

The concentration of aflatoxin G₂ decreased as well reaching a minimum value of 2.2 ppb (57.7%) by *T. viride* compared with 5.2 ppb of the control. The total aflatoxins production were found to decrease reaching a minimum concentration of 14.8 ppb (67.2%) with *T. viride* when compared with 48.2 ppb of the control. This is followed by *P. chrysogenum, P. griseofulvum, S. cerevisiae, C. utilis, P. urticae, R. nigricans* and *M. rouxii*, with total aflatoxin concentrations of 20.8 ppb (53.9%), 21.5 ppb (52.4%), 21.3 ppb (52%), 21.8 ppb (51.7%), 25.3 ppb (44.0%), 27.9 ppb (38.2%) and then 29.2 ppb (35.4%), respectively.

Compounds produced after bioremediation of aflatoxins by *Mucor rouxii*. *M. rouxii* has a unique behavior in the bioremediation of aflotoxins. In spite GC analysis showed four characterized peaks (Fig. 1). The four main peaks have mass peaks; 189, 217, 216 and 228 with molecular formula of $C_{12}H_{20}N_2O_2$, $C_{10}H_{16}O$, $C_{22}H_{46}$ and $C_{11}H_{18}O_2$, respectively. The presence of furan moiety in B and E as well as F indicates the degradation of aflatoxin molecules. The presence of ketone molecule (D) indicated the degradation of aflatoxin molecules. The presence of ketone molecule (D) indicated the degradation of aflatoxin molecules. The presence of ketone molecule (D) indicated the degradation of aflatoxin molecules. The presence of ketone molecule (D) indicated the degradation of aflatoxin molecules. The presence of ketone molecule (D) indicated the degradation of aflatoxin molecules. The presence of ketone molecule (D) indicated the degradation of aflatoxin molecules. The presence of ketone molecule (D) indicated the degradation of aflatoxin molecules. The presence of ketone molecule (D) indicated the degradation of aflatoxin molecules. The presence of ketone molecule (D) indicated the degradation of aflatoxin molecules.

Compounds produced after bioremediation of aflatoxins by *Rhizopus nigricans*. The detected cyclopentane represents the cyclopentenone ring of aflatoxin.

### Table III

<table>
<thead>
<tr>
<th>Organism name</th>
<th>Aflatoxin production (µg/l)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B₁</td>
<td>B₂</td>
</tr>
<tr>
<td>Control (A. parasiticus)</td>
<td>21.5</td>
<td>21.5</td>
</tr>
<tr>
<td><em>P. griseofulvum</em></td>
<td>7.81</td>
<td>5.2</td>
</tr>
<tr>
<td><em>P. roquefortii</em></td>
<td>12.8</td>
<td>7.4</td>
</tr>
<tr>
<td><em>P. urticae</em></td>
<td>10.0</td>
<td>6.1</td>
</tr>
<tr>
<td><em>P. nigricans</em></td>
<td>14.5</td>
<td>7.6</td>
</tr>
<tr>
<td><em>P. notatum</em></td>
<td>11.9</td>
<td>7.2</td>
</tr>
<tr>
<td><em>P. italicum</em></td>
<td>12.6</td>
<td>8.1</td>
</tr>
<tr>
<td><em>R. nigricans</em></td>
<td>10.8</td>
<td>6.9</td>
</tr>
<tr>
<td><em>S. racemosum</em></td>
<td>13.9</td>
<td>8.1</td>
</tr>
<tr>
<td>Cirinella sp.</td>
<td>15.2</td>
<td>8.3</td>
</tr>
<tr>
<td><em>P. lilacinus</em></td>
<td>8.2</td>
<td>5.9</td>
</tr>
<tr>
<td><em>C. lunata</em></td>
<td>13.1</td>
<td>7.8</td>
</tr>
<tr>
<td><em>T. viride</em></td>
<td>5.32</td>
<td>4.2</td>
</tr>
<tr>
<td><em>C. utilis</em></td>
<td>8.1</td>
<td>4.8</td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>7.8</td>
<td>4.7</td>
</tr>
<tr>
<td><em>M. rouxii</em></td>
<td>9.83</td>
<td>6.9</td>
</tr>
</tbody>
</table>
B group after bioremediation by the *R. nigricans*. While the detected benzene and cyclopetane moiety indicates the cleavage of aflatoxin structure, the detection of furan moieties as well as dioctyl phthalate confirming this degradation of aflatoxins. The chromatogram (Fig. 1) also indicated the presence of several fatty acids such as oleic, palmitic, palmitolenic, linolelaaidic and high amount of linoleic acid. Some terpines such as alpha-terpinol, farnesene, jasmon and linalol were also observed.

Compounds produced after bioremediation of aflatoxins by *Penicillium griseofulvum*. The gas
chromatographic analysis of compounds produced after the bioremediation process of aflatoxins by the *P. griseofulvum* was completely different of that of the control. The position of peaks is shifted indicating bioremediation of aflatoxins. Chromatogram shows five characteristic peaks (A-D, F) with several peak fragments. The mass spectrum exhibited 2,6-di-tert-butylphenol with a methyl group at the position 4, this butyl phenol absolutely not included in the chemical structure of the control, *i.e.* aflatoxins B$_1$, B$_2$, G$_1$ and G$_2$. The molecular weight of this compound is 220, with molecular formula C$_{15}$H$_{24}$O. On the other hand, aspergillic acid and oxyaspergillic acid (C$_{12}$H$_{20}$N$_2$O$_2$) were developed, respectively. The mass peaks are 248
and 416, respectively. The furan moiety was cleaved since it appeared as furan or within spiro structure as benzo-uran. Presumably, the appearance of dioctyl phthalate and the dihydro-dimethyl indole indicate the degradation of aflatoxins.

**Compounds produced after bioremediation of aflatoxins by *Penicillium urticae***. The GC analysis of the compounds produced after the treatment of aflatoxins by *P. urticae* is shown in Figure 1. There are several peaks present, but the most typical peaks are C and E. The last peak represents a mannosuranoside of molecular formula C_{12}H_{18}O_{2}. The total molecular weight is approximately 1210 for bioremediated aflatoxins in contrast to 1292 of the control sample. It seems that the *P. urticae* do not bioremediate aflatoxins very well as indicated from the number of hydrogen atoms. The mass peak of the value of 234 of naphthalene with phenyl- and methyl-side chain as well as the butyl hydroxyl toluene also indicate this weak tendency to cleave aflatoxin molecule.

**Compounds produced after bioremediation of aflatoxins by *Paeilomyces lilacinus***. The gas chromatographic analysis of the compounds produced after bioremediation by *P. lilacinus*, indicate the presence of four main characteristic peaks (Fig. 2). Meanwhile the positions of these peaks are completely different from peaks of control sample. The main difference is that the compounds of *P. lilacinus* have a total molecular weight of 1116 and total carbon atoms 66. However, the identification of the peaks in the mass spectra shows the dominance of benzene rings with different side chains. The presence of dioctyl phthalate on one side and the absence of furan moiety as well as coumarin and cyclopentanone on the other hand may indicate the biodegradation of aflatoxins.

**Compounds produced after bioremediation of aflatoxins by *Trichoderma viride***. *T. viride* bioremediated the aflatoxins B and G very extensively; 10 peaks in addition to peak fragments as well as very small peaks as shown in GC analysis were detected (Fig. 2). The positions of these peaks were completely different from peaks in control sample. Aspergillic acid as well as different degradation product exerted such as the diocetylphthalate, methyl jasmonate, buta-barbitol and cyclopentanone were detected. Cyclopentanetione indicate cleavage of cyclopentane ring of aflatoxins. The total molecular weight of the bioremediated aflatoxins was high (1348) in contrast to the control sample (1292), that was attributed to highly fragmented aflatoxins by *T. viride*. Consequently, carbon, hydrogen and oxygen atoms recorded 89, 136 and 9, respectively. Identification of the peaks by mass spectrometer showed the presence of benzene fused with furan moiety (C) as a dominant bioremediation product by *T. viride*. Limonene, jasmonate and other essential oil compounds as well as benzene, 3-methylbutenyl-(with molecular weight 146) and tinuvin were also detected. Furan moiety was detected in two peaks; weight peak 170 (R. time 30.64–31.25) for benzo-furanon and mass peak 221 (R. time 53.49–54.65) with androsanediene.

**Compounds produced after bioremediation of aflatoxins by *Candida utilis***. The chromatogram for bioremediation of aflatoxins by *C. utilis* shows the presence of aspergillic acid, a metabolic product of *A. parasiticus*, which is present in the control sample. The furan moiety was still present but cleaved from the aflatoxin structure since it appeared as a benzo-furan derivative. Tinuvin was present as well. Dioctyl phthalate as a degradation product was detected indicating the biodegradation process of the aflatoxins (Fig. 2).

**Compounds produced after bioremediation of aflatoxins by *Saccharomyces cerevisiae***. The GC analysis of the compounds produced after the treatment of aflatoxins by *S. cerevisiae* is given in Figure 2. Although, there are several peaks present, the most important one is the methoxycomarin. The presence of furan moiety that appeared as furan, 4, 5-diethyl-2, 3-dimethyl as well as the methoxycomarin indicate the cleavage of aflatoxins ring. Another peak produced in figure could be also regarded as a benzene ring degradation products identified as dimethyl-naphthalene, since its molecular weight 156 and molecular formula C_{12}H_{12}. Also, dioctyl phthalate was detected. The chromatogram also shows the presence of aspergillic acid, a metabolic product of *A. parasiticus* that is present in the control sample.

**Discussion**

Biological detoxification or the biotransformation of aflatoxins by microbial systems to a metabolite(s) that is either nontoxic when ingested by animals or less toxic than the original toxin can be termed as bioremediation. The goal of bioremediation is to degrade organic pollutants to concentrations that are either undetectable or, if detectable, to concentrations below the accepted limits (McKane and Kandel, 1996).

The growth of certain fungal strains (*P. griseofulvum*, *P. urticae*, *P. lilacinus*, *T. viride*, *C. utilis*, *S. cerevisiae* as well as a non-toxicogenic strain of *A. flavus*) on the three aflatoxin B concentrations used (100, 250 and 500 ppb) indicated that these fungal strains have the ability to tolerate or metabolize the toxin. Other examined fungal strains showed variable capabilities to tolerate aflatoxin B, reflected in the difference in their growth ability at the aflatoxin concentrations used (250, and/or 100 ppb) with no growth at 500 ppb. *T. viride*, *P. lilacinus*, *P. griseofulvum*, *S. cerevisiae*, *C. utilis*, *P. urticae* and *M. rouxii* were able to inhibit the growth of *A. parasiticus* and grew...
in the presence of aflatoxins in its growth medium. The organism with the least efficiency to inhibit aflatoxins was Circinella sp. having a total inhibition percentage of 23%.

The reasons for the reduction in aflatoxin levels can, therefore, be attributed to one or a combination of the following factors: (1) physical competition for space and nutrition; (2) test fungi may compete with A. parasiticus for a substrate required for toxin production but not the growth; (3) presence of test fungi might cause a change in the biochemical environment deciding, thereby, the metabolic pathway available to the toxigenic fungi and (4) degradation of aflatoxin following its formation. An inhibition in aflatoxin production by T. viride by more than 90% inhibition was also reported by Varma (1996). Shantha (1999) reported that several fungal cultures were found to prevent the synthesis of aflatoxin B₁ by A. flavus in liquid medium. Among these Phoma spp., Mucor sp., Trichoderma spp., Rhizopus sp., Alternaria sp. and Sporotrichum spp. inhibited aflatoxin synthesis by about 90% or more.

El-Sayed (1996) revealed the potential use of some phycomycetes (Absidia, Mucor, Cunninghamella, Rhizopus and Syncephalasrtium) to inhibit aflatoxins. Cole et al. (1972) and Nout (1989) studied a number of Rhizopus species and indicated the accumulation of two fluorescent metabolites of aflatoxin B₁ during its degradation. These metabolites were identified as hydroxylated stereo isomers derived from the reduction of ketone function on the cyclopentane ring of aflatoxin B₁. Weckbach and Marth (1977) and Choudhary (1992) also found that Rhizopus nigricans inhibited both the growth and aflatoxin production by A. parasiticus.

Nour et al. (1982) reported that some species of Aspergillus, Mucor, Penicillium, Rhizopus genera are antagonizing fungi that seem capable of metabolizing aflatoxin B₁ produced by A. flavus or probably producing some exudates that react with the toxin, transforming it into nontoxic compounds, degrading it or deflecting the pathway of aflatoxin B₁ synthesis. The identification of degradation products indicated that the aflatoxins were partly degraded with the examined fungal strains at different manner. The presence of furan moiety in the chromatograms of separation of most of the examined strains indicated degradation of aflatoxin. Three different furan moieties were exerted in case of M. rouxii.

The separation profile of R. nigricans exhibited high efficacy to fatty acid formation, with high percentage of linoleic acid. This result is in accordance with that of Kim et al. (2000) where linoleic acid was identified by GC-MS to be the main active component in aflatoxin degradation by soybean paste. The presence of the fatty acids and subsequent appearance of the degraded furan moiety proved this conclusion. However, in case of P. griseofulvum, two forms of furan moieties were appeared as degradation products as well as the dioctyl phthalate. The GC chromatogram also separated the antifungal metabolite of P. griseofulvum, the griseofulvin. The presence of aspergillic acid and its oxygenated form, oxyaspergillic acid, also confirm the oxidation process that cleavage hydrogen atoms and lead to liberation of H₂O and CO₂. While P. urchiae exerted two forms of furan moieties, C. utilis and P. lilacinus, show lower efficiency in the degradation of aflatoxins. The T. viride strains show different furan moieties as well as androstane-dione, a non-active molecule that have a similar structure of aflatoxin with the active bonds. The presence of benzene rings along with the dioctyl phthalate also confirms the degradation process. In case of S. cerevisiae, the mass analysis of the separated peaks indicates that degradation takes place in furan moiety as well as the coumarin moiety. These moieties represent the main skeleton of aflatoxins.

The available literature indicates only five strategies are known to reduce or detoxify aflatoxins contamination in food, food and feed processing, biocontrol and microbial inactivation, dietary modification and chemoprotection, chemical degradation, and reduction in toxin bioavailability by selective chemosorption (Smith et al., 1994). The presented results may certainly suggest a sixth strategy; application of fungal biodegradation and/or bioremediation. It is worth mentioning that the genomic mechanism controlling aflatoxin biosynthesis by the same research group is in progress and will be released shortly. T. viride, a classical fungal biocontrol agent that was used in the treatment of infected plants either in spray form or other formulation. Thus its utility can be expanded to include aflatoxin bioremediation in the infected plant, for being the most potent organism capable of decreasing aflatoxin concentration.

Hence, this study emphasizes the new value of S. cerevisiae used commercially as a rich source of protein and vitamins, which is its possible usage as a bioremediy for aflatoxins in a human being that cannot be treated with other treatments. Being used as a treatment for aflatoxins it will also provide the human body with vitamins and proteins.

### Literature


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