JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal
Published by Faculty of Biotechnology and Food Sciences

ANTIBACTERIAL ACTIVITY OF *DROSERA ROTUNDIFOLIA* L. AGAINST GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA

Dominika Ďurechová[1], Miroslava Kačániová[1], Margarita Terentjeva[2], Jana Petrová[1], Lukáš Hleba[1], Iwona Kata[3]*

Address(es):
[1]Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Microbiology, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic.
[2]Institute of Food and Environmental Hygiene, Faculty of Veterinary Medicine, Latvia University of Agriculture, K. Helmaņa iela 8, LV-004, Jelgava, Latvia.
[3]Faculty of Biology and Agriculture, University of Rzeszow, Zelwerowicza St. 4, 35-601 Rzeszow, Poland.

*Corresponding author: dominika.durechova@gmail.com

doi: 10.15414/jmbfs.2016.5.special1.20-22

ARTICLE INFO

Received 23. 12. 2015
Revised 20. 1. 2016
Accepted 24. 1. 2016
Published 8. 2. 2016

Regular article

OPEN ACCESS

ABSTRACT

The medicinal use of genus *Drosera,* as an important antitussive for different respiratory diseases, has been known for centuries. Many of extracts from carnivorous plants exhibit various antibacterial and antifungal activities. Naphthoquinones containing extracts from *Drosera* have antiviral, antibacterial, antifungal, aphrodisiac, antispasmodic, antileprosy, antisclerotic and anticancer properties. The aim of the present study was to detect antibacterial activity of *Drosera rotundifolia* against Gram-positive and Gram-negative bacteria by the testing of MIC. For the study six strains of microorganisms were selected and there were Gram-positive bacteria -*Bacillus thuringiensis* (CCM 19T), *Clostridium perfringens* (CCM 4991), and *Listeria monocytogenes* (CCM 4699), as well as and Gram-negative bacteria - *Escherichia coli* (CCM 3988), *Salmonella enterica* subsp. *enterica* (CCM 3807) and *Yersinia enterocolitica* (CCM 5671). Plant extracts were isolated from three plants of *Drosera rotundifolia* L. (S1, S2 and S3) in different time range. The most effective extract with MIC50 value of 17.07 $\mu g.ml^{-1}$ was S3, while forMIC90 of 19.05 $\mu g.ml^{-1}$ were extracts S2 and S3 exhibiting antimicrobial activity against *Bacillus thuringiensis, Clostridium perfringens* and *Listeria monocytogenes.* Extracts S1, S2 showed MIC50 value 25.53 $\mu g.ml^{-1}$for all the microorganism tested, but S3 extract revealed the same antimicrobial activity against *Yersinia enterocolitica, Salmonella enterica* subsp. *enterica* and *Escherichia coli*. Extract S1 has MIC90 value of 27.14 $\mu g.ml^{-1}$ against all the microorganism tested, but S2 and S3 shared the same MIC90 for *Yersinia enterocolitica, Salmonella enterica* subsp. *enterica* and *Escherichia coli.*

Keywords: *Drosera rotundifolia*, carnivory, minimal inhibitory concentration (MIC), antimicrobial activity

INTRODUCTION

Plants have several ways of taking up nutrients; among them one of the highly specialized way is carnivory. The more than 600 known species of carnivorous plants constitute a very diverse group, often very distantly related species originating from different systematic orders and families. The common feature of those plans is the ability to hunt and consume animals and this unites in this group the species from various climatic and geographical areas differing morphologically and ecologically (**Studnička, 2006**). Carnivorous plants capture and utilize nutrients of prey, which consists mostly from insects (**Darwin, 1875; Juniper *et al*., 1989**). These plants occur in areas such as wetlands, alpine mountain peaks, vulcanic platform. They are characterized by growth on the sunny areas, the water-rich sites, but also are able to grow in medium poor of nutrient (**Jurgens *et al*., 2012**). Plants have elaborated adaptation to prey on and use the nutrients of victim with specialized leaves, the secretion of sticky substances, digestive enzymes and nectar to attract a prey (**Thorén *et al*., 2003**). The ability of plants to catch insects was firstly observed in 1759 at Venus flytrap (*Dionaea muscipula*) by Arthur Dobbs (**Studnička, 2006**). A more detailed description of carnivory plants described by Charles Darwin (1875) and still represents the fundamental work on insectivorous plants (**Darwin, 1875**).
The genus *Drosera* represents a good model of plant evolution and functional adaptation. Importantly, extracts from numerous species of *Drosera* have been traditionally used for various medicinal purposes (**Šamaj *et al*., 1999**). The *Drosera* genus is a natural source of pharmacologically important secondary compounds used as substrates in the production of pharmaceuticals. The most important are naphthoquinones, especially plumbagin,7-methyljuglone and flavonoids (**Banasiuk *et al*., 2012**). Particularly, naphthoquinones are thought to be responsible for therapeutic effects in respiratory diseases including bronchial infections and tuberculosis. The naphthoquinones, and specially plumbagin, also inhibits a development of parasitic nematodes and insects (**Collantes *et al*., 2014**). The extracts from *Drosera* which contain naphtoquinones share medical and other valuable properties and the antiviral, antibacterial, antifungal, aphrodisiac, antispasmodic, antileprosy, antisclerotic and anticancer properties are recognized **(Juniper *et al*., 1989; Šamaj *et al*., 1999**).
The present study was focused on *Drosera rotundifolia* of genus *Drosera*, which might be a pharmacologically important plant for its antimicrobial activity. The aim of this study was to evaluate the antibacterial activity of *Drosera* plant extracts by the detection of the minimal inhibitory concentration (MIC). Antimicrobial activity of six bacteria: Gram-positive _ *Bacillus thuringiensis, Clostridium perfringens, Listeria monocytogenes* and Gram-negative bacteria *Yersinia enterocolitica*, *Salmonella enterica* subsp. *enterica* and *Escherichia coli* were evaluated

MATERIALS AND METHODS

Plant material

Plants of *Drosera rotundifolia* L. were cultivated *in vitro* on basal MS medium (DUCHEFA) supplemented with 2 % (w/v) of sucrose and 0.8 % (w/v) of agar (**Bobák *et al*., 1995**). The plantlets were cultivated at 20 ± 2 °C with a day length of 16 h under 50 μEm^{-2} s^{-1} light intensity. Plant extracts were isolated from three plants of *Drosera rotundifolia* L. in different time range.

Microorganisms

In this study the six bacteria species representing different strains were tested. Among tested were Gram-positive - *Bacillus thuringiensis* CCM 19T, *Clostridium perfringens* CCM 4991, *Listeria monocytogenes* CCM 4699 and Gram-negative bacteria - *Escherichia coli* CCM 3988, *Salmonella enterica* subsp. *enterica* CCM 3807 and *Yersinia enterocolitica* CCM 5671. All tested strains were collected from the Czech Collection of Microorganisms (Brno,

Czech republic). The bacteria were cultured in the nutrient broth for obtaining of bacterial suspension (Imuna, Slovakia) at 37 °C.

Preparation of plant extracts

Whole plants of *Drosera rotundifolia* L. were dried and crushed. Weights of plant before and after drying are showed in Table 1. Crushed plants were dissolved in 96% ethanol (Sigma, Germany) and stored at room temperature in the dark for two weeks to prevent the degradation of active components. Then, the ethanolic plant extracts were subjected to evaporation under reduced pressure at 40 °C in order to remove the ethanol (Stuart RE300DB rotary evaporator, Bibby scientific limited, UK, vacuum pump KNF N838.1.2KT.45.18, KNF, Germany). For the antimicrobial assay, the crude plant extracts were dissolved in dimethyl sulfoxide (DMSO) (Penta, Czech Republic) to equal 102.4 mg/mL as stock solution, while for chemical analysis ethanol was used as solvent.
Analysis of the essential oils was carried out with Hewlett-Packard 5890/5970 GC-MSD system.

Table1 Information about plants extracts

Sample	Weight before drying	Weight after drying	Sample in DMSO	Chemical composition
S1	8.82g	0.74g	570 µl	gallic acid 0.5%, hyperoside 0.4%, droseroside 1%, tanine 0.6%
S2	11.42g	0.61g	690 µl	gallic acid 1.7%, hyperoside 1.2%, droseroside 2.3%, tanine 1.6%
S3	15.17g	0.82 g	480µl	gallic acid 1.2%, hyperoside 0.6%, droseroside 1.3%, tanine 0.2%

Microbroth dilution method

The minimal inhibitory concentrations (MICs) *in vitro* of the compounds were determined by the microbroth dilution method according to the Clinical and Laboratory Standards Institute recommendation (CLSI, **2009**) in Mueller Hinton broth (Biolife, Italy).
The test samples were dissolved in dimethyl sulphoxide (DMSO) and the stock solutions of the serial two-fold dilutions with the final concentrations ranging between 0.5-512 µg.ml^{-1} were obtained. After that the each well was inoculated with a 100 µl volume of working microbial suspension at the final density of 0.5 McFarland. Bacterial strains were grown at 37 ± 0.5 ºC for 20-24 h. Additionally wells for positive control (wells without bacteria), inoculum viability (no extract added) and the DMSO as negative control were reserved in each plate.
The inhibition of microbial growth was evaluated by measuring the well absorbance at 450 nm in an absorbance microplate reader Biotek EL808 with shaker (Biotek Instruments, USA). The absorbance in 96 microwell plates was measured before and after experiment. Differences between both measurements - prior and after incubation were evaluated as a growth.

Statistical analysis

Measurement error was established for 0.05 values of absorbance. Differences in absorbance between the measurements before and after the analysis were expressed as a set of binary values.
These values were assigned to exact concentrations. The following formula was created for this specific experiment: value 1 (inhibitory effect) was assigned to absorbance values lower than 0.05, while value 0 (no effect or stimulant effect) was assigned to absorbance values higher than 0.05. For this statistical evaluation the probit analysis in Statgraphics software was used (**Kačániová *et al*., 2015**).

RESULTS AND DISCUSSION

The *Drosera* genus, native to Australia and New Zealand, includes multiple carnivorous species which possess substantial medicinal potential. Medicinal use of *Drosera* is convenient due to the simplicity of its cultivation *in vitro*. *Drosera* extracts owe their antimicrobial properties to secondary metabolites. Naphthoquinones, mainly plumbagin are the main active compounds produced by *D. binata* tissues. The plants are also a source of flavonoids, ellagic acid and their glycoside and methyl derivatives **(Zehl *et al*., 2011)**. It is crucial that plant extracts, unlike antibiotics, do not contribute to the emergence of resistant bacterial strains when used as antibacterial agents. Various studies showed interactions between several secondary metabolites found in plant extracts, which allowed herbal drugs to be used in lower doses of active components **(Krolicka *et al*., 2008)**.

The antimicrobial activity of *Drosera rotundifolia* L. was determined previously by the disc diffusion assay of extracts. Ethanolic extracts of *D. rotundifolia* showed an antimicrobial effect against *Yersinia enterocolitica, Bacillus thurigensis* and *Salmonela enterica* (**Kačániová *et al*., 2014**). Carnivorous plant *D. rotundifolia* assumes to be a good source of compounds which posses antimicrobial effect against different Gram-negative and Gram-positive pathogenic bacteria. In this study the minimal inhibitory concentrations (MICs) of the compounds of individual extracts from three plants of *D. rotundifolia* by the microbroth dilution assay for a quantitative determination were evaluated.
Minimum inhibitory concentrations are considered as a great method for determining the susceptibility of organisms to antimicrobials and are therefore used to judge the performance of all other methods of susceptibility testing and MIC is defined as the lowest concentration of a ,,drug,, that will inhibit the visible growth of an organisms after incubation (**Andrews, 2001**).

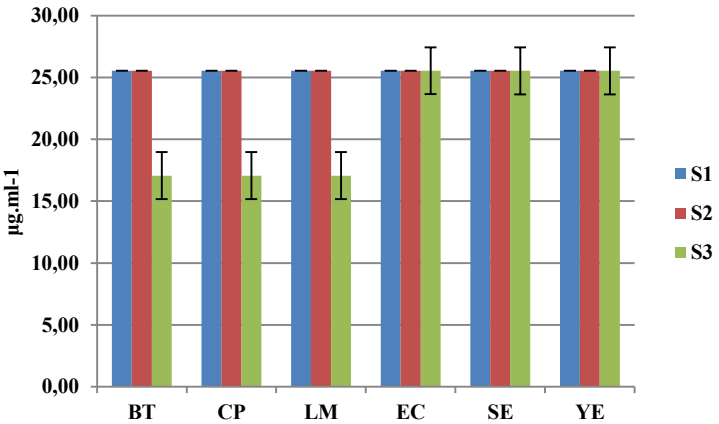

Figure 1 Antimicrobial activity MIC 50 of *Drosera rotundifolia* L.

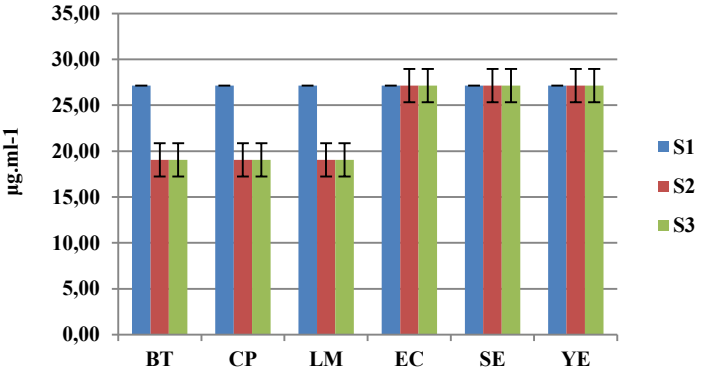

Figure 2 Antimicrobial activity MIC 90 of *Drosera rotundifolia* L extracts (S1, S2 and S3) extracts (S1, S2 and S3)

BT - Bacillus thuringiensis CCM 19T, *CP - Clostridium perfringens* CCM 4991, *LM - Listeria monocytogenes* CCM 4699, *EC - Escherichia coli* CCM 3988, *SE - Salmonella enterica* subsp. *enterica* CCM 3807, *YE - Yersinia enterocolitica* CCM 5671.

The antimicrobial activity (µg.ml^{-1}) of three extracts of *Drosera rotundifolia* L. against various strains of Gram-positive and Gram-negative bacteria are summarized in Figures 1 and 2. The most effective extract with MIC50 value of 17.07 µg.ml^{-1} was extract S3 and with MIC90 of 19.05 µg.ml^{-1} were extracts S2 and S3. The extract S3 with MIC50 was the most active against *Bacillus thuringiensis, Clostridium perfringens* and *Listeria monocytogenes*, while the extracts S2 and S3 were the most effective against *Bacillus thuringiensis, Clostridium perfringens* and *Listeria monocytogenes* at MIC90. This results correspond with the results of the previous study, where the individual differences between the extracts obtained were observed and extract DR3 showed the best antimicrobial activity against *B. thuringiensis* (**Kačániová *et al*., 2014**). Against *Yersinia enterocolitica, Salmonella enteric* subsp. *enterica* and *Escherichia coli* the extracts S1, S2 and S3 showed the same MIC50 value of 25.53 µg.ml^{-1} and MIC90 value of 27.14 µg.ml^{-1}. The extract S1 and S2 have showed the same activity against *Bacillus thuringiensis, Clostridium perfringens* and *Listeria monocytogenes* at MIC50. Our results show that Gram-positive bacteria were more susceptible to the addition of plant extract and this could be confirmed with the previous studies where *Bacillus* spp. was susceptible to active compounds of plants extracts **(Didry *et al*., 1998; Krolicka *et al*., 2009)**.

The studies on antimicrobial properties of extracts from different species of *Drosera* are still ongoing and positive antibacterial effect and determination of values MIC was done by **Taraszkiewicz *et al.* (2012)**, who demonstrated that extracts of *Drosera gigantea* contain antibacterial compounds that can be used against *Pseudomonas syringae*. Other author focused on *Drosera intermedia* extracts and this extract was the most effective against *Staphylococcus epidermidis* for which a MIC value of 13.0 $\mu g.ml^{-1}$was scored (**Grevenstuk *et al.* 2009**). Other *Drosera* was studied by **Didry *et al.* (1998)**, who revealed that extract from *Drosera peltata* showed the broad spectrum activity against numerous bacteria of the oral cavity, with greatest activity against Gram-positive bacteria *Streptococcus mutans* and *S. sobrinus* with MIC value 31.25 $\mu g.ml^{-1}$ and 15.63 $\mu g.ml^{-1}$, respectively.

Plant extracts are a very rich source of secondary metabolites with antibacterial action, and their application provides an opportunity to effectively combat also antibiotic-resistant bacterial strains **(Cuhnie, 2005).**

Previously **Krolicka *et al.* (2008)** demonstrated that extracts obtained from several other *in vitro* cultured carnivorous plants possess antibacterial activity towards various pathogens in planktonic culture such as *Escherichia coli, Enterococcus faecalis, Klebsiella pneumoniae* and *S. aureus*. The study of **Krychowiak *et al.* (2014)** was the first report describing the efficiency of the *D. binata* extract itself in eliminating the dangerous human pathogen *S. aureus,* resulting however in the increased cytotoxicity of extract on human keratinocytes. The antimicrobial effectiveness of the chloroform plant extract was similar towards all studied *S. aureus* strains, regardless of their resistance to antibiotics. However, a higher bactericidal concentration (MBEC 64 $\mu g.ml^{-1}$) was required for *in vitro* cultured biofilm.

CONCLUSION

In conclusion, the extracts of the tested *Drosera rotundifolia* exhibited good potential antibacterial activity and the potential for developing of antimicrobial agents. The active extracts should be evaluated further in-depth to isolate other active components and detect their mode of action. *Drosera* species represent a promising alternative source of material for medicinal use. *Drosera* extract in this study show very good antimicrobial activity against Gram-positive bacteria.

Acknowledgments: This work was supported by grant VEGA 1/0611/14.

REFERENCES

Andrews, J. M. (2001). Determination of minimum inhibitory concentrations. *Journal of Antimicrobial Chemotheraphy, 48*(1), 5-16. http://dx.doi.org/10.1093/jac/dkf083

Banasiuk, R., Kawiak, A. & Królicka, A. (2012). *In vitro* cultures of carnivorous plants from the *Drosera* and *Dionaea* genus for the production of biologically active secondary metabolites. *Journal of Biotechnology, Computational Biology and Bionanotechnology, 93*(2), 87-96.http://dx.doi.org/10.5114/bta.2012.46572

Bobák, M., Blehová, A., Krištín, J., Ovečka, M. & Šamaj, J. 1995. Direct plant regeneration from leaf explants of *Drosera rotundifolia* cultured in vitro. *Plant Cell Tissue and Organ Culture*, *43*(1), 43-49. http://dx.doi.org/10.1007/bf00042670

Clinical and Laboratory Standard Institute. Performance standard for antimicrobial susceptibility testing. Wayne, PA: Clinical and Laboratory Standard Institute; 2009. [Online] Available from: http://www.techstreet.com/products/1760826. [Accessed on 2nd November, 2015].

Cushnie, T. P. & Lamb, A. J. (2005). Antimicrobial activity of flavonoids. *InternationalJournal of Antimicrobial Agents, 26*(5), 343–356. http://dx.doi.org/10.1016/j.ijantimicag.2005.09.002

Darwin, C. R. (1875). Insectivorous plants. (vol. 1). London: John Murray

Didry, N., Dubreuil, L., Trotin, F. & Pinkas, M. (1998). Antimicrobial activity of aerial parts of *Drosera peltata* Smith on oral bacteria. *Journal of Ethnopharmacology*, *60*(1), 91–96. http://dx.doi.org/10.1016/s0378-8741(97)00129-3

Grevenstuk, T., Goncalves, S. Almeida, S., Coelho, N., Quintas, C. Gasper, M. N. & Romano, A. (2009). Evaluation of the antioxidant and antimicrobial properties of in vitro cultured *Drosera intermedia* extracts. *Natural Product Communications, 4*(8), 1063-1068. http://dx.doi.org/10.1055/s-0029-234359

Juniper, B. E., Robins, R. J. & Joel, D. M. (1989). The *carnivorous plants* (Vol.1). California: Academic Press.

Jurgens, A., Sciligo A., Witt, T., El-Sayed, A. M. & Suckling, D.M. (2012). Pollinator-prey conflict in carnivorous plants. *Biological Review Cambridge Philosophical Society, 87*(3), 602-615. http://dx.doi.org/10.1111/j.1469-185x.2011.00213.x

Kačániová, M., Ďurechová, D., Vuković, N., Kántor, A. Petrová, J., Hleba, L. & Vatľák, A. (2014). Antimicrobial activity of *Drosera rotundifolia* L. *Scientific Papers: Animal Science and Biotechnologies, 47*(2), 366-369.

Kačániová, M., Petrová, J., Kántor, A., Terentjeva, M. & Kluz, M. (2015). *In vitro* antimicrobial activity of four Slovak medicinal plants against different strains of bacteria. *Animal Science and Biotechnologies, 48*(1), 137-145.

Krolicka, A., Szpitter, A., Maciag, M., Biskup, E., Gilgenast, E., Romanik, G., Kaminski, M. & Wegrzyn, G. (2009). Antibacterial and antioxidant activity of the secondary metabolites from *in vitro* cultures of the Alice Sundew (*Drosera alicie*). *Biotechnology and Applied Biochemistry,* 53(3), 175-184. http://dx.doi.org/10.1111/j. 10.1042/BA20080088.x

Królicka A, Szpitter A, Gilgenast E, Romanik G, Kamiński M, et al. (2008) Stimulation of antibacterial naphthoquinones and flavonoids accumulation in carnivorous plants grown in vitro by addition of elicitors. *Enzyme Microbial Technology, 42*, 216–221. doi: 10.1016/j.enzmictec.2007.09.011

Krychowiak, M, Grinholc, M, Banasiuk, R, Krauze-Baranowska ,M, Głód, D, Kawiak, A, et al. (2014) Combination of silver nanoparticles and *Drosera binata* extract as a possible alternative for antibiotic treatment of burn wound infections caused by resistant *Staphylococcus aureus*. *PLoS ONE*, *9*(12): e115727. doi:10.1371/journal.pone.0115727

Studnička, M. (2006).Masožravérostliny. (vol.1). Praha: Academia.

Šamaj, J. ,Blehová, M., Repčák, M., Ovečka M. & Bobák, M. (1999). *Drosera* species (Sundew): *In vitro* culture and the production of plumbagin and other secondary metabolites. *Medicinal and Aromatic Plants, 43*(11), 105-136.

Taraszkiewicz, S. Jafra, A. Skrzypczak, M. Kaminski & Krolicka, A. (2012). Antibacterial activity of secondary metabolites from *in vitro* culture of *Drosera gigantean* against the plant pathogenic bacteria *Pseudomonas syringae pv. syringae* and *P. syringae pv. morsprunorum A*. *Journal of Plant Pathology, 91*(1), 63.http://dx.doi.org/10.4454/jpp.v94i1sup.011

Thoren, M., Tuomi, J., Kämäräinen, T. & Laine. K. (2003). Resource availability affects investment in carnivory in *Drosera rotundifolia*. *New Phytologist, 159*(2), 507-411.http://dx.doi.org/10.1046/j.1469-8137.2003.00816.x

Vásquez - Collantes, S. G., Rojas - Idrogo, C. & Delgado-Paredes, G. E. (2014). *In vitro* flowering and plantlets elongation in " Sundew" *Drosera capillaris. International Journal of Plant, Animal and Environmental Sciences, 4*(3), 508-517.

Zehl, M, Braunberger, C, Conrad, J, Crnogorac, M, Krasteva, S, *et al*. (2011). Identification and quantification of flavonoids and ellagic acid derivatives in therapeutically important ***Drosera*** species by LC-DAD, LC-NMR, NMR and LC-MS. *Analytical and Bioanalytical Chemistry, 400*, 2565–2576. 10.1007/s00216-011-4690-3

JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

MICROBIOLOGICAL QUALITY OF READY-TO-EAT FOODS PRODUCED IN SLOVAKIA

Ľubomír Lopašovský[1], Margarita Terentjeva[2], Simona Kunová[1], Lucia Zeleňáková[1], Miroslava Kačániová[3]

Address(es): MVDr. Ľubomír Lopašovský, PhD.,
[1]Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Food Hygiene and Safety, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic.
[2]Institute of Food and Environmental Hygiene, Faculty of Veterinary Medicine, Latvia University of Agriculture, K. Helmaņa iela 8, LV-3004, Jelgava, Latvia.
[3]Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Microbiology, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic.

*Corresponding author: lubomir.lopasovsky@uniag.sk

doi: 10.15414/jmbfs.2016.5.special1.31-35

ARTICLE INFO

Received 17. 12. 2015
Revised 13. 1. 2016
Accepted 22. 1. 2016
Published 8. 2. 2016

Regular article

OPEN ACCESS

ABSTRACT

The purpose of this study was to evaluate the microbiological quality of Ready-To-Eat (RTE) foods produced in Slovakia. A total amount of 144 samples of RTE food were tested during one-year period from January to December, 2014 and the microbiological quality of kebabs (n=30), gyros (n=10), hamburgers (n=54), cheeseburgers (n=5), hot-dogs (n=31), roasts (n=14) was analyzed. The samples were examined for the presence of: coliform bacteria, sulfite-reducing clostridia, yeast, microscopic filamentous fungi and coagulase positive staphylococci according to the ISO standards requirements. In kebab samples the counts of coliforms were from < 10 to $1.6x10^3$ cfu.g^{-1} and incompliance was found in 12/ 30 (40%) of samples. Maximum counts of coliforms and yeasts were exceeded in two and one sample of gyros and isolation range was from 10 to $1.5x10^3$ cfu.g^{-1} $3.2x10^2$ cfu.g^{-1} and from 10 to $1.4x10^2$ cfu.g^{-1}. Also 2/5 (40%) of samples of cheeseburger yielded unsatisfactory coliform counts and the isolation rates were from less than 10 to $1.5x10^3$ cfu.g^{-1}. In hot-dogs, counts of coliforms, yeasts and coagulase positive staphylococci ranged from less than 10 to $1.4x10^3$, < 10 to $2.1x10^3$ cfu.g^{-1} and <10 to $1.4x10^2$ cfu.g^{-1} and number of unsatisfactory samples were 3/31(10), 1/31(3) and 1/31 (3), respectively. In roast, the counts of coliforms, yeasts and microscopic filamentous fungi were from less than 10 to $2.1x10^2$ cfu.g^{-1}, less than 10 to $1.4x10^2$ cfu.g^{-1} and less than 10 to $3.2x10^2$ cfu.g^{-1}, respectively and the safety criteria were not met for 1/14 (7) samples for each of the bacteria group mentioned. Overall, the microbiological criteria set for RTE foods in Slovak Republic were violated in 36/ 144 (25) of samples tested. Results of the present study show that RTE foods might pose public health concerns in Slovakia and more attention on the hygienic practices should no paid.

Keywords: Microbiological quality, kebab, gyros, hamburgers, cheeseburgers, hot-dogs, roasts

INTRODUCTION

Controlling and improving of the quality and safety of chilled foods at all stages of the cold chain have always been among the main concerns in order to reduce food losses and eliminate public health hazards. Changes in microbial and physico-chemical quality of foods may occur during their shelf-life that is linked to storage temperature, as well as to the composition and properties of a product. Meat and processed meat products are ideal media for the growth of spoilage and pathogenic bacteria therefore the quality and safety of meat could change significantly during the shelf-life. Pork meat and Ready-To-Eat (RTE) pork meals are the main type of meat consumed in Europe **(Mataragas *et al.*, 2008; Verbeke *et al.*, 2010).**

RTE food is defined as food that can be consumed immediately at the point of sale without further preparation or treatment. It could be raw, partially or fully cooked, and hot, chilled or frozen **(FEHD, 2007; USFDA, 2009).** RTE food can be of animal and plant origin including fruits, vegetables and bakery products **(USFDA, 2009).** Since RTE foods are consumed without additional treatment, a risk of foodborne disease outbreaks linked to the consumption of RTE food are high if is the food was improperly handled. RTE foods were a source of bacterial foodborne outbreaks and various foodborne pathogens have been found in the RTE products in previous studies **(Castro-Rosas *et al.*, 2012; Seow *et al.*, 2012).** Methods of storage, processing, handling and distribution of RTE products can affect the numbers of microorganisms **(Christison *et al.*, 2008; Fang *et al.*, 2003).**

The catering business provides food and beverages to people and covers all sectors of society such as childcare, schools, hospitals, nursing homes, restaurants, bars, take-away and fast-food outlets **(Garayoa *et al.*, 2011).** This industry has expanded greatly and undergone profound changes in recent years. Many factors have contributed to this including changes in lifestyle, and business, travelling as well as increased purchasing power (**Garayoa *et al.*, 2011).** With globalization, the foodborne diseases (FBDs) have acquired a new dimension, as many food products are produced in one country to be imported and consumed in another **(Martins and Germano, 2008).** The increased international travel, as more affordable, has determined a FBDs globalization **(Käferstein *et al.*, 1997).** Many infectious diseases, including a variety of gastrointestinal disorders, are contracted by individuals while travelling outside their country of residence **(Evans 2006; Ravel *et al.*, 2011).** The World Tourism Organization (WTO), a specialized agency of the United Nations (UN), estimates world tourist arrivals at 940 million in 2010 **(UNWTO, 2011).** The World Health Organization (WHO) reports that up to 30% of individuals in developed countries suffer from food and water related diseases annually **(WHO, 2007).** The European Union (EU) Summary Report on foodborne outbreaks in 2009 indicated a total of 5550 foodborne outbreaks, with 48,964 human cases, 4356 hospitalizations and 46 deaths. The EU's annual report also showed that up to 63.6% of FBDs were associated with foodservice catering **(EFSA, 2011).**

In recent years, several studies **(Almualla *et al.*, 2010; Chapman *et al.*, 2010; Garayoa *et al.*, 2011; Gillespie *et al.*, 2000; Legnani *et al.*, 2004; Marzano and Balzaretti, 2011; Martinez-Tomè *et al.*, 2000; Rodríguez *et al.*, 2011 a, b; Santana *et al.*, 2009; Tessi *et al.*, 2002; Veiros *et al.*, 2009; Yoon *et al.*, 2008)** have been conducted aiming to evaluate the microbiological quality and safety of RTE foods prepared and served by catering business in many sectors of society - schools, hospitals, supermarkets, hotels, long term care facilities, canteens for workers, mass catering establishments, however, knowledge about the microbiological contamination of RTE foods in Slovakia is very limited, therefore the aim of the study was to evaluate the microbiological quality of selected ready-to-eat products. In our study we analyzed the microbiological quality of kebabs, gyros, hamburgers, cheeseburgers, hot - dogs and roasts by detection of coliform bacteria, sulfite-reducing clostridia, yeast, microscopic filamentous fungi and coagulase positive staphylococci.

MATERIAL AND METHODS

Sampling

Samples were obtained from the nearby fast food establishments in the Nitra region –shops, supermarkets and catering enterprises. After sampling, samples were delivered to the Regional Institute of Public Health of Regional Public Health Authority in Nitra accredited laboratory for microbiological testing.
A total amount of 144 samples of RTE food for the period of one year from January to December 2014 was investigated. The samples were kebabs (n=30), gyros (n=10), hamburgers (n=54), cheeseburgers (n=5), hot-dogs (n=31), roasts (n=14) were used in this study. The samples were examined for the presence of the following groups of microorganisms: coliform bacteria, sulfite-reducing clostridia, yeast, microscopic filamentous fungi and coagulase positive staphylococci.

Microbiological analysis

An amount of 5 g of ready-to-eat food was homogenized with 45 mL of sterile peptone saline solution for 1 min in order to obtain the initial dilution. For quantitative detection of microorganisms the ten-fold dilution from 10^{-2} to 10^{-4} were made. After preparation of sample, for detection of the total coliforms the suspension was plated out on Violet Red Bile agar (Merck, Darmstadt, Germany) and incubated at 37 °C for 24 h **(STN EN ISO 4832)**. Detection of coagulase positive staphylococci was done on Baird-Parker egg yolk tellurite agar incubated at 37 °C for 24 h according to **STN ISO EN 6888-1.** For testing of *Clostridium perfringens* **(STN ISO EN 13401)**, suspension was plated onto Tryptose Sulphite Cycloserine agar incubated at 37 °C for 24-48 hour in anaerobically condition. Detection of yeast and microscopic filamentous fungi was undertaken on Dichloran Rose Bengal Chlortetracycline or Dichloran glycerol agar at 25 °C for 5 days **(STN ISO EN 21527 – 2)**. After incubation the bacterial colonies with typical morphology resembling the target microorganisms were selected from each Petri dish and all typical colonies were enumerated. Evaluating of the results have been doing by applicable Codex Alimentary of the Slovak Republic reflected in Table 1 **(CA SR, 2006).**

Table 1 Criteria of production process hygiene for ready-to-eat food

Microorganisms	n	c	m	M
Coliform bacteria	5	2	10^2	$5x10^2$
Sulfite-reducing clostridia	5	2	50	10^2
Yeasts	5	2	10^3	$2x10^4$
Microscopic filamentous fungi	5	2	$2x10^2$	10^3
Coagulase positive staphylococci	5	2	10^2	$5x10^2$

m = minumum number of microorganisms in the sample size „n“, M = maximum number of microorganisms in the specified amount of sample, which allows the maximum number of samples „c“., c = the number of samples from „n“ with values between “m” and “M” of the bacterial counts in other samples do not exceed “m”

RESULTS AND DISCUSSION

Homemade foods are taken as RTE for sale on the street or for the consumption, therefore, are one of the most susceptible to microbial growth in view of the longer length of time between preparation and consumption under improper temperature conditions, besides the unusual step of reheating the foods before consumption. In the case of streetmade foods, the raw materials are usually industrially processed and either prepared in advance or on demand. Environmental conditions faced by the street vendors are an important risk factor to the safety of the food sold. Nevertheless, no difference was found between the microbial counts of home- and street-made samples in **Hanashiro *et al.* (2005)** study. It is presumable that the sanitary and structure conditions are not better at the handlers’ home and neighborhood added to the fact that food handling practices are the same no matter the place **(Hanashiro *et al.*, 2005).**

Table 2 Microbiological quality of kebab samples (n=30)

Microorganisms	Results	No. of unsatisfactory samples /No. of samples (%)
Coliform bacteria	< 10 – $1.6x10^3$ cfu.g^{-1}	12/ 30 (40)
Sulfite-reducing clostridia	< 10 cfu.g^{-1}	0/ 0 (0)
Yeasts	< 10 – $5.0x10^2$ cfu.g^{-1}	0/ 0 (0)
Microscopic filamentous fungi	< 10 – $1.8x10^2$ cfu.g^{-1}	0/ 0 (0)
Coagulase positive staphylococci	< 10 cfu.g^{-1}	0/ 0 (0)

In kebabs, the number of coliform bacteria were in the range from <10 to $1.6x10^3$ cfu.g^{-1} (Table 2) and 12 (40%) samples of kebab did not corresponding with **CA SR 2006**. Numbers of sulfite-reducing clostridia, coagulase positive staphylococci did not exceed 10 cfu.g$^{-1,}$ while the isolation range of yeasts and microscopic and filamentous fungi was from <10 to $5.0x10^2$ cfu.g^{-1} and $1.8x10^2$ cfu.g^{-1}, respectively. These microbiological quality indicators of the samples were with the requirements of Codex Alimentarius of SR **(CA SR, 2006).**
Our results indicate the microbiological quality of the tested samples was better than in previously conducted studies. In 30 samples of kebab in **Agbodaze *et al.*, 2005** research, the mean total plate count (TPC) at Osu was 5.02, Accra Central 4.08 and those from Nima 4.80 $\log_{10}$ CFU/g. The samples from Accra Central shared the highest mean coliform count - 5.12, while the samples from Osu and Nima - 4.41 and 3.70 $\log_{10}$ cfu.g^{-1}, respectively. Accra Central samples again recorded the highest fecal coliforms (4.4 $\log_{10}$ cfu.g^{-1}) as compared to 3.98 and 3.80 log10 cfu.g^{-1} for samples bought from Osu and Nima, respectively. Kebab samples from sites were contaminated with *E. coli*, other Gram-negative bacteria and *Staphylococcus* species, whose virulence factors are yet to be determined.
In study of *Ziino et al. (2013)* the microbiological quality of kebabs retailed in Palermo and Messina was evaluated and 20 raw and 22 cooked kebabs were analysed to determine the aerobic mesophilic bacteria (AMB), *Enterobacteriaceae, Escherichia coli*, sulphite reducing anaerobes, coagulase positive staphylococci, micrococci, *Bacillus cereus* and the presence of *Salmonella* spp. and *Listeria monocytogenes*. In raw kebabs, AMB ranged from 4.00 to 7.34 log cfu.g^{-1} and *Enterobacteriaceae* from 1.00 to 7.59 log cfu.g^{-1}. *Escherichia coli* and sulphite reducing anaerobe counts were from <1.00 to 6.18 and 4 log cfu.g^{-1}, respectively. Coagulase positive staphylococci ranged from <1.00 to 3.48 log cfu.g^{-1} and micrococci from <1.00 to 6.00 log cfu.g^{-1}. *Listeria* spp. was found in three raw kebab samples. In cooked kebabs, the AMB values ranged from 1.78 to 6.30 log cfu.g^{-1}, *Enterobacteriaceae* from 1.00 to 4.00 log cfu.g^{-1} and micrococci from <1.00 to 5.30 log cfu.g^{-1}. Three samples were positive for *Escherichia coli* (from 1 to 1.30 log cfu.g^{-1}) and one for sulphite reducing anaerobes (2.00 log cfu.g^{-1}). Coagulase positive staphylococci were found in two samples with loads of 2.30 and 2.78 cfu.g^{-1}, respectively.
Kebab is product, which is frequently to find to be contaminated and also the results of our study in 40% was non satisfactory and the microbiological quality of kebab should be monitored.
In gyros samples the numbers of coliform bacteria was from less to 10 to 1. $5x10^3$ cfu.g^{-1} , but the numbers of yeasts was from less than 10 to $1.4x10^2$ cfu.g^{-1} and of fungi from less than 10 to 1. $2x10^2$ cfu.g^{-1}. Counts of yeasts and coagulase positive staphylococci did not exceed 10 cfu.g^{-1} (Table 3).

Table 3 Microbiological quality of gyros samples (n=10)

Microorganisms	Results	No. of unsatisfactory samples /No. of samples (%)
Coliform bacteria	< 10 – $1.5x10^3$ cfu.g^{-1}	2/ 10 (20)
Sulfite-reducing clostridia	< 10 cfu.g^{-1}	0/ 0 (0)
Yeasts	< 10 – $1.4x10^2$ cfu.g^{-1}	1/ 10 (10)
Microscopic filamentous fungi	< 10 – $1.2x10^2$ cfu.g^{-1}	0/ 0 (0)
Coagulase positive staphylococci	< 10 cfu.g^{-1}	0/ 0 (0)

In gyros samples, we found that two samples (2/10/20) did not meet the acceptable value for coliforms bacteria and one samples of gyros (1/10/10) did not corresponding of acceptable value of yeast.
Number of unsatisfactory samples in case of gyros was higher than in case of kebab. The gyros usually is manufactured from the poultry meat and the poultry meat was found to be contaminated with high numbers of bacteria. Bacterial counts (aerobes, *Salmonella* spp., *E. coli, C. jejuni*, and *C. coli*) are higher on the breast area of broiler carcasses than on the thigh and drum areas. In addition, some microorganisms particularly *Salmonella* spp. attaches to the poultry skin and is difficult to remove **(Kotula and Davis, 1999)**. A great risk may be occurred by these pathogens, when chicken skin is added to chicken doner kebabs. Thus, some manufactures recommend that the skin using in doner kebabs can be heated to increase chemical and microbiological quality of chicken doner kebabs. Doner kebab as well as gyros are a traditional products. If it is produced properly, there have no any microbiologically serious risk. In general, the microbiological quality of gyros could be improved through implementation of the hazard analysis critical control point (HACCP) concept for the chicken doner kebab and gyros because the incompliance in case of gyros we identified more frequently than in case of kebab.

Table 4 Microbiological quality of hamburger samples (n=54)

Microorganisms	Results	No. of unsatisfactory samples /No. of samples (%)
Coliform bacteria	< 10 – 2.2x10³ cfu.g^{-1}	10/ 54 (19)
Sulfite-reducing clostridia	< 10 cfu.g^{-1}	0/ 0 (0)
Yeasts	< 10 – 8.2x10² cfu.g^{-1}	1/ 54 (2)
Microscopic filamentous fungi	< 10 – 2.0x10² cfu.g^{-1}	0/ 0 (0)
Coagulase positive staphylococci	< 10 cfu.g^{-1}	0/ 0 (0)

In **Min *et al.* (2013)** study an amount of 20 RTE hamburgers sold in Canterbury region were tested for *Listeria monocytogenes, Staphylococcus aureus*, coliforms and *Escherichia coli*. Among samples were13 chicken and 7 beef burgers, respectively, which were purchased from five fish and chip shops located in the different areas over period of 4 weeks. Overall, 16 (80%) and 4 (20%) samples were found to be of satisfactory and marginal microbiological quality, respectively. None of the samples tested was in the category of unsatisfactory or potentially hazardous levels of microbial counts. Among the 4 burger samples with marginal microbiological quality two chicken burgers were contaminated with coliforms (1.50x10^2 and 2.25x10^{2}cfu.g^{-1}), but one chicken and one beef burgers with *S. aureus* (1.05x10^2 and 2.30x10^{2}cfu.g^{-1}). *E. coli* and *L. monocytogenes* were not detected in any samples. Results indicate that the microbiological quality of burgers sold in different shops in Canterbury was satisfactory

In study of **Dinucci Bezerra *et al.*, 2010** an amount of 105 hamburgers were evaluated for facultative aerobic and/or anaerobic mesophilic bacteria, coliform counts, *Staphylococcus*, *Bacillus cereus,* sulfite-reducing clostridia and *Salmonella* spp. The hamburgers were unsuitable for human consumption in 31.4% of cases because the positive samples for coliforms and *Staphylococcus* were contaminated at unacceptably high levels set by Brazilian standards.

In cheeseburgers, sulfite-reducing clostridia and coagulase positive staphylococci were less than < 10 cfu.g^{-1} but the highest counts were detected for coliforms which were isolated from samples in up to 1.5x10³ cfu.g^{-1} high counts (Table 5).

Table 5 Microbiological quality of cheeseburger samples (n=5)

Microorganisms	Results	No. of unsatisfactory samples /No. of samples (%)
Coliform bacteria	< 10 – 1.5x10³ cfu.g^{-1}	2/5 (40)
Sulfite-reducing clostridia	< 10 cfu.g^{-1}	0/0 (0)
Yeasts	< 10 – 5.0x10² cfu.g^{-1}	0/0 (0)
Microscopic filamentous fungi	< 10 – 1.6x10² cfu.g^{-1}	0/0 (0)
Coagulase positive staphylococci	< 10 cfu.g^{-1}	0/0 (0)

Evaluating the microbiological quality of cheeseburgers, two samples (40%) were not accordance with the requirements of **CA SR 2006.** Cheesburgers and other sandwiches were found to be contaminated in previous studies and studies on the microbiological quality of sandwiches sold on the streets of São Paulo and Rio Grande do Sul showed high levels of contamination by fecal Coliforms **(Lopes, 2005).** These results probably reflect the inappropriate conditions during the preparation of food -dirtiness of the water used for hand washing by the sandwich makers, insufficient hand washing, insufficiently-heated food, and unsuitable conditions of ingredients storage. Therefore, the likelihood of contamination by fecal coliforms in the sandwiches sold on Brazilian roadsides is similar to that which occurs in other countries in Latin America **(Garin*et al.*, 2002).** Results of the present study show that the contamination of cheeseburger samples could be an actual problem for food retailed in Slovakia.

In hot-dogs, the lowest isolation rates were obtained for coliform sulfite-reducing clostridia and microscopic filamentous fungi – less than 10 cfu.g^{-1} and less than 10 to 2.4x10² cfu.g^{-1}, respectively. The highest counts were obtained for coliform bacteria, yeasts and coagulase positive staphylococci (Table 6).

Table 6 Microbiological quality of hot-dog samples (n=31)

Microorganisms	Results	No. of unsatisfactory samples /No. of samples (%)
Coliform bacteria	< 10 – 1.4x10³ cfu.g^{-1}	3/31 (10)
Sulfite-reducing clostridia	< 10 cfu.g^{-1}	0/0 (0)
Yeasts	< 10 – 2.1x10³ cfu.g^{-1}	1/31 (3)
Microscopic filamentous fungi	< 10 – 2.4x10² cfu.g^{-1}	0/0 (0)
Coagulase positive staphylococci	< 10 – 1.4x10² cfu.g^{-1}	1/31 (3)

Among the hot-dog samples three (3/ 31/ 10) shared the counts of coliform bacteria which were not in accordance with the limit set by **CA SR 2006** and the testing results ranged from less than 10 to 1.4x10³ cfu.g^{-1}. Yeasts counts ranged from less tan 10 to 2.1.10³ cfu.g^{-1} and one sample (1/ 31/ 3)had unsatisfactory results. Coagulase positive staphylococci values were from less than 10 to 1.4x10² cfu.g^{-1} and one sample had unsatisfactory results.

Hot-dogs are frequently are found to be contaminated with coliforms and staphylococci and this corresponds to our results **(Kothe *et al.*, 2016).** In **Kothe *et al.*, 2016)** study, 75% of the hot-dogs were contaminated with total coliforms, 30% of them presented fecal coliforms while 25% coagulase-positive staphylococci levels above the maximum limit permitted by Brazilian regulations. Reason for this could ne not adequate hygienic and sanitary conditions of hot-dog vendors in Southern Brazil and this results were based on questionnaire completed for evaluation of sanitary conditions of vendors. Unconformities related to storage of defrosted sausages at environmental temperature or inadequate cooling, absence of thermometer in vendors, usage of non-potable water were described. The lack of cross contamination preventive measures, lack of time and temperature controls and the use of ingredients with unknown origins were also the important factors which could influence the microbiological safety of hot-dogs in Southern Brazil (**Kothe *et al.*, 2016**).

In roasts, counts of sulfite-reducing clostridia and coagulase positive staphylococci were less than 10 cfu.g^{-1}, but coliforms, yeasts and microscopic filamentous fungi ranged from less than 10 to 2.1x10² cfu.g^{-1} , less than 10 to 3.6.x10³ cfu.g^{-1} and less than 10 to 3.2x10² cfu.g$^{-1.}$ respectively (Table 7).

Table 7 Microbiological quality of in roasts (n=14)

Microorganisms	Results	No. of unsatisfactory samples /No. of samples (%)
Coliform bacteria	< 10 – 2.1x10² cfu.g^{-1}	1/14 (7)
Sulfite-reducing clostridia	< 10 cfu.g^{-1}	0/0 (0)
Yeasts	< 10 – 3.6x10³ cfu.g^{-1}	1/14 (7)
Microscopic filamentous fungi	< 10 – 3.2x10² cfu.g^{-1}	1/14 (7)
Coagulase positive staphylococci	< 10 cfu.g^{-1}	0/0 (0)

Regarding conformity with microbiological quality criteria, in roasts in one sample 1/ 14 (7) the counts of coliforms did not meet the critera were set. The counts of yearsts and of microscopic filamentous fungi were unsatisfactory in 1/14 (7) and 1/14 (7) of samples, respectively. The safety of foods is affected by several common factors including the quality of the raw materials, food handling and storage practices. In RTE street vendors the hygiene is mostly affected, that could led to contamination of foods. In most cases, running water is not continuously supplied for hand and dishwashing, cooking or drinking, leading the street vendors to store water under vulnerable conditions subject to contamination. Street foods are exposed to aggravating environmental conditions, such as the presence of insects, rodents, other animals and air pollution studies **(Lucca and Torres, 2002; Sobel *et al.*, 1998).** Furthermore, most food vendors ignore good food handling practices, exposing foods to dangerous conditions such as cross-contamination, unsafe storage and poor time-temperature conditions **(Ekanem, 1998).**

The presence of fecal coliforms (30%) in the samples in the previous study indicates a high risk that other pathogenic organisms have also contaminated the food. *E. coli* was present in 22.5% of the samples, probably from raw vegetables and due to the lack of good hygienic practices. and 70% of tuna and 40% of chicken sandwiches were unsuitable according to the sanitary standards for fecal coliforms in study completed in the northen region of Brazil **(Damsceno and Cardonha, 1999).**

In general, the microbiological criteria set for RTE foods in Slovak Republic were violated in 36/ 144 (25). Mostly, unconformity with the microbiological criteria were observed for kebabs and cheeseburgers – 40% for each, respectively. The lowest amount of unsatisfactory samples were observed among

the hot-dogs were tested – 9.67%. Distribution of unsatisfactory samples of RTE food products produced in Slovak Republic is shown in Figure 1.

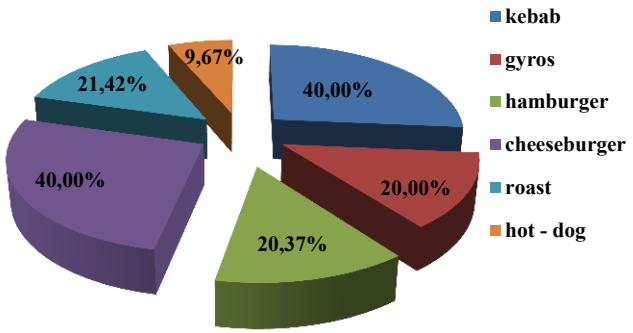

Figure 1 Distribution of unsatisfactory samples (n=36) according to the microbiological criteria set by SR CA, 2006

The results of the present study show that unconformity with the microbiological criteria was faced in 25% of all samples tested, therefore RTE foods produced in Slovakia should considered to pose a possible risk to consumers. The efficient use of HACCP can improved the microbiological safety and quality of these products **(Vazgecer *et al.*, 2004).**

CONCLUSION

Finally, we hope that the systematic microbiological testing of the RTE foods as conducted at the present study could help to assess a risk which poses each RTE product and to define better the control measures needed in order to prevent foodborne infections related to the consumption of RTE foods. This research shows that eating of RTE foods might pose public health concerns even though as there have not been any report of outbreaks related to consumption of RTE foods in Slovakia. However, to ensure the safety and health of their customers, fast-food restaurants should inculcate good hygienic practices and habits in their staff and food processing. The critical control points to preventing food borne illness such as preventing cross – contamination from the raw products to RTE foods, using adequate time and temperatures for cooking, avoiding recontamination after cooking, by surfaces previously contaminated with the raw meat, and properly chilling and storing meat after mincing should be emphasized. Food handlers should also be trained on hygienic food handling practices and safety.

Acknowledgments: This work was supported by grant VEGA 1/0611/14.

REFERENCES

Agbodaze, D., Nmai, P., Robertson, F., Yeboah-Manu, D., Owusu-Darko, K. & Addo, K. (2005). Microbiological quality of "khebab" consumed in the accra metropolis. *Ghana Medical Journal*, *39*(2), 46-9. http://dx.doi.org/10.4314/gmj.v39i2.35981

Almualla, N. A., Laleye, L. C., Abushelaibi, A. A., Al-Qassemi, R. A., Wasesa, A. A., & Baboucarr, J. (2010). Aspects of the microbiological quality and safety of ready-to-eat foods in Sharjah supermarkets in the United Arab Emirates. *Journal of Food Protection*, *73*(7), 1328-1331.

Castro-Rosas, J., Cerna-Cortés, J. F., Méndez-Reyes, E., Lopez-Hernandez, D., GómezAldapa, C. A., & Estrada-Garcia, T. (2012). Presence of faecal coliforms, Escherichia coli and diarrheagenic E. coli pathotypes in ready-to-eat salads, from an area where crops are irrigated with untreated sewage water. *International Journal of Food Microbiology*, *156*(2), 176-180. http://dx.doi.org/10.1016/j.ijfoodmicro.2012.03.025

Christison, C. A., Lindsay, D., & von Holy, A. (2008). Microbiological survey of readyto-eat foods and associated preparation surfaces in retail delicatessens, Johannesburg, South Africa. *Food Control*, *19*(7), 727-733. http://dx.doi.org/10.1016/j.foodcont.2007.07.004

Chapman, B., Eversley, T., Fillion, K., MacLaurin, T. & Powell, D. (2010). Assessment of food safety practices of food service food handlers (risk assessment data): testing a communication intervention (evaluation of tools). *Journal of Food Protection*, *73*(6), 1101-1107.

Codex Alimentorius SR 2006. Potravinový kódex SR 2006: Výnos MP SR a MZ SR zo 6. februára 2006 č. 06267/2006-SL, ktorým sa vydáva hlava PK SR upravujúca mikrobiologické požiadavky na potraviny a na obaly na ich balenie. p. 1-62. Available at: http://www.svps.sk/legislativa/legislativa_kodex.asp.

Damsceno, K. S. F. S. C., & Cardonha, A. M. S. (1999). Perfil microbiologico de ‘‘sandu ıches naturais’’ comercializados em Natal nas lanchonetes da Universidade Federal do Rio Grande do Norte. Revista Higiene Alimentar, 13(65), 47–50.

Dinucci Bezerra, A.C., Baptista dos Reis, R., Markowicz Bastos, D.H. (2010). Microbiological quality of hamburgers sold in the streets of Cuiabá - MT, Brazil and vendor hygiene-awareness. *Ciência e Tecnologia de Alimentos*, *30*(2), 520-524. http://dx.doi.org/10.1590/S0101-20612010000200035
Dinucci Bezerra, A.C., Baptista dos Reis, R., Markowicz Bastos, D.H. (2010). Microbiological quality of hamburgers sold in the streets of Cuiabá - MT, Brazil and vendor hygiene-awareness. *Ciência e Tecnologia de Alimentos*, *30*(2), 520-524. http://dx.doi.org/10.1590/S0101-20612010000200035
Ekanem, E. O. (1998). The street food trade in Africa: Safety and socio-environmental issues. *Food Control*, *9*(14), 211–215. http://dx.doi.org/10.1016/s0956-7135(97)00085-6

European Food Safety Authority, European Centre for Disease Prevention and Control. (2011). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2009. EFSA Journal, 9(3), 2090 [378 pp.]. Available online. www.efsa.europa.eu/efsajournal.

Evans, M. R., Sarvotham, T., Thomas, D. R., & Howard, A. J. (2006). Domestic and travel-related foodborne gastrointestinal illness in a population health survey. *Epidemiology and Infection*, *134*(4), 686-693. http://dx.doi.org/10.1017/s0950268805005790

Fang, T. J., Wei, Q. K., Liao, C. W., Hung, M. J., & Wang, T. H. (2003). Microbiological quality of 18 C ready-to-eat food products sold in Taiwan. *International Journal of Food Microbiology*, *80*(3), 241-250. http://dx.doi.org/10.1016/s0168-1605(02)00172-1

FEHD (Food and Environmental Hygiene Department). (2007). Microbiological guidelines for ready-to-eat food.

Garayoa, R., Vitas, A. I., Díez-Leturia, M. & García-Jalón, I. (2011). Food safety and the contract catering companies: food handlers, facilities and HACCP evaluation. *Food Control*, *22*, 2006-2012. http://dx.doi.org/10.1016/j.foodcont.2011.05.021

Garin, B., Aïdara, A., Spiegel, A., Arrive, P., Bastaraud, A., Cartel, J.L., Aissa, R.B., Duval, P., Gay, M., Gherardi, C., Gouali, M., Karou, T.G., Kruy, S.L., Soares, J.L., Mouffok, F., Ravaonindrina, N., Rasolofonirina, N., Pham, M.T., Wouafo, M., Catteau, M., Mathiot, C., Mauclere, P., & Rocourt, J. Multicenter study of street foods in 13 towns on four continents by the food and environmental hygiene study group of the international network of Pasteur and Associated Institutes. *Journal of Food Protection*, *65*(1), 146-152.

Gillespie, I., Little, C. & Mitchell, R. (2000). Microbiological examination of cold ready-to-eat sliced meats from catering establishments in the United Kingdom. *Journal of Applied Microbiology*, *88*, 467-474. http://dx.doi.org/10.1046/j.1365-2672.2000.00981.x

Microbiological quality of selected street foods from a restricted area of S~ao Paulo city, Brazil Agnes Hanashiro, Marisa Morita, Glavur R. Matte, Maria H. Matte *, Elizabeth A.F.S. Torres. Food Control 16 (2005) 439–444

Käferstein, F. K., Motarjemi, Y., & Bettcher, D. W. (1997). Foodborne disease control: a transnational challenge. *Emerging Infectious Diseases*, *3*(4), 503-510. http://dx.doi.org/10.3201/eid0304.970414

Kothe, C.I., Schild, C.H., Tondo, T.C., & da Silva Malheiros, P. (2016). Microbiological contamination and evaluation of sanitary conditions of hot dog street vendors in Southern Brazil. *Food Control, 62,* 346-350. http://dx.doi.org/10.1016/j.foodcont.2015.11.005

Kotula, K. L., & Davis, M. E. (1999). Broiler skin sampling for optimum recovery of *Salmonella* spp. *Journal of Food Protection*, *62*(3), 284–286.

Legnani, P., Leoni, E., Berveglieri, M., Mirolo, G., & Alvaro, N. (2004). Hygienic control of mass catering establishments, microbiological monitoring of food and equipment. *Food Control*, *15*, 205-211. http://dx.doi.org/10.1016/s0956-7135(03)00048-3

Lopes, G.V. (2005). Alimentos de rua: quão perigosos eles são? *Diário Popular*. 2005. Disponível em: <http://www.diariopopular.com.br/22_11_05/artigo.html>.

Lucca, A., & Torres, E. A. F. S. (2002). Condicoes de higiene de ‘‘cachorro-quente’’ comercializado em vias publicas. *Revista de Sau de Publica*, *36*(3), 350–352. http://dx.doi.org/10.1590/s0034-89102002000300015

Martinez-Tomé, M., Vera, A. M., & Murcia, M. (2000). Improving the control of food production in catering establishments with particular reference to the safety of salads. *Food Control*, *11*, 437-445. http://dx.doi.org/10.1016/s0956-7135(00)00006-2

Martins, E. A., & Germano, P. M. L. (2008). Microbiological indicators for the assessment of performance in the hazard analysis and critical control points (HACCP) system in meat lasagna production. *Food Control*, *19*, 764-771. http://dx.doi.org/10.1016/j.foodcont.2007.08.001

Marzano, M. A., & Balzaretti, C. M. (2011). Cook-serve method in mass catering establishments: is it still appropriate to ensure a high level of microbiological quality and safety? *Food Control*, *22*, 1844-1850. http://dx.doi.org/10.1016/j.foodcont.2011.04.024

Mataragas, M., Skandamis, P.N., & Drosinos, E.H., 2008. Risk profiles of pork and poultry meat and risk ratings of various pathogen/product combinations. *International Journal of Food Microbiology*, *126*(1–2), 1–12. http://dx.doi.org/10.1016/j.ijfoodmicro.2008.05.014

Min, M., Dawson, C.O., & Hussain, M.A. (2013). Microbiological Risk Assessment of Hamburgers Sold in Canterbury New Zealand. *Internet Journal of Food Safety*, 13, 99-102.

Ravel, A., Nesbitt, A., Marshall, B., Sittler, N., & Pollari, F. (2011). Description and burden of travel-related cases caused by enteropathogens reported in a Canadian community. *Journal of Travel Medicine*, *18*(1), 8-19. http://dx.doi.org/10.1111/j.1708-8305.2010.00471.x

Rodríguez, M., Valero, A., Carrasco, E., Pérez-Rodríguez, F., Posada, G. D., & Zurera, G. (2011a). Hygienic conditions and microbiological status of chilled ready-to-eat products served in Southern Spanish hospitals. *Food Control*, *22*, 874-882. http://dx.doi.org/10.1016/j.foodcont.2010.11.015

Rodríguez, M., Valero, A., Posada-Izquierdo, G. D., Carrasco, E., & Zurera, G. (2011b). Evaluation of food handler practices and microbiological status of ready-to-eat foods in long-term care facilities in Andalusia Region of Spain. *Journal of Food Protection*, *74*(9), 1504-1512.

Santana, N. G., Almeida, R. C. C., Ferreira, J. S., & Almeida, P. F. (2009). Microbiological quality and safety of meals served to children and adoption of good manufacturing practices in public school catering in Brazil. *Food Control*, *20*, 255-261. http://dx.doi.org/10.1016/j.foodcont.2008.05.004

Seow, J., Ágoston, R., Phua, L., & Yuk, H. G. (2012). Microbiological quality of fresh vegetables and fruits sold in Singapore. *Food Control*, *25*(1), 39-44. http://dx.doi.org/10.1016/j.foodcont.2011.10.017

Sobel, J., Mahon, B., Mendoza, C. E., Passaro, D., Cano, F., Baier, K., Racioppi, F., Hutwagner, L., & Mintz, E. (1998). Reduction of fecal contamination of street-vended beverages in Guatemala by a simple system for water purification and storage, handwashing, and beverage storage. *American Journal of Tropical Medicine Hygiene*, *59*(3), 380–387.

STN EN ISO 4932: 2006. Microbiology of food and animal feeding stuffs. Horizontal method for the enumeration of coliforms. Colony-count technique.

STN EN ISO 13401: 2001. Microbiology of food and animal feeding stuffs. Horizontal method for the enumeration of *Clostridium perfringens*. Colony-count technique.

STN EN ISO 6888-1: 2001. Microbiology of food and animal feeding stuffs. Horizontal method for the enumeration of coagulase-positive staphylococci (Staphylococcus aureus and other species). Part 1: Technique using Baird-Parker agar medium.

STN EN ISO 21527-2: 2010. Microbiology of food and animal feeding stuffs. Horizontal method for the enumeration of yeast and moulds. Part 1, Colony-count technique in products with water activity greater than 0,95 =

Tessi, M. A., Arìngoli, E. E., Pirovani, M. E., Vincenzini, A. Z., Sabbag, N. G., Costa, S. C., Garcia, C.C., Zannier, M.S., Silva, E.R., & Moguilevsky, M.A. (2002). Microbiological quality and safety of ready-to-eat cooked foods from a centralized school kitchen in Argentina. *Journal of Food Protection*, *65*(4), 636-642.

United Nation World Tourism Organization. (2011). UNWTO annual report 2010 a year of recovery. Madrid: Spain, Available from https://s3-eu-west-1. amazonaws.com/storageapi/sites/all/files/pdf/final_annual_report_pdf.pdf.

USFDA (United States Food and Drug Administration). (2009). Food code 2009.

Vazgecer, B., Ulu, H. & Oztan, A 2009. Microbiological and chemical qualities of chicken doner € kebab retailed on the Turkish restaurants. *Food Control*, *15*, 261–264. http://dx.doi.org/10.1016/s0956-7135(03)00065-3

Veiros, M. B., Proença, R. P. C., Santos, M. C. T., Kent-Smith, L., & Rocha, A. (2009). Food safety practices in a Portuguese canteen. *Food Control*, *20*, 936-941. http://dx.doi.org/10.1016/j.foodcont.2009.02.002

Verbeke, W., Pérez-Cueto, F.J.A., Barcellos, M.D., Krystallis, A., & Grunert, K.G. (2010). European citizen and consumer attitudes and preferences regarding beef and pork. *Meat Science*, *84*(2), 284–292. http://dx.doi.org/10.1016/j.meatsci.2009.05.001

World Health Organization. (2007). Food safety and foodborne illness e Fact sheet no. 237. Available from http://www.who.int/mediacentre/ factsheets/ fs237/en/.

Yoon, Y., Kim, S. R., Kang, D. H., Shim, W. B., Seo, E., & Chung, D. H. (2008). Microbial assessment in the school foodservices and recommendations for food safety improvement. *Journal of Food Science*, *73*(6), M304-M313. http://dx.doi.org/10.1111/j.1750-3841.2008.00828.x

Ziino, G., Gurrera, G., & Beninati, C. Microbiological quality of kebabs sold in Palermo and Messina. *Italian journal of food safety*, *2*, 277-80. http://dx.doi.org/10.4081/ijfs.2013.e23

JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

ANTIMICROBIAL ACTIVITY OF CRUDE ETHANOLIC EXTRACTS FROM SOME MEDICINAL MUSHROOMS

Lukáš Hleba[1], Matúš Kompas[1], Jana Hutková[1], Martin Rajtar[3], Jana Petrová[1], Juraj Čuboň[2], Attila Kántor[1], Miroslava Kačániová[1]

Address(es): Ing. Lukáš Hleba, PhD.,
[1]Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Microbiology, Tr. Andreja Hlinku 2, 949 76 Nitra, Slovakia.
[2]Faculty of Biotechnology and Food Sciences, Department of Animal Products Evaluation and Processing, Tr. Andreja Hlinku 2, 949 76 Nitra, Slovakia.
[3]Mykoforest Martin Rajtar, Velčice 133, 95171 Velčice, Slovakia.

*Corresponding author: lukas.hleba@gmail.com

doi: 10.15414/jmbfs.2016.5.special1.60-63

ARTICLE INFO

Received 17. 12. 2015
Revised 22. 1. 2016
Accepted 28. 1. 2016
Published 8. 2. 2016

Regular article

OPEN ACCESS

ABSTRACT

In this paper the antimicrobial activity of 1 year old crude ethanolic extracts obtained from *Cordyceps sinesis*, *Laricifomes officinalis, Oudemansiella mucida* and *Coprinus comatus* were investigated. The antimicrobial activities of extracts against two Gram-positive bacteria (*Bacillus thuringiensis, Staphylococcus aureus*) and two Gram-negative bacteria (*Klebsiella pneumoniae, Enterobacter aerogenes*) were determined by disk diffusion and microbroth dilution method according by EUCAST in 96-well microplates. Microorganisms were obtained from Czech Collection of Microorganisms. Absorbance after and before the experiment were substracted, converted to binary system and obtained values to Probit analysis were used. Not all macromycetes ethanolic extracts showed antimicrobial activity against tested bacteria. Antimicrobial activity determined by MIC methodology showed extracts from *Oudemansiella mucida, Cordyceps sinesis, Coprinus comatus* in the tested range. Conversely, the best antimicrobial activity tested by disc diffusion methods showed extract from *Laricifomes officinalis*. Equally, more better studying of antimicrobial activity in these mushrooms will needed.

Keywords: Antimicrobial activity, macromycetes ethanolic extracts, MIC, edible mushrooms

INTRODUCTION

Nature is a very good source of many medical compounds for thousands of years. In the last decades problem with antibiotic resistant bacteria has emerged. Bacterial pathogens have evolved numerous defense mechanisms against antimicrobial agents, and nowadays, the need to discover new and more potent of these agents as accessories or alternatives to antibiotic therapy is stronger (**Butler *et al*., 2004; Lam *et al*., 2007**). Macromycetes as higher fungi are rich sources of biologically active compounds with an enormous variety of chemical structures. Therefore, mushrooms could be useful in the search of new potent antimicrobial agents (**Alves *et al*., 2012**). Mushrooms need antibacterial compounds to survive in their natural environment. It is therefore not surprising that antimicrobial compounds with more or less strong activities could be isolated from many mushrooms and that they could be of benefit for human (**Lindequist *et al*., 1990**). There is many different studies about antimicrobial activity of different types of fungal extracts from India (**Seena *et al*., 2003; Quereshi *et al*., 2010**) and China (**Gao *et al*., 2005**). In this country fungi medicine has tradition for many years ago. For example, *Ganoderma lucidum* is a one of the most famous traditional medicinal fungi, being used as functional food and in preventive medicines, mostly in the form of extracts with an annual global market (**Sullivan *et al*., 2005; Pala *et al*., 2011**). But only compounds from microscopic fungi are on the market as antibiotics till now (**Lindequist *et al*., 2005**).
The present work is focus to antimicrobial activity of 1 year old medicinal mushrooms extracts isolated from *Cordyceps sinesis*, *Laricifomes officinalis, Oudemansiella mucida* and *Coprinus comatus* against some selected Gram-positive and Gram-negative bacteria.

MATERIALS AND METHODS

Fungi materials

The fungi materials used in this experiment consist from SSF (Solid State Fermentation) of *Cordyceps sinesis* and fruiting bodies (basidiocarps) of *Laricifomes officinalis, Oudemansiella mucida* and *Coprinus comatus*. Dried fungi SSF and fruiting bodies were obtained from Mykoforest company, Slovakia. Selected fungi were identified by Martin Rajtar (Mykoforest, Slovakia). Fungi were dried at the room temperature in the dark. More detailed information are showed in Table 1.

Table 1 Additional information about tested fungi

Orig. Latin title	Fungal part	Yield*	Origin	Dissolving time	Extracted by
Cordyceps sinesis	SSF	0.1006	Mykoforest	1 year at room temperature	Vacuum evaporator from methanol at room temperature at -800 mbar
Laricifomes officinalis	fruiting body	1.7269	Mykoforest		
Oudemansiella mucida	fruiting body	1.0893	Mykoforest		
Coprinus comatus	fruiting body	0.3379	Mykoforest		

* Obtained yield of evaporated ethanolic extracts in g, SSF – Solid state fermentation

Test microorganisms

Four strains of microorganisms were tested in this research. Two Gram-negative bacteria include *Klebsiella pneumoniae* CCM 2318, *Enterobacter aerogenes* CCM 2531, two Gram-positive bacteria include *Staphylococcus aureus* subsp. *aureus* ser. a5 CCM 2461, *Bacillus thiringiensis* CCM 19. All tested strains were collected from the Czech Collection of Microorganisms. The bacterial suspensions were cultured in the nutrient broth (Imuna, Slovakia) at 37 °C, expect *Bacillus thiringiensis* which was cultivated at 30°C.

Preparation of fungal extracts

After drying, the fungal materials were crushed, weighed out to 10g and soaked separately in 100 mL of ethanol p.a. (99,5 %, Sigma, Germany) during 1 year at room temperature in the dark. Why one year? The main reason was determining of antimicrobial activity after the long time of storage. Exposure to sunlight was avoided to prevent the degradation of active components. Then, ethanolic fungal extracts were subjected to evaporation under reduced pressure at 40 °C in order to remove the ethanol (Stuart RE300DB rotary evaporator, Bibby scientific limited, UK, and vacuum pump KNF N838.1.2KT.45.18, KNF, Germany). For the antimicrobial assay, the crude fungal extracts were dissolved in dimethyl sulfoxid (DMSO) (Penta, Czech Republic) to equal 102.4 mg/mL as stock solution. Stock solutions of fungal extracts were stored at -16 °C in refrigerator until use.

Preparation of discs and disc diffusion method

Synchronously with evaporation of ethanol from mushroom extract blank discs (Oxoid, UK) were added to extracts and impregnated with extracts. Discs stayed in extracts until evaporated completely. Obtained impregnated discs served as pre-determination experiment for detection of antimicrobial activity. Concentration of extracts in discs were unknown. Impregnated discs were used for disc diffusion methodology, which was done on Mueller-Hinton agar (Biolife, Italy) at 37 °C for three bacteria, expect *Bacillus thuringiensis* (30°C) during 16-20 hours. Bacterial inoculum in physiological solution at the final density of 0,5 McF° was spread out on the agar surface evenly. Impregnated discs were stacked on to the agar surface evenly with adequate spacing. Inhibition zones were read in millimeter.

Antimicrobial assay

The minimum inhibitory concentration (MIC) is the lowest concentration of the sample that will inhibit the visible growth of microorganisms. Fungal extracts dissolved in DMSO were prepared to a final concentration of 4096 µg/mL. Minimum inhibitory concentrations (MICs) were determined by the microbroth dilution method according to the Clinical and Laboratory Standards Institute recommendation (**CLSI, 2009**) in Mueller Hinton broth (Biolife, Italy) for bacteria. Briefly, the DMSO fungal extracts solutions were prepared as serial two-fold dilutions, in order to obtain a final concentration ranging between 2 – 4096 µg/mL. Each well was then inoculated with microbial suspension at the final density of 0.5 McF°. After incubation at 37 °C for three bacteria and 30 °C for *Bacillus thuringirnsis* during 16-20 hours. The inhibition of microbial growth was evaluated by measuring the well absorbance at 590 nm in an absorbance microplate reader Biotek EL808 with shaker (Biotek Instruments, USA). The 96 micro-well plates were measured before and after experiment. Differences between both measurements were evaluated as growth. Measurement error was established for 0.05 values from absorbance. Wells without fungal extracts were used as positive controls of growth. Pure DMSO was used as negative control. This experiment was done in eight-replicates for a higher accuracy of the minimum inhibitory concentrations of used fungal extracts.

Statistical analysis

Using obtained absorbance before and after the analysis, we were able to express the differences in absorbance between the measurements as a set of binary values. These values were assigned to exact concentrations. The following formula was created for this specific experiment: value 1 (inhibitory effect) was assigned to absorbance values lower than 0.05, while value 0 (no effect or stimulant effect) was assigned to absorbance values higher than 0.05. For this statistical evaluation Probit analysis in Statgraphic software was used.

RESULTS AND DISCUSSION

Disk diffusion method

Results from disc diffusion tests showed antimicrobial activity in the case of extracts from *Laricifomes officilnalis* and *Oudemansiella mucida*. Some authors (**Anke *et al*., 1979; Anke *et al*., 1990; Florianowicz *et al*., 1999**) presented results that extract from *Oudemansiella mucida* inhibited only fungal cells. In their study, scientists determined that main compounds strobilurins and oudemansins inhibited growth of yeasts like *Candida albicans*, *C. glabrata*, *C. krusei* and *C. tropicalis*. In our study we determined inhibition effect against *Staphylococcus aureus* too, which is showed in Figure 1 D, sample 9. Inhibition zone (12 mm) around *Oudemansiella* mucida extract was formed in the case of *Staphylococcus aureus* only. Extracts from *Laricifomes officinalis* had inhibitory activity against the all used bacterial strains in this experiment. Inhibition zones around the *Laricifomes officinalis* extract were formed in the case of *Staphylococcus aureus* (27 mm), *Bacillus thuringiensis* (13 mm), *Klebsiella pneumoniae* (20 mm) and *Enterobacter aerogenes* (20 mm). Other used macromycetes extracts in this study didn't showed antimicrobial activity. In the other side authors like **Demir and Yamac, 2008** tested *Coprinus comatus* basidiocarp extract dissolved in different solutions and they determined its antimicrobial activity against *Staphylococcus aureus*, *Enterococcus faecium*, *Proteus vulgaris* and *Candida glabrata*. They tested submerged mycelium and some exopolysacharides from *Coprinus comatus*, but they didn't determined so extensive activity like in previous test with basidiocarps. Inhibition zones are showed on the Figure 1.

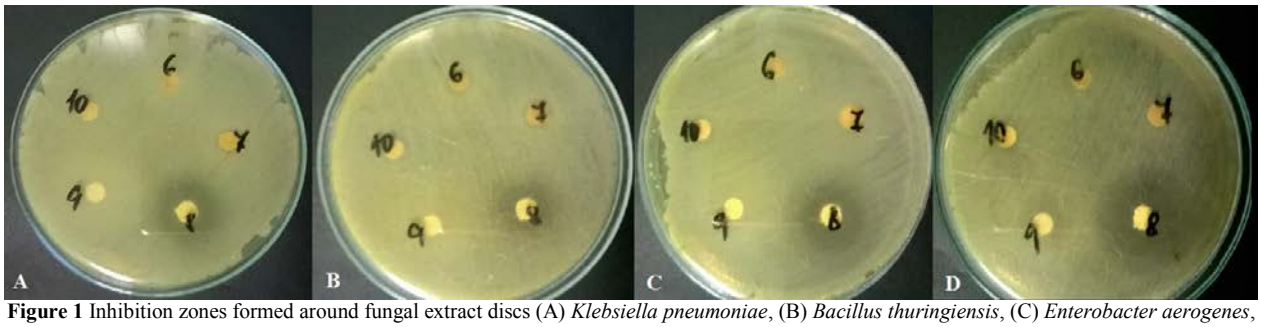

Figure 1 Inhibition zones formed around fungal extract discs (A) *Klebsiella pneumoniae*, (B) *Bacillus thuringiensis*, (C) *Enterobacter aerogenes*, (D) *Staphylococcus aureus*, (6) *Coprinus comatus*, (7) *Cordyceps sinensis*, (8) *Laricifomes officinalis*, (9) *Oudemansiella mucida* and (10) not presented extract in this study

Minimal inhibition concentration

The antimicrobial activity (expressed as µg/mL) of four ethanolic fungal extracts from *Cordyceps sinesis*, *Laricifomes officinalis, Oudemansiella mucida* and *Coprinus comatus* against four strains of bacteria are summarized in Table 2. The most effective was tested fungal extract from *Coprinus comatus* against *Enterobacter aerogenes* with a MIC 50 value 2048 ug/mL. Also, *Oudemansiella mucida* extract showed inhibitory activity against tested bacteria. Extract from *Laricifomes officinalis* inhibited the growth of all bacterial strains tested by disk diffusion method, but minimal inhibition concentration method didn't showed any inhibition activity in tested concentration range. Some studies about antimicrobial activity of *Cordyceps sinensis* determined that main compounds cordycepin had effects against *Clostridium perfringens, C. paraputrificum, Bifidobacterium* spp. and *Lactobacillus* spp. (**Kniefel *et al*., 1977; Ahn *et al*., 2000**). There are exist many studies about antimicrobial activity of higher fungi (**Yoon *et al*., 1994; Rosa *et al*., 2003; Poucheret *et al*., 2006; Molitoris 1994; Lindequist *et al*., 2005**) because is known that mushrooms need antibacterial and antifungal compounds to survive in their natural environment. It is therefore not surprising that antimicrobial compounds with more or less strong activity could be isolated from many mushrooms and that they could be of benefit for human (**Lindequist *et al*., 1990**). But only compounds from microscopic fungi are on the market and antibiotics till now (**Lindequist *et al*., 2005**).

Table 2 The minimum inhibitory concentration (MIC) of ethanolic fungal extracts on four test bacteria

Tested bacteria	Antimicrobial activity of macromycetes fungal extract (ug/mL)							
	Cordyceps sinensis		***Laricifomes officinalis***		***Oudemensiella mucida***		***Coprinus comatus***	
	MIC 50	**MIC 90**	**MIC 50**	**MIC 90**	**MIC 50**	**MIC 90**	**MIC 50**	**MIC 90**
Gram-positive bacteria								
Bacillus thuringiensis CCM	> 4096	> 4096	> 4096	> 4096	3064,03	3257,04	> 4096	> 4096
Staphylococcus aureus CCM	4096	> 4096	> 4096	> 4096	4096	> 4096	3064,03	3257,04
Gram-negative bacteria								
Klebsiella pneumoniae CCM	4096	> 4096	> 4096	> 4096	3064,03	3257,04	3064,03	3257,04
Enterobacter aerogenes CCM	3064,03	3257,04	> 4096	> 4096	3064,03	3257,04	2048	2285,99

Legend: Abr. – abbreviations,

CONCLUSIONS

In conclusion, we can state that the ethanolic fungal extracts of all fungi (*Cordyceps sinesis, Laricifomes officinalis, Oudemansiella mucida* and *Coprinus comatus*) showed antimicrobial activity in high concentration. Very interesting in this experiment was that extract from *Laricifomes officinalis* showed strongest antimicrobial activity detected by disk diffusion methodology and not by MIC methodology. We think that more studies and experiments and more range of concentration of fungal extracts are needed for better information about antimicrobial activity of macro fungal extracts.

Acknowledgements: This work was funded by VEGA 1/0611/14 and APVV-0304-12.

REFERENCES

Ahn, Y. J., Park, S. J., Lee, S. G., Shin, S. C., & Choi, D. H. (2000). Cordycepin: Selective Growth Inhibitor Derived from Liquid Culture of Cordyceps m ilitaris against Clostridium spp. *Journal of agricultural and food chemistry*, *48*(7), 2744-2748. http://dx.doi.org/10.1021/jf990862n

Al-Fatimi, M., Wurster, M., Kreisel, H., Lindequist, U. 2005. Antimicrobial, cytotoxic and antioxidant activity of selected basidiomycetes from Yemen. Die Pharmazie-An International Journal of Pharmaceutical Sciences, 60(10), 776-780.

Alves, M.J., Ferreira, I.C.F.R., Dias, J., Teixeira, V., Martins, A., Pintado, M. 2012. A review on antimicrobial activity of mushrooms (Basidiomycetes) extracts and isolated compounds. Planta Med., 78, 1707-1718. http://dx.doi.org/10.1055/s-0032-1315370

Balakumar, R., Sivaprakasam, E., Kavitha, D., Sridhar, S., Kumar, J. S. 2011. Antibacterial and antifungal activity of fruit bodies of Phellinus mushroom extract. International J. Biosc., 1(3), 72-77.

Butler, M.S. 2004. The role of natural product chemistry in drug discovery. Journal of Natural Products, 67, 2141-2153. http://dx.doi.org/10.1021/np040106y

Clinical and Laboratory Standard Institute. Performance standard for antimicrobial susceptibility testing; informational supplement M100-S21. 21th ed. Wayne, PA: CLSI; 2009.

Demir, M. S., & Yamac, M. (2008). Antimicrobial activities of basidiocarp, submerged mycelium and exopolysaccharide of some native Basidiomycetes strains. *Journal of Applied Biological Sciences*, *2*(3), 89-93.

Gao, Y., Tang, W., Gao, H., Chan, E., Lan, J., Li, X., Zhou, S. 2005. Antimicrobial activity of medical mushrooms *Ganoderma*. Food Rev. Int., 21, 211-229. http://dx.doi.org/10.1081/fri-200051893

Heleno, S.A., Ferreira, I.C.F.R., Esteves, A.P., Ćirić, A., Glamočlija, J., Martins, A., Soković, M., Queiroz, M.J.R.P. 2013. Antimicorbial and demelanizing activity of Ganoderma lucidum extract, p-hydroxybenzoic and cinnamic acids and their synthetic acetylated glucuronide methyl esters. Food and Chemical Toxicology, 58, 95-100. http://dx.doi.org/10.1016/j.fct.2013.04.025

Kavitha, D., Balakumar, R., Sivaprakasam, E., Sridhar, S., Suresh, J. 2011. Antibacterial and antifungal potential of fruit body extracts from daldinia concentrica (bolton) cesati & de notaris. International journal of pharmaceutical sciences and research, 2(9), 2376-2379.

Keller, C., Maillard, M., Keller, J., Hostettmann, K. 2002. Screening of European fungi for antibacterial, antifungal, larvicidal, molluscicidal, antioxidant and free-radical scavenging activities and subsequent isolation of bioactive compounds. Pharmaceutical biology, 40(7), 518-525. http://dx.doi.org/10.1076/phbi.40.7.518.14680

Keypour, S., Riahi, H., Moradali, M. F., Rafati, H. 2008. Investigation of the Antibacterial Activity of a Chloroform Extract of Ling Zhi or Reishi Medicinal Mushroom, Ganoderma lucidum (W. Curt.: Fr.) P. Karst. (Aphyllophoromycetideae), from Iran. International Journal of Medicinal Mushrooms, 10(4), 345-349. http://dx.doi.org/10.1615/intjmedmushr.v10.i4.70

Kneifel, H., Konig, W. A., Loeffler, W., & Müller, R. (1977). Ophiocordin, an antifungal antibiotic of Cordyceps ophioglossoides. *Archives of microbiology*,*113*(1-2), 121-130. http://dx.doi.org/10.1007/bf00428591

Lam, K.S. 2007. New aspects of natural products in drug discovery. Trends in Microbiology, 15, 279-289. http://dx.doi.org/10.1016/j.tim.2007.04.001

Lindequist, U., Niedermeyer, T. H., & Jülich, W. D. (2005). The pharmacological potential of mushrooms. *Evidence-Based Complementary and Alternative Medicine*, *2*(3), 285-299. http://dx.doi.org/10.1093/ecam/neh107

Lindequist, U., Teuscher, E., & Narbe, G. (1990). Neue Wirkstoffe aus Basidiomyceten. *Z Phytother*, *11*, 139-49.

Molitoris, H. P. (1994). Mushrooms in medicine. *Folia microbiologica*, *39*(2), 91-98. http://dx.doi.org/10.1007/bf02906801

Nikolovska-Nedelkoska, D., Atanasova-Pančevska, N., Amedi, H., Veleska, D., Ivanova, E., Karadelev, M., Kungulovski, D. 2013. Screening of antibacterial and antifungal activities of selected Macedonian wild mushrooms. *Zbornik Matice srpske za prirodne nauke*, (124), 333-340. http://dx.doi.org/10.2298/zmspn1324333n

Öztürk, M., Duru, M. E., Kivrak, Ş., Mercan-Doğan, N., Türkoglu, A., Özler, M. A. 2011. In vitro antioxidant, anticholinesterase and antimicrobial activity studies on three Agaricus species with fatty acid compositions and iron contents: A comparative study on the three most edible mushrooms. Food and Chemical Toxicology, 49(6), 1353-1360. http://dx.doi.org/10.1016/j.fct.2011.03.019

Pala, S.A. and Wani, A.H. 2011. Mushrooms: the entities with multifarious medicinal properties. J. Pharm. Res., 165, 496-500.

Poucheret, P., Fons, F., & Rapior, S. (2006). Biological and pharmacological activity of higher fungi: 20-year retrospective analysis. *Cryptogamie Mycologie*,*27*(4), 311.

Quereshi, S., Pandey, A.K., Sandhu, S.S. 2010. Evaluation of antibaterial activity of different Ganoderma lucisum extracts. J. Sci. Res., 3, 9-13.

Reinoso, R., Cajas-Madriaga, D., Martínez, M., San Martín, A., Pérez, C., Fajardo, V., Becerra, J. 2013. Biological Activity Of Macromycetes Isolated From Chilean Subantarctic Ecosystems. Journal of the Chilean Chemical Society, 58(4), 2016-2019. http://dx.doi.org/10.4067/s0717-97072013000400024

Rosa, L. H., Machado, K. M. G., Jacob, C. C., Capelari, M., Rosa, C. A., & Zani, C. L. (2003). Screening of Brazilian basidiomycetes for antimicrobial activity. *Memórias do Instituto Oswaldo Cruz*, *98*(7), 967-974. http://dx.doi.org/10.1590/s0074-02762003000700019

Sheena, N., Ajith, T. A., Mathew, A.T., Janardhanan, K.K. 2003. Antibacterial activity of three macrofungi, Ganoderma lucidum, Navesporus floccosa and Phellinus rimosus occuring in South India. Pharm. Biol., 41, 564-567. http://dx.doi.org/10.1080/13880200390501226

Suay, I., Arenal, F., Asensio, F.J., Basilio, A., Cabello, A.M., Díez, M.T., García, J.B., González del Val, A., Gorrochategui, J., Hermández, P., Peláez, F., Vincente, M.F. 2000. Screening of basidiomycetes for antimicrobial activities. Antoine van Leeuwenhoek Journal of Microbiology, 78(2), 129-140.

Sullivan, R., Smith, J.E., Rowan, N.J. 2006. Medicinal mushrooms and cancer therapy: translating a traditional practise into Western medicine. Perspect. Biol. Med., 49, 159-170. http://dx.doi.org/10.1353/pbm.2006.0034

Venturini, M.E., Rivera, C.S., Gonzalez, C., Blanco, D. 2008. Antimicrobial activity of extracts of edible wild and cultivated mushrooms against foodborne bacterial strains. Journal of Food Protection, 8, 1701-1706.

Yoon, S. Y., Eo, S. K., Kim, Y. S., Lee, C. K., & Han, S. S. (1994). Antimicrobial activity of Ganoderma lucidum extract alone and in combination with some antibiotics. *Archives of pharmacal research*, *17*(6), 438-442. http://dx.doi.org/10.1007/bf02979122

Zheng, H., Maoqing, Y., Liqiu, X., Wenjuan, T., Lian, L., Guolin, Z. 2006. Purification and characterization of an antibacterial protein from the cultured mycelia of Cordyceps sinensis. Wuhan University J. Nat. Sci., 11, 709-714. http://dx.doi.org/10.1007/bf02836695

JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

CHARACTERISTIC OF SELECTED SOIL STREPTOMYCETES WITH ANTIMICROBIAL POTENTIAL AGAINST PHYTOPATHOGENIC MICROORGANISMS

Ivana Charousová[1], Soňa Javoreková[1], Juraj Medo[1], Romy Schade[2]*

Address(es): Dipl.-Ing. Ivana Charousová,
[1] Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Microbiology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, +421 376414433.
[2] Helmholtz Centrum for Infection Research, Microbial Strain Collection Group, Inhoffenstraβe 7, 38124 Braunschweig, Germany.

*Corresponding author: ivanacharousova@gmail.com

doi: 10.15414/jmbfs.2016.5.special1.64-68

ARTICLE INFO

Received 26. 12. 2015
Revised 13. 1. 2016
Accepted 21. 1. 2016
Published 8. 2. 2016

Regular article

OPEN ACCESS

ABSTRACT

The main objective of the present study was isolation and identification of soil streptomycetes, having antibacterial and antifungal activity against 8 selected phytopathogenic microorganisms, which attack crops in Slovakia. Out of 27 morphologically different streptomycete isolates, 14 of them demonstrated antimicrobial activity against at least two tested microorganisms in primary screening. Ethylacetate extract used for secondary screening showed different inhibitory pattern in comparison with primary screening. The tested isolates were mostly active against Gram-positive bacterium *Clavibacter michiganensis subps. sepedonicus,* the causal agent of bacterial canker of tomaten and against fungus *Fusarium poae*, pathogen of cereals, which can also infest stored grains. Only three isolates, namely VY59, VY87 and VY47 showed broad spectrum of inhibition activity and therefore were used for further identification. On the basis of various morphological (color of aerial and substrate mycelium, growth, production of pigments and melanin on ISP media), physiological (optimal pH, temperature, NaCl tolerance and C-utilization) and biochemical (ApiZym and ApiCoryne stripes) methods these strains belonged to the genus *Streptomyces*. In addition to the antimicrobial profile, the strains differed in API ZYM test results, which imply that the selected strains might produce different antimicrobial substances. Throught the comparative analysis of 16S rRNA gene, the most active isolates contained different nucleotide sequences for the 16S rRNA gene. Sequence similarity search by BLAST program revealed that they show sequence similarities to *Streptomyces somaliensis* (VY59), *Streptomyces* sp. (VY87) and *Streptomyces albidoflavus* (VY47). These three isolates with broader spectrum of antimicrobial activity can be used in the development of substances for agriculture purposes.

Keywords: Soil, *Streptomyces* sp., primary and secondary screening, phytopathogenic microorganisms, characterization

INTRODUCTION

The growing human population will call for a significant increase in agricultural production. This challenge is made more difficult by the fact that changes in the environmental conditions under which crops are grown have resulted in the appearance of plant diseases **(Boyd *et al.*, 2013)**. Losses in crop production due to plant disease average 13% worldwide and severely limit production, quality, and safety food **(Oskay, 2009).** The application of fungicides and chemicals can control crop diseases to a certain extent, however, it is expensive and public concern for the environment has led to alternative methods of disease control to be sought, including the use of microorganisms as biological control agents **(Dhanasekaran *et al.*, 2012).** Because of this problem, many researchers are working on isolating actinomycetes which have the ability to degrade these harmful chemicals and also those with ability to act as bio control agents **(Prabhakar *et al.*, 2014).** Actinomycetes are the group of gram positive filamentous bacteria which are ubiquitous various natural and man-made environments. Actinomycetes are the most economically valuable prokaryotes **(Balagurunathan and Radhakrishnan, 2007)** producing antibiotics of agricultural and medical importance **(Tanaka and Omura, 1993).** It is well known that actinomycetes produce 70% to 80% of bioactive secondary metabolites, where approximately 60% of antibiotics development for agricultural use **(Ilic *et al.*, 2007).** Among the genera of actinomycetes, the genus *Streptomyces* is represented in nature by the largest number of species and varieties, which differ greatly in their morphology, physiology and biochemical activities **(Suneetha and Zaved, 2011).** Interestingly, the majority of the antibiotic-producing actinomycetes are found among these species, which led to a growing economic importance for this group of organisms **(Kumar *et al.*, 2012).** Genus *Streptomyces* produces and secrete a wide array of biologically active compounds including antibiotics, hydrolytic enzymes and enzyme inhibitors **(Singh *et al.*, 2006)** enhances soil fertility and have been proved to possess antagonistic activity against wide range of soil-borne plant pathogens **(Aghighi *et al.*, 2004).** Several studies have been focused on identifying biocontrol agents which could be used as an alternative to agrochemicals in plant protection **(Saravanamuthu *et al.*, 2010)**.
Therefore, in this study an effort was made to screen soil actinomycetes for inhibition activity against phytopathogenic microorganisms, which can play an important role in plant growth promotion. This study is also an attempt to identify and characterize the most effective isolates by morphological, biochemical, physiological and molecular methods.

MATERIAL AND METHODS

Isolation of streptomycetes

Streptomycetes were isolated from arable soil collected in Východná, Slovakia. Soil sample was air dried for 2 days, crushed, and sieved prior to use for isolation purpose. Twenty seven actinomycete strains were isolated as pure cultures by using standard microbiological methods. An aliquot of 0.1 ml of each soil solution (10^{-1}, 10^{-2}, 10^{-3}) was taken and spread evenly over the surface of streptomycete selection Pochon medium **(Korzeniewska *et al.*, 2009)** complemented with nystatin (50 µg/ml). Plates were incubated at 28 ºC for 7 days. Suitable colonies those showed *Streptomyces* like appearance were recultivated several times for purity on yeast-malt extract medium **(Shirling and Gottlieb, 1966)**. The purified actinomycetes were preserved at -20 ºC in the presence of glycerol (30% v/v) for longer period.

Test organisms

Eight test microorganisms were used for detection of streptomycete antibiotic activity. Test organisms used in this study include Gram-positive bacterium

Clavibacter michiganensis subsp. sepedonicus (CCM 7014), Gram-negative bacteria *Xanthomonas campestris* (CCM 22), *Pseudomonas syringae* (CCM 2868), *Erwinia amylovora* (CCM 1114) from Czek Collection of Microorganisms, and fungi *Alternaria tenuissima* (16A6), *Fusarium poae* (12A18), *Penicillium expansum* (KMH5) and *Aspergillus niger* (KMH12) from Microbial Collection of Department of Microbiology, SUA, Slovakia.

Antimicrobial activity of pure cultures

Primary screening

Preliminary screening for antibiotic activity of the isolates was done by using agar plug method on Sabouraud agar (SigmaAldrich, USA) (fungi), tryptic soy agar (Sigma Aldrich, USA) (Gram-negative bacteria) and glucose yeast extract agar agar (Sigma Aldrich, USA) (Gram-positive bacteria). Agar discs were prepared using a sterile cork borer from well grown actinomycetes culture and placed on fresh lawn culture of test microorganisms. Plates were incubated at 25 ºC for fungi and 30 ºC for bacteria. The zones of inhibition were determined after 2-3 days (fungi) or after 1-2 days for bacteria.

Secondary screening

Active isolates in primary screening were subjected to secondary screening using well-dillution method with actinomycete extracts. For preparation of extracts we used liquid cultivation of actinomycetes in medium supporting metabolite production (starch-15g, yeast extract-4g, K2HPO4-1,0g, MgSO4.7H2O-0,5g, destilled water-1000ml, pH-7,0). After five days of incubation we mixed 20 ml of culture with 20 ml of ethyl acetate (Sigma Aldrich, USA). After a 12 min shaking step the sample was centrifuged at 9000 rpm for 10 min and the upper phase was transferred into a round bottom flask. At 40°C the ethyl acetate was evaporated in a rotary evaporator (Stuart, UK). Finally, the extract was solved in 1 ml of ethyl acetate: acetone: methanol (1:1:1) and centrifuged at 14000 rpm for 10 min. We added 50µl of extracts to wells bored into freshly inoculated plates.The plates were incubated and zones of inhibition was recorded like above. For negative control we added 50 µl of ethyl acetate: acetone: methanol (1:1:1) solution to the wells.

Phenotypic characterization

Aerial mass color and reverse side pigments

The mature sporulated aerial and substrate mycelium color was recorded in yeast-malt extract agar (ISP2), oat meal agar (ISP3), inorganic salt starch agar (ISP4), glycerol asparagine agar (ISP5), peptone yeast etract iron agar (ISP6) and tyrosine agar (ISP7) **(Shirling and Gottlieb, 1966)**. The colors were described by the RAL-code. Production of melanoid pigments was tested on ISP6 and ISP7 media.

Spore chain morphology

The spore bearing hyphae was determined by direct examination of culture under microscope (OLYMPUS CX22LED, Japan) 1000 x magnification using a well grown sporulated culture plates.

Physiological characterization

Utilization of different carbon sources were studied by the method recommended in International Streptomycete Projects using a microplate technique with twelve well plates. Carbon sources like glucose, mannitol, arabinose, inositol, lactose, mannose, fructose, galactose, rhamnose, sucrose and xylose were tested on the carbon utilization agar (ISP9) **(Shirling and Gottlieb, 1966)** supplemented with 1% carbon sources. Sodium chloride tolerance level of the isolates was evaluated on medium (casein peptone – 10.0 g/L, yeast extract - 5.0 g/L, agar – 20.0 g/L, deionized water – 1000 m) supplemented with graded doses of NaCl (0, 2, 5, 5, 7, 5 and 10% of sodium chloride), maximum NaCl tolerance concretation in the medium allowing any growth was recorded. Sodium chloride tolerance was tested on six-well microtiter plates. Physiological characterization such as the effect of pH (5-9) and temperature (25, 30, 45 and 60 ºC) were also tested.

Biochemical characterization with Api stripes

For biochemical identification we used ApiZym and ApiCoryne systems (BioMérieux, USA). After week of actinomycete inbubation in shaking flasks with GYM medium (**Větrovský *et al*., 2014)** were the strains inoculated followed by manufacturer's manual. Incubation time was 24 hours at 30 ºC. After incubation period we added reagents to each cupule and let the colors develop. After five minutes we evaluated stripes according to manual criteria.

16S rRNA sequencing

Molecular taxonomy, sequencing and phylogenetic analysis

The genomic DNA isolation of actinomycetes was done by the method described by **Deininger *et al.* (1989)** and amplified by PCR using primers according to **Cook and Meyers (2003)**. The PCR reaction ran in thermo cycler Biometra T Personal (Germany). (Reaction mixture contained 5 µl of 10 × DreamTaq Green PCR buffer, 5 µl of 2 mmol.dm^{-3}dNTP, 2 µl of each 10 µmol.dm^{-3} primer, 0,3 µl Taq DNA polymerase and 0,5 µl of template DNA (approximately 20 ng). The PCR reaction ran under the following conditions: 95 °C for 3 min, 40 cycles of 95 °C for 30 sec, 56 °C for 30 sec, 72 °C for 90 sec and final extension at 72 °C for 10 min. Purification of PCR products were done using Exonuclease I and Thermosensitive Alkaline Phosphatase. The sequencing was carried out in both sense and antisense direction in MacroGen Company, South Korea. The similarity and homology of the 16S rRNA partial gene sequence was analyzed with the similar existing sequences available in the data bank of NCBI using BLAST search. The DNA sequences were aligned and phylogenetic tree was constructed with PhyML software using Maximum likehood tree. A bootstrap analysis of 100 replicates was carried out.

RESULTS AND DISCUSSION

A total of 27 morphologically different actinobacterial colonies were isolated from collected soil and made pure culture. According to Bergey´s Manual of Determinative Bacteriology by **Holt *et al.* (1994)** the organisms were identified as *Streptomyces* species based on the morphological characteristics. The colonies were slow growing, aerobic, glabrous or chalky, heaped, folded and with aerial and substrate mycelia of different colors. In addition, all colonies possessed an earthy odour (**Suneetha *et al.*, 2011).** The prevalence of *Streptomyces* species over other actinomycetes was likely due to screening conditions (media and cultivation).

In the present study, agar plug method was used for primary screening of antimicrobial activity. This method allowed utilizing of very small amount of medium for the culturing and production of bioactive compounds and also for the detection of antimicrobial activity of more number of actinobacterial isolates against wide range of microorganisms with less investment costs **(Mohanraj *et al.*, 2011).** The results of primary testing indicated that 14 of the total isolates demonstrated antimicrobial activities against two or more tested microorganisms, the remaining 13 isolates showed meagre activity. Antibacterial activity of soil actinomycetes against various phytopathogenic bacteria was determined by many researchers (**Muangham *et al.*, 2015 and Encheva-Malinova *et al.*, 2014).** The highest inhibition activity was measured against gram-positive bacterium *Clavibacter michiganensis subps. sepedonicus* in comparison with Gram-negative bacteria. Similar findings concur with the findings by various researchers, where they observed that antagonistic reaction against the Gram positive bacteria were much higher than the Gram negative (**Basilio *et al.*, 2003; Kumar *et al.*, 2012; Sacramento *et al.*, 2004**).

In case of antifungal activity, the highest inhibited fungus was *Fusarium poae*. The mechanism of antifungal antagonists can be due to the secretion of hydrolytic enzymes which degrade the fungal cell wall, or the secretion of antifungal compounds **(Yuan *et al.*, 1995)**. But it is not known whether the zone of inhibition, caused by the our *Streptomyces* strains occurs as a result of hydrolytic enzymes or antifungal metabolites. To determine whether our strains produced antifungal metabolites, crude extracts were prepared. Using a well diffusion method inhibition of mycelial growth was clearly observed in the presence of extracts and therefore it is possible that these strains are producing antifungal metabolites.

Further work carried by the leading 14 isolates. After cultivation, the ethylacetate extracts from these positive isolates were prepared and subjected to secondary screening using the same test pathogens. Solvent extraction is usually employed for the extraction of antibiotics from the culture filtrates. Organic solvents with different polarities have been used by many researchers for the extraction of antimicrobial compounds from actinomycetes (**Selvameenal *et al.*, 2009**). This result clearly indicated that the antimicrobial activity of potential strains is due to the production of extracellular bioactive compounds. The published literature stated that most of the antibiotics from actinomycetes are extracellular in nature **(Valanarasu *et al.*, 2008**). In well diffusion method, extracts of active actinobacterial strains showed different activity from that of primary screening; some of the active isolates didn't show the activity (2 isolates) in the secondary screening, perhaps due to the inconvenient liquid growth medium, while some showed only little activity (8 isolates) and some showed similar activity (VY59, VY47, VY87). Such results had been reported from other scientists too, which had found the activity reducing in comparison with that showed by the method of agar plug method. Actinomycete isolates namely VY59, VY47 and VY87 showed broad spectrum of antimicrobial activity against test phytopathogens (Table 1) and therefore, they were selected and characterized for further study.

Table 1 Size of inhibition zones (mm) of the most active isolates

Strain	*Xanthomonas campestris*	*Pseudomonas syringae*	*Erwinia amylovora*	*Clavibacter michiganensis*	*Alternaria tenuissima*	*Fusarium poae*	*Penicillium expansum*	*Aspergillus niger*
VY59	0*/0**	12/0	0/0	20/19	11/0	18/14	12/12	10/10
VY9	0/0	0/0	0/0	28/26	18/12	0/0	20/20	0/0
VY11	0/0	0/0	0/0	16/14	12/11	12/11	0/0	0/0
VY31	11/0	0/0	0/0	12/11	14/0	0/0	0/0	11/0
VY87	12/12	13/11	11/10	20/22	10/8	24/16	12/0	0/0
VY3	0/0	11/0	11/0	15/17	0/0	0/0	0/0	11/0
VY7	0/0	0/0	0/0	24/24	0/0	11/12	0/0	0/0
VY53	12/12	0/0	12/0	26/24	0/0	12/0	0/0	0/0
VY47	12/12	11/0	14/14	30/26	22/14	12/0	12/11	12/12
VY26	0/0	14/12	0/0	12/11	12/12	11/0	0/0	28/20
VY53	0/0	0/0	0/0	12/0	0/0	0/0	0/0	12/11
VY43	0/0	0/0	0/0	22/16	0/0	12/12	0/0	0/0
VY28	11/0	0/0	0/0	18/18	14/11	0/0	11/0	0/0
VY14	0/0	11/0	0/0	30/22	0/0	14/12	0/0	0/0

*Primary screening / ** secondary screening

It was possible to identify these actinomycete isolates based on pigment production, morphological and physiological characteristics and biochemical properties, which can provide more details that can be used for identification purposes as reported by **Oskay *et al.* (2004),** but other advanced methods such as gene analysis of 16S rRNA are more reliable **(You *et al.*, 2005).**

Morphological characterization of the isolates

Many characteristics of actinomycetes have been employed for the purpose of easy classification and ideally, these should be constant under the same cultural conditions **(Sathi *et al.*, 2001).** All selected strains were Gram-positive, very long, rod shaped and possessing an earthy odour characteristic for actinomycetes. Morphological characteristics of the active strains on different ISP specific media are shown in table 2.

Table 2 Morphological identification of the selected active strains

Medium	Color of Aerial mycelium			Color of Substrate mycelium			Production of soluble pigments		
	VY59	**VY87**	**VY47**	**VY59**	**VY87**	**VY47**	**VY59**	**VY87**	**VY47**
ISP2	Lemon yellow	Curry	Sand yellow	Oyster white	Platinum grey	Oyster white	Lemon yellow	Sandy yellow	-
ISP3	No growth	Pale brown	Brown beige	No growth	Platinum grey	No growth	-	-	Brown beige
ISP4	Ivory	Green beige	Ivory	No growth	Pure white	No growth	-	-	-
ISP5	Ivory	Yellow grey	Sand yellow	Oyster white	Granite grey	Light ivory	-	-	-
ISP6	No growth	Brown beige	No growth	No growth	Pearl light grey	No growth	-	Ochre yellow	Broom yellow
ISP7	Honey yellow	Sepia brown	Sand yellow	Stone grey	Light ivory	Oyster white	-	Black brown	Sandy yellow

Under microscopic observation all strains showed the presence of substrate and aerial mycelium. Using an identification guide by the International Steptomyces Project **(Getha *et al.*,)**, the characteristic of the sore bearing hyphae of the isolates VY59 and VY87 was found to be rectus flexibilis, whereas that of VY47 was spira. The different media did not have an effect on micro-morphological characteristics of the isolates but it had an effect on the melanin production. Only isolate VY87 showed melanin production and this was determined on ISP7 agar medium. **Mutitu *et al.* (2008)** also found that a variety of pigments and colony types are produced by the same organism on different media.

Table 3 Physiological characteristics of active isolates

Parameter	Carbon sources			Parameter	pH range		
	VY59	**VY87**	**VY47**		**VY59**	**VY87**	**VY47**
Arabinose	+	+	+	**2**	-	-	-
Cellulose	+	+	+	**3**	-	-	-
Fructose	(+)	+	(+)	**4**	-	-	-
Glucose	+	+	+	**5**	-	-	-
Inositol	(+)	-	-	**6**	-	(+)	-
Mannitol	+	+	+	**7**	+	+	(+)
Raffinose	+	+	+	**8**	(+)	+	+
Rhamnose	-	+	(+)	**9**	-	-	-
Sucrose	(+)	+	(+)	**10**	-	-	-
Xylose	(+)	-	(+)				
NaCl concrentation (%)				**Growth at (ºC)**			
0	**(+)**	**+**	**(+)**	**25**	(+)	+	+
2,5	+	+	+	**28**	+	+	+
5	-	(+)	(+)	**35**	(+)	-	(+)
7,5	-	-	-	**45**	-	-	-
10	-	-	-	**60**	-	-	-

+ good growth, (+) moderate growth, - no growth

Physiological characterization

In general, biochemical and physiological characteristics of the actinomycetes vary from isolate to isolate depending on the growth conditions. The present investigation concluded that the physiological characteristics of actinomycetes varied depending on the avaliable nutrients in the medium and the physical conditions. Thus, it was concluded on the basis of the present and previous studies that the nutrient composition of the medium greatly influence the growth and morphology of organisms **(Gesheva *et al.*, 1993).** Tested isolates showed variable results, in the utilization of the carbon sources tested, isolate VY59 utilized all the carbon sources except rhamnose, isolate VY47 did not utilize inositol and VY51 cellulose, raffinose and sucrose (Table

3). Studies on the requirement of carbon sources for growth showed that cellulose, arabinose, glucose, manitol and raffinose are needed as carbon sources for abundant growth of the isolates. Slight or poor growth is an indication that, the particular carbon source is not an adequate source of carbon or the material may contain traces of other compounds **(Sathi *et al.*, 2001).** Optimal growth of all strains was observed at 2.5% NaCl, but maximum tolerance of chlorid concentration was exhibited up to 5% in case of strain VY47. All the isolates could grow at pH 7. **Park *et al.* (1991)** rewieved that neutrophiles *Streptomycecs* species are able to grow between pH 5 and 9 with optimum growth close to neutrality. Temparature range of isolates was from 25 to 28 ºC, with the optimum conditions at 28 ºC. Isolates VY59 and VY47 exhibited moderate growth at 35 ºC.

Biochemical tests with Api-stripes

Activity of the extracellular enzymes was quantified using API ZYM. During the incubation period, the products of the end metabolism produced and detected as color reaction **(Aljassim, 2015).** It was found that all isolates showed good phosphatase alcaline, leucinearylamidase, phosphatase acid, and N-acetyl-glucoseamidase activity and glucosidase. Contrary, the least occurring enzyme was galactosidase and glucuronidase (Table 4).

Table 4 Detection of various enzymes using ApiZym tests

Enzyme	VY59	VY87	VY47
Phosphatase alcaline	(5) +	(5) +	(5) +
Esterase (C4)	(3) +	(3) +	(2) +
Esterase lipase (C8)	(4) +	(2) +	(3) +
Lipase(C 14)	(0) -	(0) -	(2) +
Leucinearylamidase	(5) +	(5) +	(5) +
Valinearylamidase	(3) +	(5) +	(5) +
Cystinearylamidase	(1) +	(3) +	(1) +
Trypsin	(0) -	(5) +	(0) -
Chymotrypsin	(1) +	(1) +	(3) +
Phosphatase acid	(5) +	(5) +	(5) +
Naphtol-AS-BI-phosposfohydrolase	(5) +	(5) +	(3) +
Galactosidase	(0) -	(0) -	(0) -
Galactosidase	(0) -	(1) +	(0) -
Glucuronidase	(0) -	(0) -	(0) -
Glucosidase	(1) +	(3) +	(0) -
Glucosidase	(4) +	(5) +	(5) +
N-acetyl-glucoseamidase	(5) +	(5) +	(5) +
Mannosidase	(0) -	(5) +	(0) -
Fucosidase	(0) -	(2) +	(0) -

Numbers indicate colour intensity which is proportional to concentration of respective enzyme presence, + enzymatic activity was detected, - enzymatic activity was non-detected

Similar results obtained **Jiang *et al.* (2013)**, who found out, that the tested isolates showed alkaline phosphatase, acid phosphatase, leucinearylamidase, naphtol-AS-BI-phosphatase and B-glucosidase and α-glucosides activity and none strains showed β-glucuronidase activity. According to API CORYNE system we detected nitrate reduction, production of esculin, gelatin hydrolysis, production of alkaline phosphatase and N-acetyl-β- glucosamidase and urease activity. The rest of tested activities were not positive (Table 5)

Table 5 Enzymatic and fermentation tests using Api Coryne system

Parameter	VY59	VY87	VY47
Nitrate reduction	+	-	-
pyrrolidonyl arylamidase	-	-	-
β – glucuronidase	-	-	-
α- glucosidase	-	-	-
esculin	-	+	+
gelatine (hydrolysis)	+	+	+
Ribose fermentation	-	-	-
Mannitol fermentation	-	-	-
Sucrose fermentation	-	-	-
pyrazinamidase	-	-	-
Alkaline phosphatase	+	+	+
β –galactosidase	-	-	-
N-acetyl-β- glucosamidase	-	+	-
Urease	-	-	+
Glucose fermentation	-	-	-
Xylose fermentation	-	-	-
Lactose fermentation	-	-	-
Glycogen fermentation	-	-	-

Results indicates that actinomycetes possess the potential to secrete broad range enzymes, which maybe the results from natural selection of the microorganisms in order to survive in a competing environment.

Molecular identification

Although various morphological and biochemical tests were performed, to identify the *Actinomycetes* up to species level, for proper identification of genera and species of *Actinomycetes*, molecular identification is necessary. Identification of using molecular tools proved to be faster and least tedious compared to classical microbiological methods. Initial morphological characterization using light microscope showed that all the 3 isolates belong to the genus *Streptomyces* spp. The results obtained from the direct sequencing of purified PCR products confirm this suggestion. The nucleotide sequences for a section of the 16S rRNA gene from 3 selected strains were subjected to BLAST analysis using NCBI database for identification at the genus level. All three isolates contained different nucleotide sequences for the 16S rRNA gene, indicating that they were different strains. VY59 was most closely related to *Streptomyces somaliensis* (similarity index 99%), VY87 to *Streptomyces* sp. (similarity index 99%) and VY47 to *Streptomyces albidoflavus* with similarity index 99%. The results described above were supported by phylogenetic analysis base on the neighbor-joining tree.

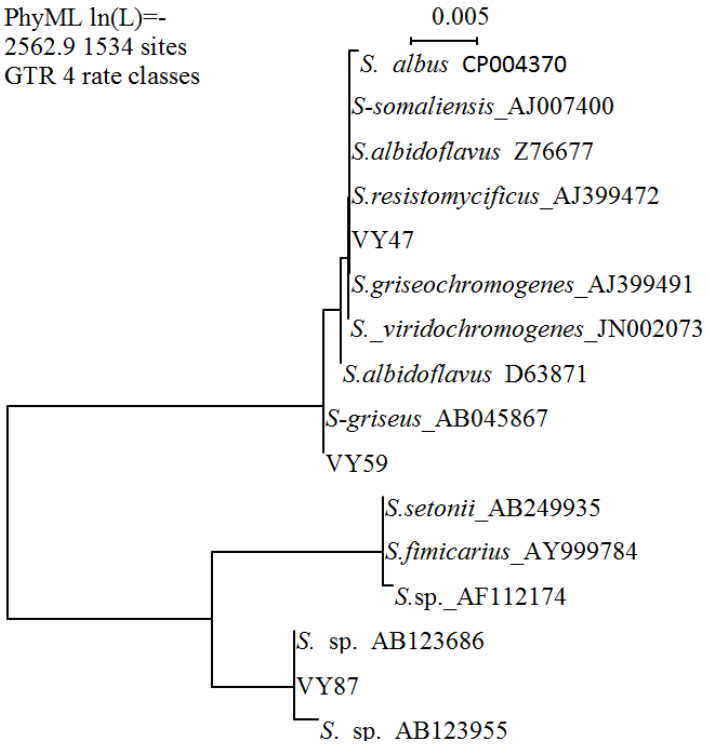

Figure 1 Phylogenetic relationships based on neighbour-joining analysis of 16S rRNA gene sequence of the most active strains and closely related *Streptomyces* species

CONCLUSION

In conclusion, the three streptomycete strains, *S. somaliensis* (VY59), *S.* sp. (VY87) and *S. albidoflavus* (VY47) exhibited interesting antimicrobial activity against phytopathogenic Gram-positive, Gram-negative bacteria and against fungi. This study showed that the test actinomycetes isolates have the potential to act as sources of antimicrobial compounds against phytopathogenic microorganisms which attack crops. It is suggested that these strains of soil streptomycetes be further studied in search for some novel antibiotics, which could be effective in the protection of crop production. Thus our study brings forward a good promise for future drug development and agricultural programs.

Acknowledgement: The author would like to thank PD Dr. Joachim Wink, head of the Microbial Strain Collection Group of Helmholtz Centrum of Infection Research in Braunschweig, Germany for an Erasmus+ intership and supporting of this results. This work was partially co-funded by Europien Community under project no. 26220220180: Building Research Centre Agrobiotech.

REFERENCES

Aghighi, S., Shahidi Bonjar, G. H., Rawashdeh, R., Batayneh, S., & Saadoun, I. (2004). First report of antifungal spectra of activity of Iranian actinomycetes strains against Alternaria solani, Alternaria alternate, Fusarium solani, Phytophthora megasperma, Verticillium dahliae and Saccharomyces cerevisiae. *Asian Journal of Plant Sciences*, *3*(4), 463-471. http://dx.doi.org/10.3923/ajps.2004.463.471

Aljassim, Z. (2015). *Enzymatic and immunological comparison of Mycobacterium tuberculosis and a clinical isolate of streptomyces* (Doctoral dissertation, Laurentian University of Sudbury).
Balagurunathan, R., & Radhakrishnan, M. (2007). Actinomycetes: diversity and their importance. *Microbiology–Applications and Current Trends. PC Trivedi (editor), Pointer publishers, Jaipur, India*, 297-329.
Basilio, A., Gonzalez, I., Vicente, M. F., Gorrochategui, J., Cabello, A., Gonzalez, A., & Genilloud, O. (2003). Patterns of antimicrobial activities from soil actinomycetes isolated under different conditions of pH and salinity. *Journal of applied microbiology*, *95*(4), 814-823. http://dx.doi.org/10.1046/j.1365-2672.2003.02049.x
Boyd, L. A., Ridout, C., O'Sullivan, D. M., Leach, J. E., & Leung, H. (2013). Plant–pathogen interactions: disease resistance in modern agriculture. *Trends in genetics*, *29*(4), 233-240. http://dx.doi.org/10.1016/j.tig.2012.10.011
Cook, A. E., & Meyers, P. R. (2003). Rapid identification of filamentous actinomycetes to the genus level using genus-specific 16S rRNA gene restriction fragment patterns. *International journal of systematic and evolutionary microbiology*, *53*(6), 1907-1915. http://dx.doi.org/10.1099/ijs.0.02680-0
Deininger, P. (1990). Molecular cloning: A laboratory manual: Edited by J. Sambrook, EF Fritsch, and T. Maniatis. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989 (in 3 volumes). http://dx.doi.org/10.1016/0003-2697(90)90595-z
Dhanasekaran, D., Panneerselvam, A., & Thajuddin, N. (2012). *Applications of Actinobacterial Fungicides in Agriculture and Medicine*. INTECH Open Access Publisher. http://dx.doi.org/10.5772/25549
Encheva-Malinova, M., Stoyanova, M., Avramova, H., Pavlova, Y., Gocheva, B., Ivanova, I., & Moncheva, P. (2014). Antibacterial potential of streptomycete strains from Antarctic soils. *Biotechnology & Biotechnological Equipment*, *28*(4), 721-727. http://dx.doi.org/10.1080/13102818.2014.947066
Getha, K., Vikineswary, S., Wong, W. H., Seki, T., Ward, A., & Goodfellow, M. (1970). Characterization of Selected Isolates of Indigenous Streptomyces Species and Evaluation of their Antifungal Activity against Selected Plant Pathogenic Fungi. *Malaysian Journal of Science*, *23*(2).
Gesheva, V., & Gesheva, R. (1993). Structure of the Streptomyces hygroscopicus 111-81 population and characteristics of its variants. *Actinomycetes*, *4*, 65-72. http://dx.doi.org/10.1007/s00284-008-9165-1
Holt, J. G., Krieg, N. R., Sneath, P. H., Staley, J. T., & Williams, S. T. (1994). International edition: Bergey's manual of determinative bacteriology. http://dx.doi.org/10.1002/jobm.3620251024
Ilic, S. B., Konstantinovic, S. S., Todorovic, Z. B., Lazic, M. L., Veljkovic, V. B., Jokovic, N., & Radovanovic, B. C. (2007). Characterization and antimicrobial activity of the bioactive metabolites in streptomycete isolates. *Microbiology*, *76*(4), 421-428. http://dx.doi.org/10.1134/s0026261707040066
Jiang, Y., Han, L., Chen, X., Yin, M., Zheng, D., Wang, Y., ... & Huang, X. (2013). Diversity and bioactivity of cultivable animal fecal actinobacteria. http://dx.doi.org/10.4236/aim.2013.31001
Korzeniewska, E., Filipkowska, Z., Gotkowska-Płachta, A., Janczukowicz, W., Dixon, B., & Czułowska, M. (2009). Determination of emitted airborne microorganisms from a BIO-PAK wastewater treatment plant. *Water research*, *43*(11), 2841-2851.
http://dx.doi.org/10.1016/j.watres.2009.03.050
Kumar, P. S., Raj, J. P. P., Duraipandiyan, V., & Ignacimuthu, S. (2012). Antibacterial activity of some actinomycetes from Tamil Nadu, India. *Asian Pacific journal of tropical biomedicine*, *2*(12), 936-943.
http://dx.doi.org/10.1016/s2221-1691(13)60003-9
Mohanraj, D., Bharathi, S., Radhakrishnan, M., & Balagurunathan, R. (2011). Journal of Chemical and Pharmaceutical Research. *J. Chem*, *3*(3), 439-446.
Muangham, S., Pathom-aree, W., & Duangmal, K. (2014). Melanogenic actinomycetes from rhizosphere soil—antagonistic activity against Xanthomonas oryzae and plant-growth-promoting traits. *Canadian journal of microbiology*, *61*(2), 164-170.
http://dx.doi.org/10.1139/cjm-2014-0645
Mutitu, E. W., Muiru, W. M., & Mukunya, D. M. (2008). Evaluation of antibiotic metabolites from actinomycete isolates for the control of late blight of tomatoes under greenhouse conditions. *Asian J. Plant Sci*, *7*, 284-290.
http://dx.doi.org/10.3923/ajps.2008.284.290
Oskay, M. (2009). Antifungal and antibacterial compounds from Streptomyces strains. *African Journal of Biotechnology*, *8*(13).
Oskay, A. M., Üsame, T., & Cem, A. (2005). Antibacterial activity of some actinomycetes isolated from farming soils of Turkey. *African journal of Biotechnology*, *3*(9), 441-446.
Park, Y. H., Yim, D. G., Kim, E., Kho, Y. H., Mheen, T. I., Lonsdale, J., & Goodfellow, M. (1991). Classification of acidophilic, neutrotolerant and neutrophilic streptomycetes by nucleotide sequencing of 5S ribosomal RNA. *Microbiology*, *137*(9), 2265-2269.
http://dx.doi.org/10.1099/00221287-137-9-2265
Prabhakar, K. A., Karad, D. D., & Kulkarni, S. W. (2014). PESTICIDE RESISTANT AND ANTIMICROBIAL ACTIVITY OF SOME STREPTOMYCES ISOLATED FROM SOIL UNDER CULTIVATION OF CURCUMA LONGA L.
Sacramento, D. R., Coelho, R. R. R., Wigg, M. D., Linhares, L. F. D. T. L., dos Santos, M. G. M., Semêdo, L. T. D. A. S., & da Silva, A. J. R. (2004). Antimicrobial and antiviral activities of an actinomycete (Streptomyces sp.) isolated from a Brazilian tropical forest soil. *World Journal of Microbiology and Biotechnology*, *20*(3), 225-229.
http://dx.doi.org/10.1023/b:wibi.0000023824.20673.2f
Saravanamuthu, R. (2010). *Industrial Exploitation of Microorganisms*. IK International Pvt Ltd.
Sathi, Z. S., Rahman, M. A. A., & Gafur, M. A. (2001). Identification and in vitro anti microbial activity of a compound isolated from Streptomyces species. *Pak. J. Biol. Sci*, *4*(12), 1523-1525. http://dx.doi.org/10.3923/pjbs.2001.1523.1525
Selvameenal, L., Radhakrishnan, M., & Balagurunathan, R. (2009). Antibiotic pigment from desert soil actinomycetes; biological activity, purification and chemical screening. *Indian journal of pharmaceutical sciences*, *71*(5), 499.
http://dx.doi.org/10.4103/0250-474x.58174
Shirling, E. T., & Gottlieb, D. (1966). Methods for characterization of Streptomyces species1. *International Journal of Systematic and Evolutionary Microbiology*, *16*(3), 313-340. http://dx.doi.org/10.1099/00207713-16-3-313
Singh, L. S., Baruah, I., & Bora, T. C. (2006). Actinomycetes of Loktak habitat: isolation and screening for antimicrobial activities. *Biotechnology*, *5*(2), 217-221.
http://dx.doi.org/10.3923/biotech.2006.217.221
Suneetha, V., & Khan, Z. A. (2010). Screening, characterisation and optimization of microbial pectinase. In *Soil Enzymology* (pp. 329-337). Springer Berlin Heidelberg. http://dx.doi.org/10.1007/978-3-642-14225-3_18
Suneetha, V., & Raj, K. (2011). Isolation and identification of Streptomyces ST1 and ST2 strains from Tsunami affected soils: Morphological and biochemical studies. *Journal of Oceanography and Marine Science*, *2*(4), 96-101.
Tanaka, Y., & Omura, S. (1993). Agroactive compounds of microbial origin. *Annual Reviews in Microbiology*, *47*(1), 57-87.
http://dx.doi.org/10.1146/annurev.micro.47.1.57
Valan, A. M., Asha, K. R. T., Duraipandiyan, V., Ignacimuthu, S., & Agastian, P. (2012). Characterization and phylogenetic analysis of novel polyene type antimicrobial metabolite producing actinomycetes from marine sediments: Bay of Bengal India. *Asian Pacific journal of tropical biomedicine*, *2*(10), 803-810.
http://dx.doi.org/10.1016/s2221-1691(12)60233-0
Větrovský, T., Steffen, K. T., & Baldrian, P. (2014). Potential of cometabolic transformation of polysaccharides and lignin in lignocellulose by soil Actinobacteria. *PloS one*, *9*(2), e89108.
http://dx.doi.org/10.1371/journal.pone.0089108
You, J. L., Cao, L. X., Liu, G. F., Zhou, S. N., Tan, H. M., & Lin, Y. C. (2005). Isolation and characterization of actinomycetes antagonistic to pathogenic Vibrio spp. from nearshore marine sediments. *World Journal of Microbiology and Biotechnology*, *21*(5), 679-682.
http://dx.doi.org/10.1007/s11274-004-3851-3
Yuan, W. M., & Crawford, D. L. (1995). Characterization of streptomyces lydicus WYEC108 as a potential biocontrol agent against fungal root and seed rots. *Applied and Environmental Microbiology*, *61*(8), 3119-3128.
http://dx.doi.org/10.1016/s0141-0229(96)00175-5