

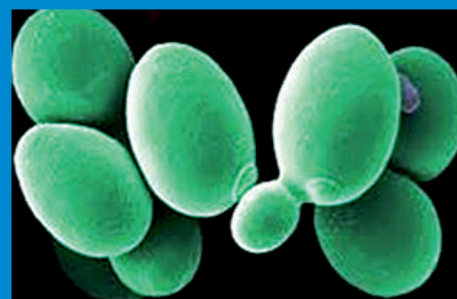
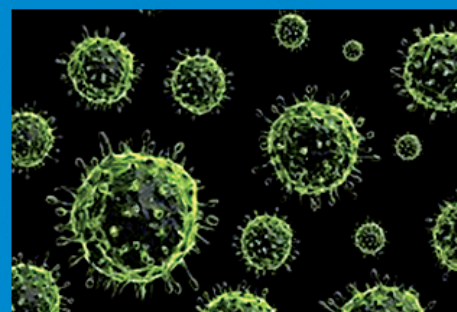


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Review

Potential of Halotolerant and Halophilic Fungi as a Source of New Extracellular Enzymes and Antimicrobial Compounds

Yana Gocheva, Maria Angelova, Ekaterina Krumova*

The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences

Abstract

The scope of this review is the literature published over the last 10 years and presents information on studies conducted on microscopic fungi isolated from various natural saline ecosystems (aquatic and terrestrial). Another interesting point is that it presents a classification of halophilic fungi and the concepts of halotolerant and halophilic microorganisms. The main aim of this review is to show fungal diversity of different natural salinity ecosystems and the biotechnological potential of halophilic and halotolerant fungi as producers of biologically active substances (antibiotics, enzymes, polysaccharides). A careful study on halophilic and halotolerant fungi could offer new biologically active compounds that could be used in many industrial processes taking place in unfavorable conditions, such as high salt concentration, low water activity, high pressure or high temperature, etc.

Keywords: halophilic fungi, salt-adaptation, biodiversity, enzyme production, antimicrobial compounds

Резюме

Настоящият обзор представя преглед на литературата, публикувана през последните 10 години относно изследванията върху микроскопични гъби, изолирани от различни естествено солени екосистеми (водни и сухоземни). Едновременно с това, той запознава читателите с класификацията на тези гъби и концепцията за халотолерантни и халофилни микроорганизми. Проследено е разнообразието им в различни екосистеми с естествена соленост, както и техния биотехнологичен потенциал като продуценти на биологично активни вещества (антибиотици, ензими, полизахариди). Внимателното проучване на халофилните и халотолерантни гъби е предпоставка за получаването на нови биологично активни съединения с цел използването им в редица индустриални процеси, протичащи при неблагоприятни условия, като напр. висока концентрация на сол, ниска водна активност, високо налягане или висока температура и други.

Introduction

In recent years, there has been increasing interest in the study of extremophilic microorganisms, including halophiles. Intensified studies of halophilic eukaryotic microorganisms appear to have several advantages for science and society, as they: provide clues in our understanding of the targets, processes and networks involved in the complex field of salt tolerance; increase our understanding of stress responses; identify genes that might enhance the properties of industrial microorganisms and of plants used in agriculture; contribute to a more complete understanding of 'simple' ecosystems that are characterized by the small diversity of their

occupants and relatively few physical and chemical variables. As large areas throughout the world are exposed to salinisation, a better understanding of extremophilic fungi will help us to adapt to Earth's changing climate (Gunde-Cimerman *et al.*, 2009). The study of halophilic fungi contributes to the elucidation of the biodiversity of different ecosystems and clarifies the mechanisms of adaptation to adverse environmental conditions, such as increased salt concentration.

Halotolerant and halophilic microorganisms capable of living in a saline environment offer many actual or potential applications in various fields of biotechnology such as producers of new biologi-

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cally active substances (enzymes, antibiotics, polysaccharides, etc.). To date, the biotechnological applications of halophilic microorganisms have been associated mainly with the use of halophilic bacteria, while the broad potential of halophilic fungi is still poorly understood and therefore poorly used in practice (Ali *et al.*, 2014).

A large number of known microorganisms that grow and survive under normal conditions cannot give the desired results when applied in biotechnology due to the unfavourable conditions for their survival or for the activity required in this operation. Thus, understanding the hypersaline environment and the adaptation in their microbial inhabitants could potentially lead to new biotechnological applications or help to find resistance genes that can then be incorporated into food crops or other organisms to allow their optimal growth, or their products (such as enzymes) to be used in more extreme conditions (temperature, pressure, pH, and salinity) (Gunde-Cimerman *et al.*, 2009). Halotolerant microorganisms play an essential role in food biotechnology for the production of fermented foods and food additives. Other areas of application of these groups of extremophiles are the decomposition and/or transformation of a number of organic pollutants, as well as their participation in the production of alternative energy from bio-waste (Margesin and Schinner, 2001).

One of the main reasons for studying extremophiles, including halophilic microorganisms, is to understand their mechanisms of stress adaptation, as well as the possibilities for the biotechnological application of their metabolites capable of acting in extreme conditions. The low water activity and high concentration of salts in the hypersaline environment make these habitats an important source of halophilic microorganisms that can provide enzymes of industrial interest (Oren *et al.*, 2010).

There is a growing interest in the production of enzymes with extremophilic characteristics, as they can find application in industrial processes that take place under specific, adverse conditions, such as high temperature, acidic or alkaline pH, and high salt concentrations. Therefore, attempts are being made to discover or develop new extracellular enzymes, amylases, proteases, xylanases, cellulases and others with desired characteristics in order to meet the variety of applications (Ghadikolaei *et al.*, 2019). The ever-increasing antibiotic resistance determines the constant need for new antibiotics. Some of the halophilic fungi isolated so far show high antibacterial activity, which makes them at-

tractive as potential producers of antibiotics. The existing information about halophilic fungi is still very fragmentary.

The aim of this review is to present the natural habitats and biodiversity of halophilic and halotolerant fungi, as well as their potential as sources of new biologically active substances with application in industrial processes taking place in unfavorable conditions such as increased salt concentration.

Halophilic and halotolerant fungi – natural habitats and biodiversity

For a long time, places with extreme living conditions were considered uninhabitable. Intensive research on these habitats has begun recently and a wide variety of extremophilic microorganisms have been found (Turk *et al.*, 2011). Saline ecosystems (aquatic and terrestrial) are characterized by the presence of specific flora, fauna, and microorganisms. There are two major types of biologically important environments in which the salt factor will interact with the microbial populations, soil and water. Soils containing >0.2% soluble salts are considered saline (Kaurichev, 1980). The range of salinities that can be found in soils is very wide and complex microflora develops within most of this range (Quesada *et al.*, 1982). Saline waters are much better studied and known than saline soils. Besides the ocean proper, there are many waters with salinity similar to that of seawater. Lakes are considered saline if they have >0.3% salinity (De Dekker, 1983).

Classification of halophilic fungi

Microbes that inhabit high-salt environments are adapted to high levels of ions, as well as to low water activity (w_a), and are described as halophilic or halotolerant, while the term xerophilic or xerotolerant is restricted to describing those organisms growing at w_a values imposed by ions other than inorganic ions (Grant, 2004). Halophiles are basically salt-loving organisms that inhabit hypersaline environments. In a review article “Microbial life at high salt concentrations: phylogenetic and metabolic diversity”, Oren *et al.* (2008) wrote: “Salt dependence and salt tolerance are phenotypic characteristics generally included in the ‘polyphasic’ characterization of newly discovered microorganisms toward their description as new taxa with new names and the determination of their position within the microbial taxonomy”. According to the same publication, there is no precise definition of the term ‘halophilic’. Some authors use the term for all organisms that require some level of salt for

growth, including concentrations around 35 g/L as found in seawater.

There are various definitions of halophilic fungi in the literature according to their relationship to salt, and there is still a debate as to which is most appropriate. The term halophilic for fungi was introduced as late as 1975 for a few foodborne species that showed superior growth on media with NaCl as the controlling solute (Pitt and Hocking, 1985). Some authors state that it is quite difficult to characterize the clear boundaries between the concepts: halotolerant and halophile fungi. Larsen (1986), and Kushner and Kamekura (1988) introduced three important definitions: moderate halophiles, extreme halophiles, and halotolerant microorganisms. Halophilic fungi can be classified according to their salt requirement and growth pattern. Gunde-Cimerman *et al.* (2005), proposed an operative definition of halophilic fungi according to their relationship to salt. In this study, halophilic fungi are defined as those fungi that can be cultured *in vitro* at a salt concentration of 3 M and can be isolated from natural habitats of salinity above 1.7 M.

Halophilic microorganisms, including microscopic fungi, have different mechanisms of adaptation and can multiply at high osmotic pressure and low level of w_a . They are able to maintain a balance between high osmotic pressure in the environment and low level of w_a outside the cell, compared to that within the intracellular (in the case of prokaryotes) or intercellular (in the case of eukaryotes) spaces (Le-Borgne *et al.*, 2008). Fungi living in different saline environments are generally adapted to extreme conditions of low w_a , temperature, pH and salinity (Griffith, 1994).

Natural habitats and biodiversity of halophilic fungi

Fungal diversity of different saline environments has been studied by many researchers. Species diversity and potential application in the industry of halophilic and halotolerant fungi has aroused increasing interest among scientists in the last decade. Halophilic fungi have been reported to exist in hypersaline and polyhaline marine environments, such as solar salterns, in different continents (Gunde-Cimerman *et al.*, 2000; Nayak *et al.* 2012; Ali *et al.*, 2018), Dead Sea (Buchalo *et al.*, 1998; Kis-Papo *et al.*, 2003) desert soil (Gunde-Cimerman *et al.*, 2005) and some sebkha (Jaouani *et al.*, 2014), estuary and salinity lakes (Kopytina *et al.*, 2019), and mangroves (Nayak *et al.*, 2012).

The Dead Sea is an extremely hypersaline environment hostile to microorganisms. There is

a high concentration of about 340‰ of total dissolved salts in its water, with levels of divalent ions such as magnesium and calcium being higher than those of monovalent ions. This specific ionic composition could be responsible for the low biodiversity of the Dead Sea biota (Buchalo *et al.*, 1998; Kis-Papo *et al.*, 2003). Buchalo *et al.* (1998) were the first to report *Gymnascella marismortui* species isolated from surface water samples from the Dead Sea as obligate halophilic fungi, and *Penicillium westlingii* and *Ulocladium chlamydosporum* isolated from deep waters at Ein Gedi as halotolerant (Buchalo *et al.*, 1998). Later, different authors published information for many more halotolerant and halophilic fungal species in Dead Sea samples, such as *Aspergillus* and *Cladosporium* spp., *Penicillium westlingii*, *G. marismortui* (Molitoris *et al.*, 2000; Wasser *et al.*, 2003; Nazareth *et al.*, 2012; Nazareth *et al.*, 2014). Fungal seasonal dynamics and interannual changes of the quantity and qualitative composition of filamentous fungi inhabiting the Dead Sea have been analyzed by Tova *et al.* (2017). Evidence that within 15 years (2000-2015) the turnover of fungal species diversity decreased from 34 species in winter 2000 to 2 species in summer 2015 was published in their article. Species diversity steadily decreased and highly and significantly correlated with declining water level as well as increasing density and salinity stress, which is currently 348 gL⁻¹. The two surviving species, *Aspergillus amstelodami* and *A. ruber*, increased in frequency across all sampling sites, down to the Dead Sea bottom (-291 m), due to their evolutionary adaptations to tolerate hypersalinity. However, even the species best adapted to highly increased salinity have decreased in frequency in the current extremely harsh conditions.

The Black Sea is an interesting saline ecosystem still poorly studied. In the literature there is scarce information about microbial and particular fungal diversity in some regions of the Russian, Georgian, Romanian, Turkey, Ukrainian and Bulgarian coastal waters (Kopytina, 2019). Micromycetes are represented by two ecological groups: 121 species of obligate water fungi (51 species of lower fungi, and 70 species of filamentous fungi (higher), 323 species of facultative water micromycetes (terrestrial fungi, which are able to function in marine water) (Dudka and Kopytina, 2011). The identification of isolated fungi from different geographical regions proved the existence of large amounts of fungi of the genera *Aspergillus*, *Penicillium*, *Alternaria* and *Cladosporium* (Kopytina, 2019).

Salt lakes are another natural habitat of halotolerant fungi. Episodic studies of the mycobiota inhabiting the salty estuaries (lakes) in the Black Sea basin have been carried out at different times, for example, on the Crimean Peninsula, Lake Pomorie in Bulgaria, Tekirgela estuary in Romania. Their salinity varies from 77.8 up to 340‰. In the Kuyalnitsky estuary, 44 species of fungi were found (in water - 5, in peloids - 42, on a tree blue - 14). In the bottom sediments of Lake Pomorie, 36 species have been identified (Smolyanyuk *et al.*, 2011). In the peloids of the Tekirgela estuary (Romania), species of the families *Traustochytriaceae* and *Chytridiaceae* have been identified (Kopytina, 2019). To our knowledge, there is no available information about the fungal biodiversity of Pomorie Lake water (Bulgaria). Mono Lake is a closed salt lake located in central California, particularly rich in sodium, potassium, phosphorus and boron. Soil and sediments contain very high levels of calcium and magnesium, but also barium, boron and strontium. In total, sixty-seven fungal species belonging to various taxonomic groups were isolated from sediment, soil and tufa samples, while fungi were not isolated from water samples. The highest number of species was found in the genera *Acremonium* (four species) and *Penicillium sodium*. Authors concluded that no very specific fungal flora was found in hypersaline environments (Steiman *et al.*, 2004).

The structure and diversity of fungal communities was documented in young transient and mature hypersaline microbial mats from a tropical region (Puerto Rico). Traditional and molecular techniques revealed strong spatial and temporal heterogeneities in both microbial mats. As shown in the paper of Cantrell *et al.* (2013), some of the species isolated belong to the genera *Aspergillus*, *Cladosporium*, *Hortaea*, *Pichia* and *Wallemia*, which are often isolated from hypersaline environments. The most abundant clones belong to *Acremonium strictum* and *Cladosporium halotolerans*, which were not isolated in pure culture.

Another natural habitat in which halophilic fungi are found is solarsalters - hypersaline extreme environments with unique physicochemical properties such as a salinity gradient. The hypersaline waters of solar salters are referred to as thalassohaline, having salt concentrations greater than 3.5%, the concentration of salt in seawater (Gunde-Cimerman *et al.*, 2000). Fungi isolated from salters are represented by black yeasts (*Hortaea werneckii*, *Phaeotheca triangularis*, *Aureobasidium pullulans*, and *Trimmatostroma salinum*), *Cladosporium*, *Aspergillus*, and *Penicillium* species. Most studies on saltern-derived fungi focused

on black yeasts and their physiological characteristics, including growth under various culture conditions (Chunget *et al.*, 2019).

A new halophilic fungal species, *P. imranianum*, isolated from a man-made solar saltern, located at Phetchaburi province, Thailand, has been recently reported. The authors claim that to the best of their knowledge, it is the most extremely halophilic *Penicillium* ever isolated (Ali *et al.*, 2018). Studies by other authors have reported *G. marismortui* (Buchalo *et al.*, 1998), *Wallemia ichthyophaga* (Zalar *et al.*, 2005; Gunde-Cimerman *et al.*, 2009), *Trichosporium* (El-Meleigy *et al.*, 2010), *A. penicillioides* and *A. unguis* (Nazareth *et al.*, 2012) as obligate halophiles.

Mangroves and salters of Goa, India, are typical saline ecosystems where halophilic fungi have been found. According to authors, *Aspergillus* and *Penicillium* species predominate in the studied regions. The isolates are reported to have high levels of tolerance to sodium chloride and showed resistance to salts of heavy metals Pb^{2+} , Cu^{2+} , and Cd^{2+} . Species belonging to *Penicillium* showed the highest levels of halotolerance as well as resistance to heavy metals, the single isolate of a triverticillate morphotype of *Penicillium* obtained showing the highest resistance. *Aspergilli* also displayed fairly high halotolerance as well as metal resistance, with isolates of *A. niger* and *A. flavus* showing higher levels of resistance. Other isolates belong to the genera *Paecilomyces*, *Fusarium*, *Alternaria* and *Cladosporium*. Among these, *Paecilomyces* showed a fair amount of halotolerance; the others showed lower tolerance (Nazareth *et al.*, 2012). During his study of mangroves and solar salters of Goa (Nayak *et al.*, 2012), isolated and identified (characterized) high salt tolerant fungi belonging to the genus *Aspergillus*; some to *Penicillium*, and a few to *Eurotium* and *Hortaea*. Only one species, *A. penicillioides*, strongly required addition of salt to the growth medium and was termed as an obligate halophile. However, most isolates do not have an absolute requirement for added salt for growth. One hundred soil samples were collected from the Miani-Khor mangrove ecosystem of Balochistan, Pakistan, to study the microbial diversity of this salt area. Isolated halotolerant and halophilic fungi were identified by classical and molecular methods. Different fungal species were found to belong to the genera *Aspergillus*, *Penicillium*, *Alternaria*, *Fusarium*, and *Pleurospora* (Khan *et al.*, 2020).

Sebkhas are salt flat areas, coastal or interior, where, as the result of evaporation, salt and

other evaporite minerals precipitate near or at the surface (Neuendorf *et al.*, 2005). There are several publications about halotolerant and halophilic fungi isolated from The Great Sebkha of Oran (the largest sebkha in north-western Algeria), whose salt concentration is estimated at more than 100 g/L⁻¹ of dissolved salts (Benziane, 2013), Sebkha El Melah in Tunisia (Jaouani *et al.*, 2014), and the sabkha in Kuwait (Al-Musallam *et al.*, 2011). Fungal isolates were identified by morphological and microscopical observations and by molecular techniques. Halotolerant and halophilic fungi belonging to the genera *Penicillium*, *Trichoderma*, *Alternaria*, *Chaetomium* and *Wallemia* (showing high similarity ($\geq 97\%$), *Chrysosporium*, *Tritirachium*, *Lecanicillium* and *Pleospora* showing a high similarity (100%) to unidentified species of these genera were described. Also, some fungi were found to belong to the genera *Aspergillus*, *Arachnomycetes*, *Fusarium* and *Chaetomium*. Several species of the genera *Aspergillus* and *Penicillium* have already been reported as the most frequently isolated species in hypersaline environments. Surprisingly, the results show that only one strain of *Wallemia* sp. and the two strains of *G. halophilus* are obligatorily halophilic. Most of the strains could grow at 12.5% NaCl and 5 strains (*A. subramanianii* strain A1, *Aspergillus* sp. strain A4, *P. vinaceum* and the two strains of *G. halophilus*) at 17.5%. The only strain that could grow at 20% was *Wallemia* sp. Taxonomic characterization of the isolates showed that the most dominant genera found in the sebkha were *Penicillium*, *Aspergillus*, their teleomorphic forms and *Fusarium* (Rajaa Chamekhet *et al.*, 2019). From Sebkha El Melah in Tunisia, Jaouani *et al.* (2014) isolated and characterized 21 fungi. They reported that this fungal community has similar composition to those reported in inhospitable habitats characterized by limitation of nutrients, moisture deficit, and exposure to high solar radiation. Alkali-halotolerant fungi have been isolated and assigned to 15 taxa belonging to 6 genera of Ascomycetes.

The Great Salt Plains (GSP) of Oklahoma was studied as a natural salinity habitat for halophilic and halotolerant fungi. This region is an inland terrestrial hypersaline environment where saturated brines leave evaporite crusts of NaCl (Evans *et al.*, 2013). It was found that all of the isolates fall within the ascomycetes, with a predominance of *Trichocomaceae*, represented by *Aspergillus*, *Eurotium*, and *Penicillium* species. Representatives of *Anthrinxium*, *Cladosporium*, *Debaryomyces*, *Fusarium*, and *Ulocladium* also were isolat-

ed. Overall, the isolates were widely halotolerant, with best growth observed at lower salinities and no halophilism. The fungal genera observed were all cosmopolitan, without strong specialization. The dominance of cosmopolitan fungal genera, such as *Aspergillus* and *Penicillium*, suggests that the hypersaline fungal community is a halotolerant subset of common soil fungi in these areas. The culturable fungal community at the GSP appears to follow the same trends, being composed mainly of *Aspergillus*, *Cladosporium*, *Fusarium*, and *Penicillium* species.

In the literature, there are several publications dedicated to ecology and fungal diversity in cold extreme habitats such as glaciers, the Arctic shores, and the Antarctic region (Abyzov 1993; Buchalo *et al.*, 1998; Ma *et al.*, 1999; Gunde-Cimerman *et al.*, 2000; Ruissi *et al.*, 2006). Gunde-Cimeran *et al.* (2005) surmise that fungal adaptations to low temperatures can be related to salt stress, suggesting a link between psychrophily and halophily. Recently published scientific papers indicate that fungal strains isolated from cold habitats such as glaciers have been observed to grow at diverse salt concentrations (NaCl 18-24%) (Hasan *et al.*, 2018). For example, the fungal species most frequently isolated from ice, sediments and water samples collected from the Siachen glacier, Pakistan, were *Leotiomycetes* sp., followed by *Thelebolus*, *Penicillium*, *Cladosporium*, *Trichoderma*, *Periconia*, *Geomyces*, *Cryptococcus* and *Pueraria*. All isolates were found to be halophilic and able to tolerate NaCl concentration up to 10-20% (Hassan *et al.*, 2017). From Tirich Mir glacier 46 fungal strains were isolated and characterized. Among them, one isolate of genus *Davidiella*, identified as *Davidiella tassiana* HTF9, showed growth in the presence of 18% NaCl (Rafiq *et al.*, 2020). The investigation of the fungal biodiversity and halotolerant and halophilic fungal abundance of cold regions are only just beginning.

Extracellular enzymes and antimicrobial activity

The ability of many microorganisms to grow at different concentrations of NaCl and low a_w has been of great interest for microbiologists and biochemists in the last decade. Like each of their cellular components, their proteins are inherently more stable to salinity than those of conventional organisms. Microbiome of saline environments, especially halophilic fungi, has become one of the richest bioresources for industrially important molecules and enzymes. Halophilic microorganisms, including different groups of fungi, are recognized producers of secondary metabolites under harsh

saline conditions, such as carotenoid pigments, retinal proteins, hydrolytic enzymes, and compatible solutes, such as macromolecules stabilizers, biopolymers, and biofertilizers (Amoozegar *et al.*, 2017). Table 1 presents some of possible areas of applications of halophilic fungi.

Table 1. Potential biotechnological applications of halophilic fungi (Irman *et al.*, 2016)

Application	Resources
Antibiotics producers	Sepcic <i>et al.</i> , 2010
Sorce of antioxidants	Ghosh <i>et al.</i> , 2010; Ravindran, 2012
Environmental pollution indicators	Ali <i>et al.</i> , 2014
Enzymes producers	Dalboge, 1997; Karbalaee-Heidari <i>et al.</i> , 2011; Chakraborty <i>et al.</i> , 2009; Vidyasagar <i>et al.</i> , 2009

Extracellular enzymes produced by halophilic and halotolerant fungi

One of the important requirements for enzymes to find application in industry is that these bioactive catalysts maintain their stability and activity in an unfavorable environment, such as high temperature, presence of different salts, alkaline or acidic pH. Previous studies have shown that halophilic fungi are an important source of polyextremophilic metabolites. Their thermotolerant and halophilic properties allow them to be stable and applicable within wide ranges of pH and temperature in industrial processes (Dalboge, 1997; Setati, 2010).

As previously reported in the literature, extremozymes from halophilic fungi (such as *T. salinum*, *W. ichthyophaga*, *H. werneckii*, and *P. triangularis*) are interesting for industrial and biotechnological applications, since they can act at harsh conditions. Extremozymes from halophilic fungi, e.g. amylases, cellulases, lipases, and proteases, which possess the so-called polyextremophilicity (they are thermostable, tolerant to a wide range of pH, less susceptible to denaturation and tolerant to high salt concentrations), present a novel catalytic alternative for biotechnological applications (Delgado-García, 2012). Physiological activities providing energy and metabolites for growth and other processes require the enzymes to be active under the prevailing conditions.

There are many scientific data in the literature on halophilic enzymes isolated from halophilic bacteria, while halophilic fungi are still poorly

studied as sources of halophilic enzymes, but interest in them has grown in recent years (Mukhtar and Haq, 2012). Halophilic and halotolerant fungi isolated from different natural habitats can provide thermostable and salt-tolerant enzymes of industrial interest. Potential industrial applications of enzymes from halophilic fungi are in waste management (reduction of mixed waste or waste water remediation), textile, pharmaceutical, and food industries. For example, the demand for salt-tolerant lignocellulolytic enzymes for development of various industrial processes such as production of biofuels is currently growing. Freshwater is a rare and valuable resource that could be replaced by sea water in certain biotechnological applications using salt tolerant enzymes (Arfi *et al.*, 2013). Some enzymes of biotechnological interest isolated from halophilic fungi are presented in Table 2.

Laccases are phenoloxidases involved in the transformation of the recalcitrant fraction of organic matter in soil. These enzymes are also able to transform certain aromatic pollutants such as polycyclic aromatic hydrocarbons (PAHs) and are known to be inhibited by chloride ions. *Chaetomium sp.*, *Xylogone sphaerospora*, and *Coprinopsis sp.* fungi isolated from the Mediterranean coastal area have been found to produce halotolerant laccase (Qasemian *et al.*, 2012).

Aspergillus caesiellus, a moderate halophilic thermotolerant fungal strain isolated from sugarcane fermentation in the presence of 2 M NaCl, has been reported as a producer of thermostable and salt-tolerant cellulases (Batista-García *et al.*, 2014). Also, some unidentified halophilic fungal strains isolated from Great Sebkha of Oran from two species, *Aspergillus sp.* and *Chaetomium sp.*, were described as good producers of cellulose in the study of Chamekh *et al.* (2019). Some authors demonstrated that the marine white-rot fungus *Phlebia sp.*, when grown in the presence of 3% sea salt, secretes two different manganese peroxidases (MnP) distinct from the one produced in the absence of salt, suggesting that marine fungi may possess sets of alternative lignocellulolytic enzymes adapted to salt (Kamei *et al.*, 2008).

To date, however, very little is known about the mechanisms employed by mangrove fungi to breakdown the available lignocellulosic biomass and how their enzymes has adapted to marine conditions, especially to the presence of high concentrations of salt. It has been shown that several marine ascomycetes encountered in mangrove are capable of wood decay and

Table 2. Examples of some valuable enzymes produced by halophilic fungi and their application in different industrial processes

Enzyme	Application	Halophilic fungal source	References
Proteases	degradation of plant and animal proteins; peptides synthesis	<i>P. chrysogenum</i> , <i>C. halotolerans</i> <i>C. sphaerospermum</i> , <i>C. cladosporioides</i> , <i>Engyodontium album</i>	Chamekhet <i>et al.</i> , 2019 Jaouani <i>et al.</i> , 2014
Amylases	textile, laundry, pharmaceutical and food industries, wastewater remediation, degradation of starch	<i>A. gracilis</i> , <i>A. penicilliioides</i> , <i>P. chrysogenum</i> , <i>C. cladosporioides</i> , <i>Engyodontium album</i> , <i>Alternaria alternata</i>	Chakraborty <i>et al.</i> , 2009 Ali <i>et al.</i> , 2014; Jaouani <i>et al.</i> , 2014; Ali <i>et al.</i> , 2015
Laccase	plant material delignification, lignin-degrading enzyme	<i>C. halotolerans</i> , <i>C. sphaerospermum</i> , <i>Penicillium sp.</i>	Jaouani <i>et al.</i> , 2014
Lipases	Food and agro industries, cleaning, biofuel, pharmaceuticals, textile cosmetic, perfumery, flavour industry, bioremediation	<i>P. vinaceum</i> , <i>U. cynodontis</i> Wallemia sp. <i>C. sphaerospermum</i> <i>E. album</i>	Jančič <i>et al.</i> , 2016 Chamekh <i>et al.</i> , 2019
Pectinases	Fruit juice industries, paper and pulp industries, food industries	<i>Penicillium sp.</i> K 3-17 (extreme halophile), <i>Penicillium sp.</i> K-5 <i>A. terreus</i>	Helanet <i>et al.</i> , 2013
Cellulases	Hydrolyses the cellulose, production of biofuel, waste management, paper industry	<i>A. calidoustus</i> , <i>G. halophilus</i> , <i>P. vinaceum</i> , <i>Aspergillus sp.</i> , <i>Chaetomium sp.</i> <i>Ustilago cynodontis</i> , <i>Fusarium species</i> , <i>Chrysosporium sp.</i> , <i>Arachnomyces sp.</i> , <i>S. strictum</i> , <i>Aspergillus glaucus CCHA</i> , <i>Pestalotiopsis sp.</i> NCi6, <i>Aspergillus caesiellus</i>	Chamekh <i>et al.</i> , 2019; Li <i>et al.</i> , 2018; Arfi <i>et al.</i> , 2013; Batista-García <i>et al.</i> , 2014
Xylanases	Production of biofuel, waste management, paper industry, as a supplement in animal feed, for the manufacture of bread, food and drinks, textiles, bleaching of cellulose pulp, and xylitol production	<i>Pestalotiopsis sp.</i> NCi6	Wang <i>et al.</i> , 2009; Arfi <i>et al.</i> , 2013

show cellulose and xylan degradation activities (Bucher *et al.*, 2004). Given the presence of sea water in these ecosystems, mangrove fungi are adapted to high salinity. *Pestalotiopsis sp.* NCi6, a halotolerant and lignocellulolytic mangrove fungus showed the ability to grow on a complex lignocellulosic substrate regardless of the presence of high concentrations of salt, but with variations in lignocellulolytic activities (Arfi *et al.*, 2013). *A. caesiellus* H1 isolated from sugarcane bagasse fermentation in the presence of 2 M NaCl was characterized as a producer not only of thermostable cellulases but also of xylanases, MnP, and esterases. No laccase activity was detected in the tested conditions (Batista-García *et al.*, 2014). The biotechnological potential and ability to produce extracellular enzymes (i.e., lipase, amylase, protease, and cellulase) of the 50 halophilic fungal strains isolated from Great Sebkha of Oran in north-western Algeria was tested by Chamekh *et al.* (2019). *Aspergillus sp.* strain A4, *Chaetomium sp.* strain H1, *P. vinaceum*, *G. halophilus*, and the two basidiomycetous *Wallemia sp.* and *U. cynodontis* have been reported as the most interesting species presenting the highest enzymatic index. *Wallemia sp.* has high lipase activity but no cellulolytic, amylolytic or proteolytic activity was detected. This result was also obtained by Jančič *et al.* (2016), who studied the enzymatic profile of the four species of *Wallemia* (*W. sebi*, *W. ichthyophaga*, *W. muriae* and *W. hederiae*). They found that *Wallemia spp.* secretes several enzymes including lipase and esterase, but no cellulolytic, amylolytic, and proteolytic activities were observed. Amylase, cellulase, invertase and xylanase were found to be secreted in significant amounts (creating clear zones) by one strain of *P. imranianum* (extreme halophile) isolated by Ali *et al.* (2018), from a man-made solar saltern in Thailand. Polyextremophilic α -amylases obtained from halophilic *Engyodontium album*, obligate halophilic *A. gracialis* and from obligate halophilic *A. penicillioides* were purified and characterized (Ali *et al.*, 2014; Lotrakul and Punnapayak *et al.*, 2014; Ali *et al.*, 2015).

Fungi elaborate a wide variety of proteolytic enzymes than bacteria. Filamentous fungi have the potential to grow under varying environmental conditions such as time course, pH and temperature, utilizing a wide variety of substrates as nutrients (Haq *et al.*, 2006).

Halophilic fungi showing antimicrobial activity

Antimicrobial resistance is a growing problem in the world today. Nearly three-quarters of all

clinically relevant antibiotics are natural substances produced by bacteria or fungi. However, the antibiotics that are currently available are losing their effectiveness and increasing numbers of pathogens are becoming resistant. This means there is an urgent need for new antibiotics and new antimicrobial substances (Katz and Baltz, 2016). Drug and antibiotic resistance is an issue that has developed over decades and determines the constant need for new antibiotics. Some of the halophilic fungi isolated so far show high antibacterial activity, which makes them attractive as potential producers of antibiotics. A series of recent studies has indicated that antibiotic activity from halophilic/halotolerant fungi is more effective at low water content or increased NaCl concentration (Sepcic *et al.*, 2011). Quite recently, microbiologists have started to take a new and serious interest in the antimicrobial activity of fungi isolated from hypersaline ecosystems. Still there are very few reports related to antimicrobial agents produced by halotolerant and halophilic fungi. Table 3 presents information about the antimicrobial activity of some halophilic and halotolerant fungi.

Lebogang *et al.* (2009), carried out a preliminary study of fungi isolated from the soil of the salt pans in Botswana (saline and alkaline environment) and their anti-microbial properties. The most prevalent fungi were *Aspergillus*, *Fusarium*, *Dendryphiopsis* and *Phoma* species. Surprisingly, only one species of *Penicillium* was found. Bioactivity results indicate that all fungal isolates produce substances that inhibit the growth of at least one test microorganisms (*B. megaterium*, *S. aureus*, *E. coli*, *C. albicans*, *A. niger*). Isolated *A. terreus* was found to exhibit the highest antimicrobial activity inhibiting the growth of all test microorganisms. The antimicrobial activity of *Phoma* species was observed to be the lowest. Other strains of the genus *Aspergillus* were also reported to have antimicrobial activity (Ali *et al.*, 2014). It was demonstrated that the extremely halophilic fungal strain *P. imranianum* isolated from a man-made solar saltern in Phetchaburi province, Thailand, possessed antibacterial potential against gram-negative bacteria (*Acinetobacter spp.* and *E. coli*) compared to gram-positive ones (*S. aureus*). Its antibacterial activity was demonstrated by plate screening method (Ali *et al.*, 2018).

A study of halophilic fungi from solar saltern (Helan Soundra Rani and Kalaiselvam, 2013) recorded *Cladosporium sp.*, *H. werneckii*, *A. alternata* isolates grown at 15% NaCl concentration. All isolates were screened for secondary metabolite production. Then, the potential of a secondary me-

Table 3. Antimicrobial activity of some halophilic and halotolerant fungi

Fungi	Antibacterial activity/test microorganisms	References
<i>Cladosporium</i> sp	<i>Salmonella typhi</i> , <i>Klebsiella pneumoniae</i>	Helan Soundra Rani and Kalaiselvam, 2013
<i>H. werneckii</i>	<i>Escherichia coli</i> , <i>Vibrio parahaemolyticus</i>	Helan Soundra Rani and Kalaiselvam, 2013
<i>A. alternata</i>	<i>E. coli</i>	Helan Soundra Rani and Kalaiselvam, 2013
<i>A. protuberus</i>	<i>Staphylococcus aureus</i>	Corral <i>et al.</i> , 2018
<i>A. flocculosus</i> PT05-1	<i>Enterobacter aerogenes</i> , <i>Pseudomonas aeruginosa</i> , <i>Candida albicans</i>	Zheng <i>et al.</i> , 2013 Wang <i>et al.</i> , 2011
<i>A. terreus</i> PT06-2	<i>E. aerogenes</i> , <i>P. aeruginosa</i> , <i>C. albicans</i>	Zheng <i>et al.</i> , 2013 Wang <i>et al.</i> , 2011
<i>P. imranianum</i>	gram negative bacteria (<i>Acinetobacter</i> spp. and <i>E. coli</i>) gram positive one (<i>S. aureus</i>).	Ali <i>et al.</i> , 2018

tabolite extract from *A. alternata* was observed by testing with human pathogens and it showed maximum inhibitory effect against *E. coli*. Extracts of *Alternaria alternata* were highly active against the pathogens *E. coli*, *S. typhi*, *V. parahaemolyticus*, *K. oxytoca*. Crude extracts from *Cladosporium* sp showed activity against *S. typhi*, *K. pneumoniae*, and *H. werneckii* fungus also showed inhibition against two pathogens, *E. coli* and *V. parahaemolyticus*. It was concluded that halophilic fungi from solar salterns are diverse, many produce compounds with antimicrobial activity, and could be suitable sources of new antimicrobial natural products (Helan Soundra Rani and Kalaiselvam, 2013).

The halophilic species of the genus *Aspergillus* are the most prolific and several strains of *Aspergillus* sp. have been isolated from Arctic sub-sea sediments from the Barents Sea. In particular, strain 8Na identified as *A. protuberus*, a polyextremophilic fungus able to grow in a wide range of pH, temperature and salinity (up to 25% (w/v)) showed antimicrobial efficacy against human pathogens. The strongest inhibitory action was observed against *S. aureus*. The molecule responsible for the activity was identified as *Bisvertinolone*, a compound member of the family Sorbicillinoid (Corral *et al.*, 2018). *A. flocculosus* PT05-1 and *A. terreus* PT06-2, both isolated from sediment of Putian sea saltern of Fujian, China, showed antimicrobial activity against *E. aerogenes*, *P. aeruginosa*, and *C. albicans*. Strain PT05-1 produces 11 metabolites, among which two are new ergosteroids and pyrrole derivative compounds (Zheng *et al.*, 2013), and strain PT06-2 produces the novel compounds Terrelactone A and Terremides A and B (Wang *et al.*, 2011). All studies of

halophilic and halotolerant fungi until now indicate their great potential as producers of enzymes and antimicrobial metabolites.

Conclusions and perspectives

An attempt has been made to add new information on the biodiversity of salt-adapted fungi and their potential in biotechnology. Data persuasively prove the remarkable diversity of halophilic and halotolerant fungi. Published results demonstrate that species of a wide range of fungal genera can grow and form spores in saline environments. These extremophiles are ubiquitously present in natural ecological niches, such as salt lakes, inland seas, evaporation ponds of seawater, salterns, glaciers, the Arctic and Antarctic regions, etc. Taxonomic studies with culturable diversities of halophiles have revealed that the dominant genera are *Aspergillus*, *Cladosporium*, *Penicillium*, *Alternaria*, *Fusarium*, and *Chaetomium*. Many new species have been reported from various saline and hypersaline habitats located in different countries. The investigations of new natural sources of salt-adapted fungi as well as new information about their abundance will expand our knowledge of possible life performance under diverse and most extreme environmental parameters. In addition, understanding fungal adaptation to high salinity will provide new insight into the mechanisms that help organisms to survive under such extreme environmental conditions.

In this review, we have also focused on the capability of halophilic and halotolerant fungi to produce bioactive molecules. All data presented suggest that fungi isolated from different saline habitats are promising producers of extremozymes and new biologically active and antimicrobial sub-

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Mycosynthesis and Characterization of Gold and Silver Nanoparticles Using Biomolecules of *Pleurotus ostreatus* as Antibacterial Agent against Some Selected Pathogens

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Abstract

The study aims to investigate the biosynthesis and characterization of gold (AuNPs) and silver nanoparticles (SNPs) using biomolecules: exopolysaccharides (EPS), culture-free filtrate (CFF) and fruiting body extract (FBE) of *Pleurotus ostreatus* (PO), and to evaluate the antibacterial potential of the nanoparticles against some pathogens. The nanoparticles were characterized by UV-visible spectra, FTIR and SEM. Nanoparticles formation was confirmed by changes in colour; colour changes from pale yellow to purple, cloudy colourless to purple and yellow to dark purple indicate, respectively, POEPSAuNPs, POCFFAuNPs and POFBEAuNPs formation. Colour changes from pale yellow, cloudy colourless and yellow to dark brown indicate POEPSSNPs, POCFFSNPs and POFBESNPs formation, respectively. Surface plasmon resonance (SRP) peaks were observed at 400 nm. SEM revealed POEPSAuNPs, POCFFAuNPs and POCFFAuNPs as polymorphic with sizes ranging from 0.5 – 2.6 μm , 0.08 – 1.00 μm , and 0.2 – 2.4 μm . POEPSSNPs, POCFFSNPs and POFBESNPs were aggregate particles with sizes of 0.2 – 3.0 μm , 0.2 – 2.8 μm , and 0.1 – 3.2 μm . FTIR showed that amide, carboxyl and hydroxyl groups contributed to nanoparticles biosynthesis. The AuNPs exhibited a broad spectrum of activity against the tested pathogens compared to the SNPs. POEPSAuNPs and POFBEAuNPs had the highest inhibitory activity against *E. coli* while POFBEAuNPs had the highest inhibitory activity against *Micrococcus* sp. with MIC of 0.2 at 200 $\mu\text{g/mL}$, and POEPSSNPs and POCFFSNPs had the highest inhibitory activity against *S. aureus* with MIC of 0.05 at 50 $\mu\text{g/mL}$. In conclusion, biomolecules from *P. ostreatus* supported the biosynthesis of nanoparticles with enhanced therapeutic properties.

Keywords: *Pleurotus ostreatus*, exopolysaccharides, culture-free filtrate, gold and silver nanoparticles, antibacterial activity, pathogen.

Резюме

Целта на това проучване е да се изследва биосинтезата и охарактеризирането на златни (AuNPs) и сребърни наночастици (SNP) с помощта на биомолекули: екзополisahариди (EPS), отдекантирана културелна супернатанта (CFF) и екстракт от плодно тяло (FBE) на *Pleurotus ostreatus* (PO), както и да се направи оценка на антибактериалния потенциал на тези наночастици срещу някои патогени. Наночастиците са охарактеризирани с UV/Vis спектрофотометър, FTIR и SEM. Образоването на наночастици се потвърждава от промени в цвета от бледожълто до лилаво, от безцветно облачно до лилаво, както и от жълто до тъмно лилаво, което съответства на образуването на POEPSAuNPs, POCFFAuNPs и POFBEAuNPs. Промените в цвета от бледожълто, безцветно облачно и жълто до тъмнокафяво показват съответно образуването на POEPSSNPs, POCFFSNPs и POFBESNPs. Наблюдавани се пикове на повърхностния плазмен резонанс (SRP) при 400 nm. Чрез SEM POEPSAuNPs, POCFFAuNPs и POCFFAuNPs се визуализират като полиморфни частици с размери, вариращи от 0.5 – 2.6 μm , от 0.08 – 1.00 μm и от 0.2 – 2.4 μm . POEPSSNPs, POCFFSNPs и POFBESNPs се виждат като агрегирани частици с размери от 0.2 – 3.0 μm , от 0.2 – 2.8 μm и от 0.1 – 3.2 μm . Чрез FTIR се доказва, че amidните, карбоксилните и хидроксилните групи участват в биосинтезата на наночастиците. AuNPs показва широк спектър на антибактериална активност срещу тестваните патогени в сравнение с SNPs. POEPSAuNPs и POFBEAuNPs имат най-висока инхибиторна активност срещу *E. coli*, докато POF-

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BEAuNPs проявяват най-висока инхибиторна активност срещу *Micrococcus sp.* с MIC 0,2 при 200 µg/ml, а POEPSSNPs и POCFFSNPs имат най-висока инхибиторна активност срещу *S. aureus* с MIC 0.05 при 50 µg/ml. В заключение, изследването демонстрира, че биомолекулите от *P. ostreatus* поддържат биосинтезата на наночастици с повишени терапевтични свойства.

Introduction

The increased resistance of pathogenic bacteria to conventional antibiotics has led to an inroad into a novel field called nanotechnology. This is a science that combines biology, chemistry, and physics for the synthesis of nano-scale particles that have numerous applications (Rezaei-Zarchi *et al.*, 2012; Demir *et al.*, 2014). The field of nano-science and technology have gained great importance because of its potential applications in various areas such as chemicals, textile industries, materials industry, medical diagnostic (future nanobots), drug and gene delivery and electronics, diagnosis, artificial implants, tissues engineering, computing, biochemical sensors, medical imaging and so on (Huang *et al.*, 2003; Ghosh *et al.*, 2008; Pissuwan *et al.*, 2010; Pissuwan *et al.*, 2011; Saha *et al.*, 2012). Moreover, it provides a platform to modify and develop metals of unique properties to form nanoparticles having promising applications in diagnostics, biomarkers, cell labelling, contrast agents for biological imaging, antimicrobial agents, drug delivery systems and nano-drugs for treatment of various diseases (Coster *et al.*, 2010). They have been used to produce nanoparticles and these are referred to as the building blocks of nanotechnology. Nanoparticles can be produced using physical, chemical and biological methods and the latter method is considered to be safe and eco-friendly.

Although biological methods involve the use of bacteria, fungi, yeast, actinomycetes and higher plants, fungi are considered a reliable source of metabolites that can be used to biosynthesize nanoparticles (Phanjom *et al.*, 2015). Mushrooms, a higher fungus, are gourmet cuisine with a high nutritional value and therapeutic properties, and also known to contain metabolites that have been confirmed to have antimicrobial effect, a good example being *P. ostreatus*, a cosmopolitan mushroom. Metabolites such as polysaccharides and cell-free filtrates of mushrooms may act as both reducing and capping agents in nanoparticle biosynthesis, thus producing bio-compatible and bio-stable nano-sized particles. The use of mushrooms in nanotechnology derives from the large production of bioactive compounds that are eco-friendly bio-reductants, such as proteins, polysaccharides and enzymes (Castro-Longoria *et al.*, 2011). These have been used for pharmaceutical purposes due to their diverse biological

activities that include anti-tumour and immunomodulation activities. It is usually secreted into the environment and is known to reduce heavy metals in their surroundings because of the functional groups (hydroxyl groups) and metal salt precursors they possess (Mata *et al.*, 2004). Since fungi are very effective secretors of extracellular compounds, therefore, achieving a vast production of bioactive compounds. Also, the economic viability and facility of employing biomass are another merit of the utilization of a green fungal-mediated approach to synthesize metallic nanoparticles. Moreover, a number of species grow fast and therefore culturing and keeping them in the laboratory are very simple (Castro-Longoria *et al.*, 2011). In the present scenario, nanomaterials emerged as novel antimicrobial agents and this is due to their high surface area to volume, unique chemical and physical properties, and ability to concentrate conventional antibiotics when used in synergy (Kim *et al.*, 2003).

The most studied aspect of nanotechnology is its ability to offer an opportunity to fight drug resistance. The mechanism of action of nanoparticles is quite different from that of antibiotics, thus nanoparticles are safe, economical and cost effective to combat the challenges in future (Fayaz *et al.*, 2011).

Biologically inspired nanoparticles synthesis is currently a rapidly expanding area of research in nanotechnology owing to their many advantages, with silver nanoparticles (SNPs) and gold nanoparticle (AuNPs) being the most studied of the metals. Among nanoparticles, SNPs possess higher antibacterial activity against gram-negative and gram-positive microorganisms. Factors such as solvent choice, non-toxic stabilization material and environmental benign reducing agent are the factors that are responsible for the biosynthesis of biologically safe nanoparticles. Also, gold nanoparticles, apart from their antibiotic capabilities, have been used in nano-imaging due to their bio-compatibility, stability and bio-reduction ability. Gold nanoparticles in peptidoglycan will disturb the crystalline process of peptidoglycan by forming an amorphous region around them, which will reduce the capacity to resist pressure from the inside of cell walls. Usually, peptidoglycan has a crystalline structure that is resistant against the high osmotic pressure inside the cell, which may reach 20

atmospheres in the case of gram-positive bacteria. The nanoparticles will act as destruction triggering points of cell walls. In addition, gold nanoparticles can be utilized as energy sources of physical energy under the fields of electromagnetic waves, X-ray or ultrasound to damage cell walls or chromosomes, leading to the suppression of bacteria growth.

Silver nanoparticles (SNPs) attached to the surface of bacteria based on size. All SNPs were found to be highly toxic to the bacterial strains and their antibacterial efficacy increased by lowering particle size. This effect was significantly enhanced as the size of nanoparticles approached the sub-10nm range and 5nm SNPs demonstrated the fastest bactericidal activity as compared to 7nm and 10nm SNPs at their respective, minimum bactericidal concentration. The bacterial membrane contains sulphur-containing proteins, and the silver nanoparticles interact with these proteins in the cell as well as with the phosphorus-containing compounds like DNA (Marini *et al.*, 2007). Silver has a greater affinity to bind with the sulphur- and phosphorus-containing bio-molecules of the cell. Therefore, sulphur-containing proteins in the cell membrane, inside the cells and phosphorus-containing elements like DNA are likely to be the preferred sites for binding of silver nanoparticles. If silver nanoparticles enter the bacterial cell, they form a low molecular weight region in the centre of the bacteria to which the bacteria conglomerates, thus protecting the DNA from the silver ions. The nanoparticles preferably attack the respiratory chain, cell division finally leading to cell death. The nanoparticles release silver ions in the bacterial cells, which enhances their bactericidal activity (Rai *et al.*, 2009).

Increasing resistance of pathogenic strains to antibiotics has led to failure in the treatment of infectious diseases and this is a major challenge in the medical and pharmaceutical fields. These major strains include *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Klebsiella pneumonia* (Fayaz *et al.*, 2011). Different infections by drug resistant strains have been reported worldwide and are considered major pathogen of community and hospital-acquired infections. These have resulted into high mortality and morbidity rate and the treatments are getting difficult as a result of high resistance to antibiotics. Drug resistance, especially in recent decades, has led to the search for different approaches and methods for finding new compounds against bacteria and fungi. Nanoparticles have dimensions of 100 nm or less. They

have gained remarkable attention because of their unusual properties and the various applications they are suited for, when compared to their bulkier counterparts (Kato *et al.*, 2011). These features have helped spread the use of nanomaterials at a faster rate day by day. They can be used to fight against germs, in diagnostics and treatment of diseases, water and air purification, food production, cosmetics, and clothing (Aitken *et al.*, 2006). Silver is the most commonly used engineering nanomaterial in all consumer products (Akinoglu *et al.*, 2014). Mycosynthesis and characterization of gold and silver nanoparticles using EPS and CFF and FBE of *P. ostreatus* and its antibacterial potential against some selected pathogens was investigated.

Materials and Methods

Sample collection and culture preparation

P. ostreatus samples were purchased from FoodCo in Ibadan, Oyo state, Nigeria. Mycelium was obtained by tissue culturing, pure cultures were obtained by sub-culturing and the stock culture was maintained on PDA supplemented with 0.5% yeast extract stored at 4°C on PDA slants (Jonathan and Fasidi, 2003).

Clinical isolates of *K. pneumonia*, *Bacillus cereus*, *P. aeruginosa*, *Micrococcus* sp., *E. coli* and *S. aureus* were collected from the culture collection of the Environmental Unit of the Department of Microbiology and Department of Pharmacy, University of Ibadan.

Production of biomolecules (CFF, EPS and FBE) from *P. ostreatus* mycelium

P. ostreatus mycelium was cultured in Potato Dextrose Broth (PDB) for CFF production. The sterile PDB was inoculated with the mycelial culture and incubated at 28°C for 7 days. The fermentation broth was filtered to remove the mycelium. The mycelium was washed 3 - 4 times to remove any attached media component. Ten g of the mycelial biomass was suspended in 100 ml sterilized distilled water and kept for 3 days at 28°C under shaking conditions at 150 rpm. The biomass was filtered using Whatman No.1 filter paper to obtain CFF. The CFF was kept at 4°C for further use.

EPS production was done using Mushroom Complete Medium (MCM) containing glucose (20 g/L), peptone (3 g/L), yeast extract (5 g/L), KH_2PO_4 (0.2), K_2PO_4 and NH_4SO_4 using the modified method of Cui *et al.* (2007) with some modifications. Sterile MCM was inoculated with young *P. ostreatus* culture (3-day-old) and incubated at 28°C for 20 days. The fermentation broth was filtered

and the EPS was precipitated using cold absolute ethanol at 4°C for 24 hrs. The precipitate EPS was obtained by centrifugation at 12,000 rpm for 15 mins. The total sugar concentration was determined by the phenol-sulphuric acid method using glucose as a standard (Dubois *et al.*, 1956). The exopolysaccharide production was expressed as mg/L. The EPS was further dried at 60°C to remove the ethanol content.

Production of fruiting body extracts from the fruiting body of P. ostreatus

The fruiting body extract was obtained from the fruiting body of *P. ostratus* according to the method of Felek *et al.* (2015). 5 g of oven dried milled sample was soaked in sterile distilled water. The soaked sample was boiled for 30 mins and then allowed to cool. The cooled sample was incubated for 24 hrs at 35°C. The resulting leachate was filtered and labeled FBE and stored at 4°C for further use. The POEPS, POCFF and POFBE were used for nanoparticles biosynthesis.

Biosynthesis of AuNPs and SNPs using POEPS

AuNPs biosynthesis using POEPS was done by adding 30 mL of 1 mg/mL solution of EPS to 30 mL of 10^{-3} M (1 mM) of HAuCl_4 solution. The mixture was incubated at 35°C for 24 hrs in the dark (Sathiyarayanan *et al.*, 2014).

SNPs biosynthesis using POEPS was done by adding 20 ml of EPS solution to 20 ml of 10 mM aqueous solution of freshly prepared silver nitrate (AgNO_3) (Kanmani and Lim, 2013). The mixture was incubated at room temperature in the dark for 24-48 hrs.

Biosynthesis of AuNPs and SNPs using POCFF and POFBE

AuNPs biosynthesis using POCFF and POFBE was done by adding equal volumes of 1mM HAuCl_4 solution with CFF and POFBE, respectively. The mixture was incubated at 35°C in the dark (Nadaf and Kanase, 2016). Colour change from pale yellow to bluish purple indicated AuNPs formation.

SNPs biosynthesis using POCFF and POFBE was done by adding 50 mL of AgNO_3 solution (10 mM) to 10 mL of the POCFF and POFBE, respectively. The mixture was incubated at room temperature for 48 hrs in the dark (Zonooz and Salouti, 2011).

Characterization of the biosynthesized AuNPs and SNPs

The formation of AuNPs and SNPs was monitored by visual observation for a change in col-

our of the solutions in comparison to the control. Bio-oxidation and bio-reduction of gold and silver was measured periodically using UV-Vis spectrophotometer (a Lambda 25-Perkin Elmer, Waltham, MA, USA) with a resolution of 0.5 nm. The absorbance of the sample was read at the wavelengths of 200-800 nm.

Scanning Electron Microscopic was used to determine the size, shape and morphology of the biosynthesized nanoparticles. The aqueous solution of NPs synthesized were lyophilized and subjected to Scanning Electron Microscopy (SEM).

The functional groups present in the nanoparticles samples were determined using FTIR. The FTIR spectra of the dried KBr powdered nanoparticles samples were analysed using FTIR spectroscopy (Shimadzu) operated at resolution of 4 cm^{-1} . The spectra were recorded at a wave range of 500-4000 cm^{-1} .

Antibacterial potential of the nanoparticles against some pathogens

The antibacterial activity of the POEP-SAUNPs, POCFFAuNPs and POFBEAuNPs POEPSSNPs, POCFFSNPs and POFBESNPs against some pathogens was determined using the agar well diffusion method (Shivashankar *et al.*, 2013). The test pathogens used were: *K. pneumonia*, *Bacillus cereus*, *P. aeruginosa*, *Micrococcus* spp. *E. coli* and *S. aureus*). Suspension of 24-hour-old cultures of the test pathogens was done using saline. A lawn of the indicator strain was made by spreading the cell suspension over the surface of Mueller - Hinton agar plates with a sterile cotton swab. The plates were allowed to dry and wells were bored on the agar using a sterile cork borer of 7 mm diameter. Each well was filled with 20 μL of the biosynthesized AuNPs and SNPs. HAuCl_4 and AgNO_3 solution (1 mM), POEPS, POCFF and POFBE were used as negative controls. The seeded plates were incubated at 37°C for 24 hrs, the incubated plates were observed for zones of inhibition (ZOI) around the wells. Zone diameters (mm) greater than 1 mm were considered positive (Prabhu *et al.*, 2014).

Statistical analysis

The statistical results are presented as the mean $\pm$ standard deviation (SD) at $P < 0.05$.

Results

Production of biomolecules from P. ostreatus

Biomolecules (EPS and CFF and FBE) from *P. ostreatus* were used for mycosynthesis of gold

and silver nanoparticles. The nanoparticles were characterized and their antibacterial potential against some selected pathogens was investigated. Exopolysaccharide production by *P. ostreatus* (PO) is shown in Fig. 1. There was a significant difference ($P \geq 0.05$) in EPS production by PO on different days of incubation. EPS production by PO ranged from 300 - 3675.57 mg/L and the highest production was recorded on the 15th day.

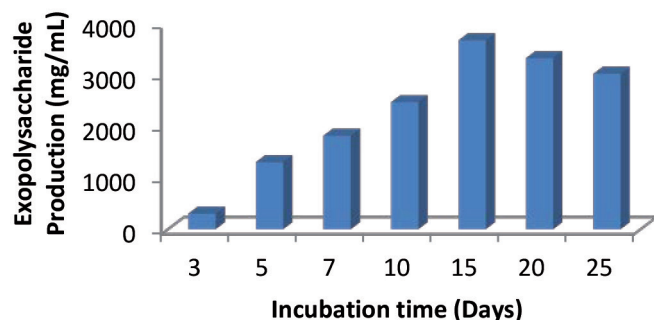


Fig. 1. Production of exopolysaccharides by *P. ostreatus*

Biosynthesis and characterization of the nanoparticles

Changes in colour were observed after the addition of the biomolecules to gold and silver nitrate solution. The visual observation of the biosynthesized POEPSAuNPs, POCFFAuNPs and POFBEAuNPs after incubation for 72 hrs is shown in Fig. 2a -2c. Colour changes from pale yellow to purple, cloudy colour and yellow colour to dark purple indicated the formation of POEPSAuNPs, POCFFAuNPs and POFBEAuNPs. The visual observation of the biosynthesized POEPSSNPs, POCFFSNPs and POFBESNPs is shown in Fig. 2d – 2f. Colour changes from pale yellow to dark brown indicated formation of POEPSSNPs while changes in colour from cloudy and pale yellow to dark brown indicated POCFFSNPs and POFBESNPs formation.

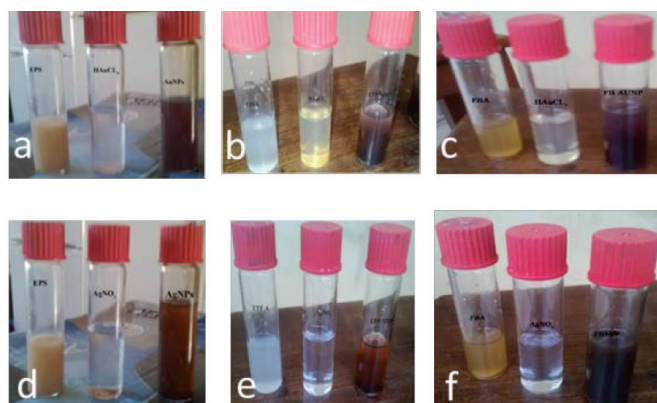


Fig. 2. Visual observation of (a) POEPSAuNPs, (b) POCFFAuNPs, (c) POFBEAuNPs (d) POEPSSNPs (e) POCFFSNPs and (f) POFBSNPs.

The absorption spectrum of the biosynthesized POEPSAuNPs, POCFFAuNPs and POFBEAuNPs after 72 hrs of incubation is shown in Figures 3a-c. The AuNPs gave absorbance peaks within the range of 200 - 800 nm after different hours of incubation. A strong SPR peak was observed at 500 nm and 600 nm.

The absorption spectra obtained from biosynthesized POEPSSNPs, POCFFSNPs and POFBESNPs at 72 hrs of incubation is shown in Figures 3 d - f. A sharp peak was recorded at 450 nm.

Fourier Transform Infrared (FT-IR) analysis of the nanoparticles

Figure 4a shows the spectra of biosynthesized AuNPs from the EPS of *P. ostreatus*. Eleven peaks were observed. The peak at 3429.17 cm^{-1} gave a broad stretch of hydroxyl (O-H) while the peak at 2363.66 and 2335 cm^{-1} corresponds to strong stretching carbon dioxide group ($\text{O}=\text{C}=\text{O}$). The peak at 1641.08 cm^{-1} gave a medium conjugated alkene ($\text{C}=\text{C}$). A medium bending alkane from the methyl group of alkane ($\text{C}-\text{H}$) was seen at 1384.04 cm^{-1} . The peak at 1073.49 cm^{-1} and 993.35 cm^{-1} indicates a medium stretched amine group ($\text{C}-\text{N}$) and strong bending monosubstituted alkene ($\text{C}=\text{C}$), respectively. The peaks from 553.12 cm^{-1} to 362.45 cm^{-1} indicate the presence of alcohol (OH) bonds.

Figure 4b shows the spectra of AuNPs biosynthesized from the CFF of *P. ostreatus*. 8 peaks were observed. A broad stretching peak of carboxyl(O-H) was seen at 3418.68 cm^{-1} while the peak at 2096 cm^{-1} corresponds to a strong stretching isothiocyanate group ($\text{N}=\text{C}=\text{S}$). The peak at 1633.72 cm^{-1} indicates an aromatic ring stretch with a peak at 1446.4 cm^{-1} and 1086.14 cm^{-1} represents CO stretch from the COO^- group. The peaks at 605.66 cm^{-1} , 467.3 cm^{-1} and 390.51 cm^{-1} indicate O-H alcohol group.

Figure 4c shows the FTIR spectra of AuNPs from the fruiting body of *P. ostreatus*. Sixteen (16) peaks were observed. The peak at 3428.85 indicates the hydroxyl stretching functional group while the peak at 2936.2 cm^{-1} indicates weak CH stretch of methyl group. The peak at 2099.83 cm^{-1} represents a strong stretch of isothiocyanate group of ($\text{N}=\text{C}=\text{S}$). The peak at 1641.84 cm^{-1} shows the aromatic ring stretch and the peak at 1559.1 cm^{-1} indicates a medium stretching cyclic alkene. The peaks at 1455.1 cm^{-1} and 1402.42 cm^{-1} could be assigned $\text{C}=\text{O}$ stretch from the COO^- group with the peak of 1306.5 cm^{-1} indicating an aromatic amine? CN stretch. The peak at 1238.8 cm^{-1} indicates phosphate group ($\text{P}=\text{O}$). The peak at 1084.15 cm^{-1} cor-

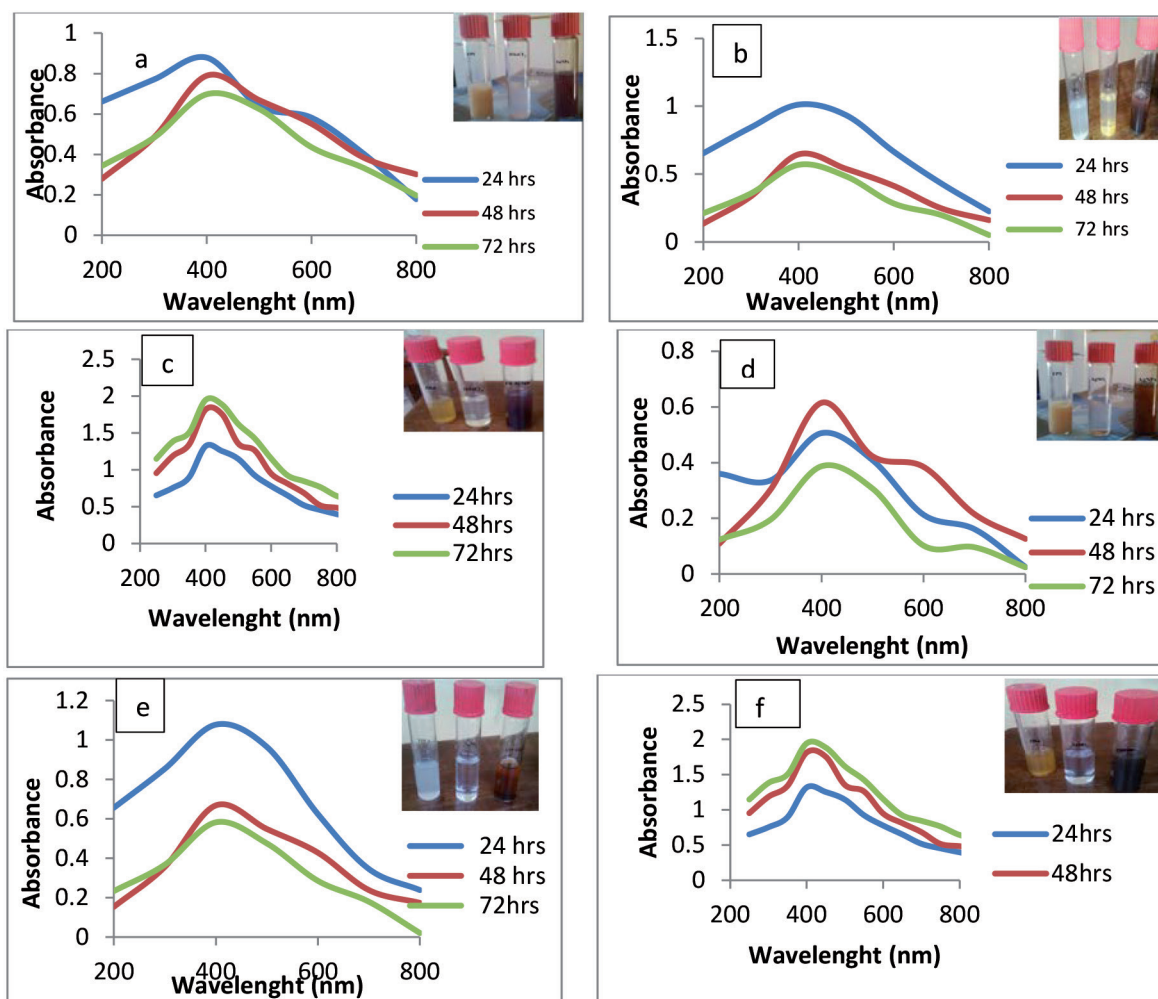


Fig. 3. UV-vis spectra of (a) POEPSAuNPs, (b) POCFFAuNPs, (c) POFBEAuNPs (d) POEPSSNPs (e) POCFFSNPs and (f) POFBSNPs.

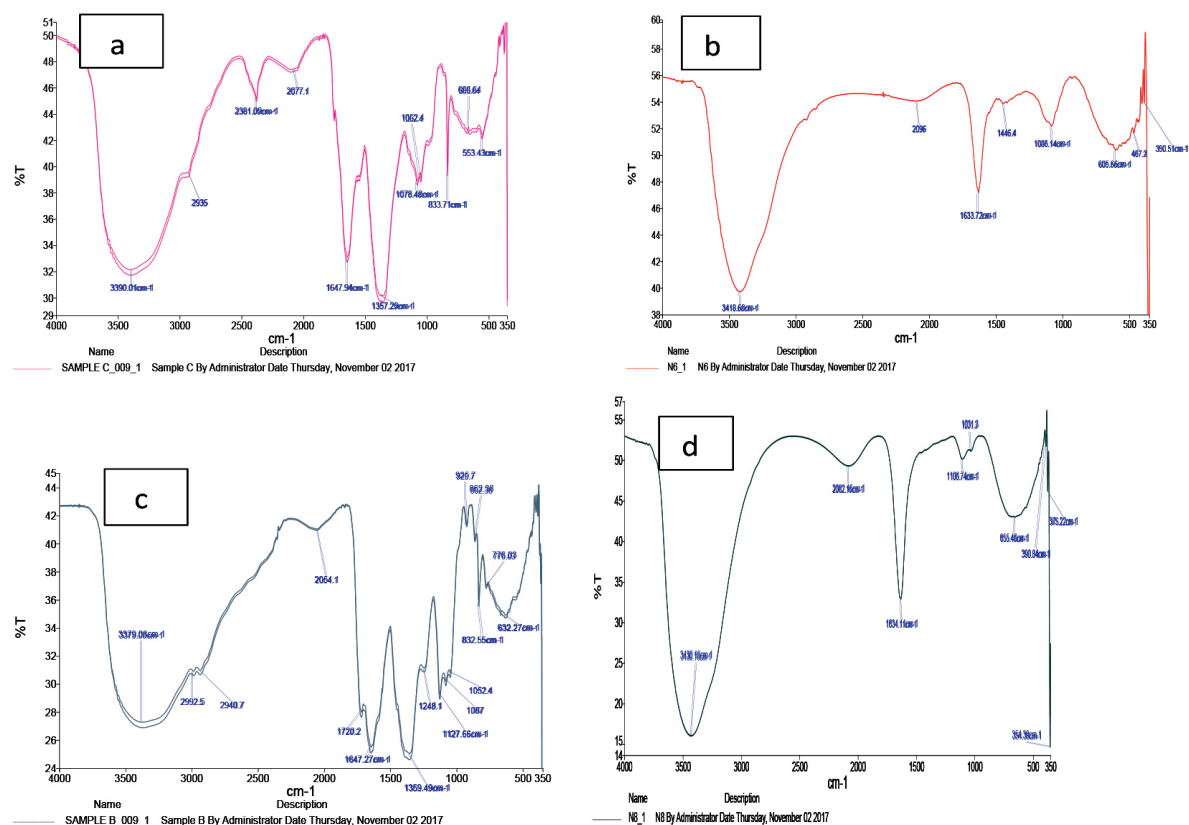


Fig. 4. FT-IR spectrum of (a) POEPSAuNPs (b) POCFFAuNPs, (c) POFBEAuNPs (d) POEPSSNPs

responds to the C=O=C vibration in the glucopyranose ring. The peaks appearing from 999.7 cm^{-1} are associated with the aromatic phosphate (P-O-C) stretch. The peaks appearing from 616.77 cm^{-1} to 363.59 cm^{-1} represent the alcohol OH group.

Figure 4d shows the spectra of the biosynthesized SNPs from EPS of *P. ostreatus*. Nine (9) peaks were observed in which the peak at 3430.18 cm^{-1} indicates hydroxyl stretch of O-H group and the peak at 2082.16 cm^{-1} indicates strong stretching isothiocyanate group (N=C=S). The peaks observed at 1106.74 and 1031.3 cm^{-1} correspond to C-O-C vibration in the glucopyranose ring with the peaks at 655.46 cm^{-1} , 390.84 cm^{-1} and 375.22 cm^{-1} indicating an alcohol O-H group.

Scanning Electron Microscopic analysis of AuNPs and SNPs from the biomolecules

Figures 5 a-d show the micrograph of POEPSAuNPs, POCFFAuNPs, POEPSSNPs and POCFFSNPs. POEPSAuNPs was polymorphic in shape with $0.5 - 2.6\text{ nm}$ in size and $0.08 - 1.0\text{ nm}$ for POCFFAuNPs, POEPSSNPs and POCFFSNPs were aggregate with $2.2 - 3.0\text{ nm}$ and $0.2 - 2.8\text{ nm}$ in size, respectively.

Antibacterial potentials of the biosynthesized nanoparticles and the biomolecules against some test pathogens

Table 1a shows the antibacterial activity of the AuNPs and the biomolecules from *P. ostrateus* against the test pathogens. There was a significant difference in the antibacterial activity of the AuNPs

against the test pathogens. The antibacterial activity of POEPSAuNPs, POCFFAuNPs and POFBEAuNPs against the test pathogens ranged from $21 - 28\text{ mm}$, $8 - 22\text{ mm}$ and $9 - 29\text{ mm}$. POEPSAuNPs, POCFFAuNPs and POFBEAuNPs exhibited the highest activity against *E. coli*, *Micrococcus* sp. and *P. aeruginosa*, respectively.

The antimicrobial activity of POEPS and POCFF ranged from $7 - 10\text{ mm}$ and $4 - 6\text{ mm}$, with the highest activity recorded against *Micrococcus* sp. and *B. cereus*. POFBE did not have any observable antibacterial activity against the test pathogens. The antimicrobial activity of HAuCl_4 and ceftriaxone ranged from $1 - 7\text{ mm}$ and $16 - 26\text{ mm}$, with the highest activity achieved against *B. cereus*, *K. pneumonia* and *E. coli*.

Table 1b shows the antibacterial activity of the SNPs and biomolecules from *P. ostrateus* against some selected test pathogens. A significant difference was observed in the antibacterial activity of the SNPs against the test pathogens. The antibacterial activity of POEPSSNPs, POCFFSNPs and POFBESNPs against the test pathogen ranged from $11 - 25\text{ mm}$, $9 - 24\text{ mm}$ and $3 - 6\text{ mm}$. POEPSSNPs, POCFFSNPs and POFBESNPs exhibited the highest activity against *B. cereus*, *S. aureus* and *E. coli*, respectively.

The antimicrobial activity of AgNO_3 and ceftriaxone against the test pathogens ranged from $4 - 9\text{ mm}$ and $16 - 26\text{ mm}$. The highest activity was achieved against *P. aeruginosa*, *K. pneumonia* and *E. coli*.

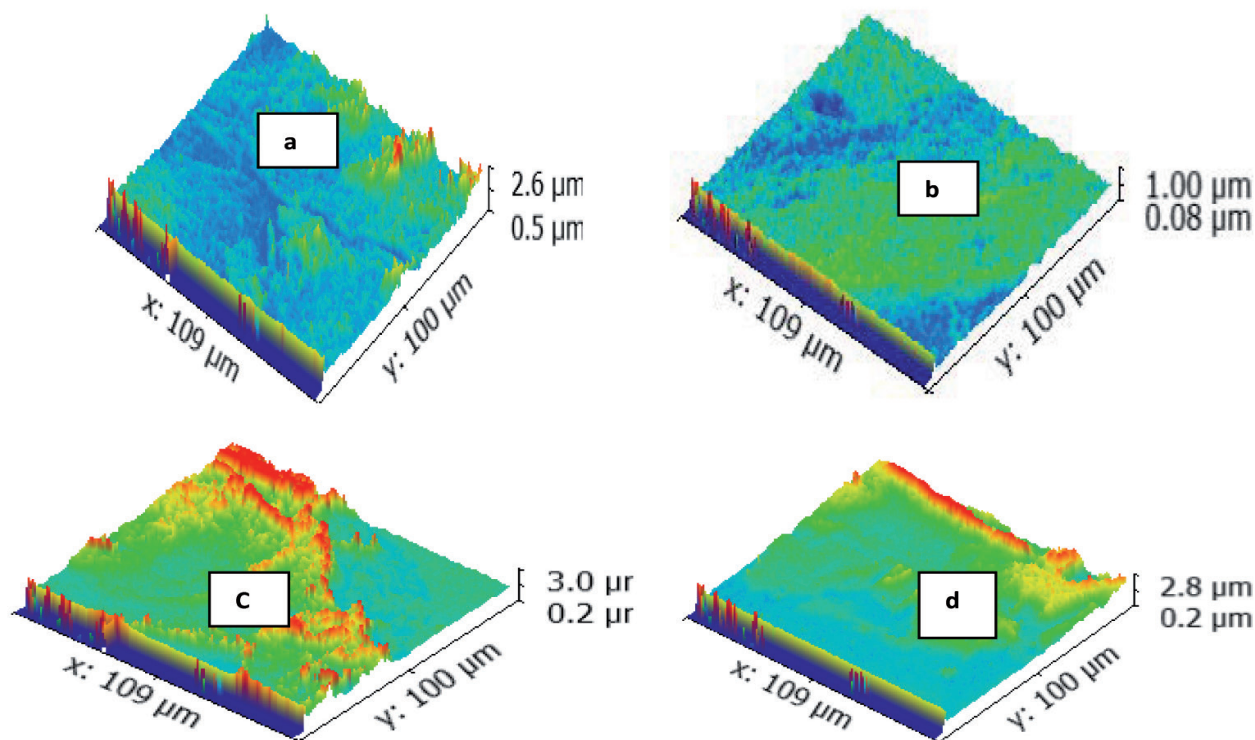


Fig. 5. SEM of micrograph of (a) POEPSAuNPs, (b) POCFFAuNPs, (c) POEPSSNPs and (d) POCFFSNPs

Table 1a. Antimicrobial activity of the biosynthesized AuNPs and the biomolecules against some pathogenic strains

Test pathogen	Zone of inhibition (mm)							
	POEPS AuNPs	POCF AuNPs	POFB AuNPs	POEPS	CFF	FB	HAuCl ₄	CEFTR
<i>K. pneumonia</i>	26	8	13	9	4	-	2	26
<i>B. cereus</i>	27	18	16	7	6	-	7	16
<i>P. aeruginosa</i>	22	12	29	6	4	-	5	21
<i>Micrococcus</i> sp.	27	22	23	10	5	-	5	21
<i>E. coli</i>	28	12	28	7	5	-	0	26
<i>S. aureus</i>	21	11	9	6	4	-	1	22

Table 1b. Antimicrobial activity of the biosynthesized SNPs and the biomolecules against some pathogenic strains

Test pathogen	Zone of inhibition (mm)							
	POEPS SNPs	POCFF SNPs	POFBE SNPs	POEPS	CFF	FB	AgNO ₃	CEFTR
<i>K. pneumonia</i>	14	19	-	9	4	-	6	26
<i>Bacillus cereus</i>	25	22	-	7	6	-	6	16
<i>P. aeruginosa</i>	11	9	-	6	4	-	9	21
<i>Micrococcus</i> sp.	19	23	-	10	5	-	8	21
<i>E. coli</i>	19	11	6	7	5	-	4	26
<i>S. aureus</i>	25	24	3	6	4	-	7	22

Minimum Inhibitory Concentration (MIC) of the nanoparticles

The minimum inhibitory concentration (MIC) of the biosynthesized nanoparticles is shown in Table 2. The MIC of 200 µg/mL was observed for all the test pathogens when the biosynthesized POEP-SAuNPs and POCFFAuNPs were used as antimicrobial agents. There was variation in the MIC of the biosynthesized SNPs against the test pathogens. POEPSSNPs had a MIC of 6.25 µg/mL against *E. coli*, 50 µg/mL against *P. aeruginosa*, *B. cereus*, *S. aureus* and *Micrococcus* sp. and 100 µg/mL against *K. pneumoniae*.

POCFFSNPs had a MIC of 12.5 µg/mL against *E. coli* while the MIC of 50 µg/mL as recorded against *P. aeruginosa*, *B. cereus*, *S. aureus* and *Micrococcus* sp. Ceftriaxone had an MIC of 5 µg/mL against *P. aeruginosa*, *B. cereus*, *E. coli*, *S. aureus* and *Micrococcus* sp. An MIC of 10 µg/mL was observed against *K. pneumoniae*.

The EPS and CFS from *P. ostreatus* were used for the biosynthesis of AuNPs and SNPs. The biosynthesized nanoparticles were characterized and their antibacterial activities against the test pathogens were evaluated.

Table 2. Minimum Inhibitory Concentration of biosynthesized nanoparticles from *P. ostreatus*

Nanoparticle samples	Minimum Inhibitory Concentration (µg/ml)					
	<i>P. aeruginosa</i>	<i>B. cereus</i>	<i>K. pneumonia</i>	<i>Micrococcus</i> sp.	<i>E. coli</i>	<i>S. aureus</i>
POEPSAuNPs	200	200	200	200	200	200
POCFF AuNPS	200	200	200	200	200	200
POFB EAuNPS	100	200	200	200	200	200
POEPSSNPs	50	50	100	50	6.25	50
POCFFSNPs	50	50	100	50	12.5	50
POFBESNPs	25	25	50	50	12.5	50
Ceftriaxone	5	5	10	5	5	5

Discussion

EPS-production by P. ostreatus

P. ostreatus produced a reasonable quantity of exopolysaccharides. Reduction in EPS- production after 15th day of incubation may be due to the exhaustion of the available nutrient in the production medium. This was also recorded by Olfat and Sadik (2014), who worked on the production of EPS and bioactive metabolites in a submerged culture of *P. ostreatus*. A different report was obtained in the work of Adebayo-Tayo *et al.* (2011), where there were fluctuations in EPS production by *P. ostreatus* during optimization of the growth conditions, which was ascribed to the different media composition.

Biosynthesis of AuNPs and SNPs by P. ostreatus biomolecules

Biosynthesis of silver nanoparticles using EPS from *Pleurotus* sp. was achieved via the bio-reduction method. Other methods of biosynthesis have also been reported. Ravishankar *et al.* (2011) used photo-irradiation method to biosynthesize SNPs from an aqueous extract of *Pleurotus florida*; Sujatha *et al.* (2013) also synthesized SNPs from edible mushroom extracts, such as *Agaricus bisporus*, *Calocybe indica*, *P. florida* and *P. platypus*. CFF was also used in the biosynthesis of NPs. Abdelmageed *et al.* (2016) synthesized NPs using an extracellular supernatant of *Penicillium politans*; Soheyila *et al.* (2013) reported a green process for production of silver (Ag) nanoparticles using *Penicillium chrysogenum*; Kumar and Ghosh (2016) biosynthesized silver nanoparticles from silver nitrate using *Bacillus* sp. (*Brevibacillus borstelensis* MTCC10642).

Biosynthesis of AuNPs from EPS produced by *Pleurotus* sp. has not been reported though CFF has been used in the biosynthesis of nanoparticles. Michael *et al.* (2015) reported the biosynthesis of AuNPs from fungi and their protein extracts.

Characterization of the biosynthesized nanoparticles

The appearance of a pale yellow to purple colour indicated the oxidation of the chloroauric acid ions by the fungal biomolecules, which resulted in the formation of gold nanoparticles. Changes in colour were due to the excitation of surface plasmon vibrations, which is a characteristic feature of synthesized nanoparticles (Song *et al.*, 2009). This was in agreement with other studies. Gitanjali and Ashok (2014) reported the biosynthesis of AuNPs from observing the gradual change in colour thus

concluding that the colour of nanoparticles from *Herbarium tetramera* periodically change from different shades to purple, which indicated preliminary test in the formation of AuNPs.

The yellowish brown colour formation by SNPs has been confirmed by many researchers (Hany *et al.*, 2014). The metal salt bio-reduction ability of the fungal filtrate for nanoparticles biosynthesis with an observable colour change has been attributed to the surface plasmon resonance of deposited SNPs (Mulvaney, 1996). Balakumaran *et al.* (2016) also reported that the change of colour to brown was a result of SPR vibration excitation. Characteristic changes in colour may be attributed to the alteration in surface plasmon resonance (SPR) when positive ions (Ag⁺) are transformed into nano-forms (Mital, 2013).

Characterization of metal ions exposed to the biomolecules of *P. ostreatus* by UV-Vis spectrophotometer confirmed the reduction of metal ions to metal nanoparticles. Metallic nanoparticles exhibit an intense UV absorption band due to SPR effect. Mie's theory proposed SPR arose from the interaction of small metallic particles with an external electromagnetic field induced by light, resulting in a coherent oscillation of the conductor free electrons on the surface (Mie, 1908). These confirm the formation of NPs. The SPR peak obtained for the AuNPs synthesized using the different samples in this work was observed at 500 nm. Typically, AuNPs have a maximum wavelength in the visible range of 500 nm - 600 nm (Oba *et al.*, 2009; Barabadi *et al.*, 2014; Shivaji *et al.*, 2014). The SNPs formed from other biomolecules was also confirmed using the UV spectrophotometer and are seen in the range of 400 -500 nm. This is referred to as the SPR of the SNPs with strong peaks between 400 nm – 450 nm. This result agrees with the report of Gannamani *et al.* (2014), who biosynthesized SNP from aqueous seed extract of *Protorhus longifolia*. Hany *et al.* (2014) produced SNP from the CFF of different fungi with peaks, which was due to the surface resonance and vibration of electrons. These peaks are known as SPR (Mansoori *et al.*, 2007). Raza *et al.* (2016) reported that the presence of a single SPR peak indicates that the particles were spherical with a wide range of size distribution. Formation of an SPR peak in the range of 410–450 nm has been attributed to the biosynthesis of AgNP. This occurs as a result of alteration in the surface plasmon resonance (SPR) when positive ions (Ag⁺) are transformed into nano-forms.

The active compounds in the biomolecules

involved in the reduction of metal ions for metal nanoparticle formation were investigated using FTIR. It confirmed that biomolecules such as EPS, CFF and FBE were responsible for capping and efficient stabilization of the metal nanoparticles synthesized. This makes fungi extremely good candidates in the synthesis of metal nanoparticles that are safe and eco-friendly. The spectrum of the biomolecules extract exhibited intense and distinct absorption bands at 1720 and 3317 cm^{-1} , which correspond to the amide, carboxyl and hydroxyl bands. Formation of stronger bonds between the biomolecules (EPS, CFF and FBE) and the metal ions may be as a result of the presence of the functional groups (carboxylic, hydroxyl and amide groups) in the biomolecules. The presence of the amide linkage suggests that there are some proteins in the reaction mixture which may be responsible for nanoparticles formation and may play an important role in the stabilization of the formed nanoparticle. The bands of aromatic and aliphatic amines, respectively, are also observed, which is similar to the earlier reports of Ashajyothi *et al.* (2014) on biosynthesis and characterization of NPs from gram-negative bacteria biomass. These bands are close to those reported for natural phytochemicals in the *Agaricus biporus* extract. Thus, nano capping of the particles from EPS, CFF and FBE of *P. ostreatus* are responsible for reduction and subsequent stabilization of Au-NPs.

The SEM micrograph of the gold and silver nanoparticles shows polymorphic and aggregated particle shapes due to the capping agent. This result agrees with the work of Adebayo-Tayo *et al.* (2017), who reported on the bacterial synthesis of nanoparticles from the supernatant of lactic acid bacteria.

The antimicrobial potential of nanoparticles was also examined in this report. The activity of gold chloride (positive control) on bacteria isolate was minimal compared to the nanoparticles. Gold ions as well as AuNPs are known to have strong antimicrobial activities. The antimicrobial ability of Au nanoparticles might be due to their small size (which is 250 times smaller than a bacterium), which makes their adhesion to the cell wall of microorganisms easier, thus leading to destruction and cell death (Chwalibog *et al.*, 2010). Metal nanoparticles are also harmful to bacteria due to their ability to disrupt the membrane protein and sulphur components. They bind closely to the surface of microorganisms causing visible damage to the cells, thus demonstrating good self-assembling ability. Gold nanoparticles possess well-developed surface

chemistry, chemical stability and appropriate smaller size, which facilitate their interaction with microorganisms (Nirmala and Pandian, 2007). Also, the particles interact with the building elements of the outer membrane and might cause structural changes, degradation and finally cell death. The test pathogens were susceptible to the NPs biosynthesized using biomolecules of *P. ostreatus*.

Conclusion

EPS, CFS and FBE of *P. ostreatus* were able to bio-reduce and bio-oxidize silver and gold for the biosynthesis of SNPs and AuNPs, respectively. The AuNPs had a broad spectrum of activity against the test pathogenic strains compared to the SNPs. These nano-biocomposites can be used in the formulation of novel antibacterial agents and can be applied in biomedical fields.

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***Micrococcus luteus* Strain in Free and Immobilized Form is Capable of Simultaneous Utilization of Aromatic and Aliphatic Xenobiotics**

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Abstract

Aliphatic and aromatic xenobiotics can cause serious ecological problems due to their widespread occurrence and toxicity. The investigated strain *Micrococcus luteus* BN56 appeared to be capable of degrading both aromatic and aliphatic xenobiotics simultaneously as carbon and energy sources. Adaptation of the investigated strain was carried out with repeated additions of xenobiotic to the medium. The cells showed the highest capacity for biodegradation after 10 passages simultaneously degrading both xenobiotics - 10 g/l (100%) phenol and 10 g/l (100%) n-hexadecane, in 20 active cycles of operation (duration of 12 - 36 h). The simultaneous biodegradation was not hindered by either of them. By applying an *in situ* immobilization procedure using poly(acrylamide) (PAAm) and poly(ethylene oxide) (PEO) cryogel matrices, these processes were intensified, stabilized and reached 40 cycles of repeated operation of successful simultaneous biodegradation of the aromatic and the aliphatic xenobiotics (20g/l + 20g/l). This capability can be further implemented in different real-world bioremediation processes.

Keywords: *Micrococcus luteus*, biodegradation, immobilization, cryogel, phenol, n-hexadecane

Резюме

Алифатните и ароматните ксенобиотици могат да причинят сериозни екологични проблеми поради широкото им разпространение, наличност и токсичност. Изследваният щам *Micrococcus luteus* BN56 се оказва способен да разгражда едновременно ароматни и алифатни ксенобиотици като въглеродни и енергийни източници. Адаптация на изследвания щам е извършена с многократно добавяне на ксенобиотик към средата. Клетките показаха най-висок капацитет за биоразграждане след 10 пасажи, реализирайки едновременно разграждане и на двата ксенобиотика - 10 g/l (100%) фенол и 10 g/l (100%) n-хексадекан, разградени в 20 активни цикъла на работа с продължителност 12 - 36 часа. Едновременната биодegradация не е възпрепятствана от никой от тях. Прилагайки *in situ* процедура за имобилизация, с използване на матрици от поли (акриламид) (PAAm) и поли (етилен оксид) (PEO), тези процеси бяха интензифицирани, стабилизиращи и достигнаха 40 цикъла на повтарящо се успешно едновременно биоразграждане на ароматния и алифатния ксенобиотик (20 g/l + 20 g/l). Тази способност може да бъде приложена в последствие в различни реални процеси на биоремедиация.

Introduction

Aliphatic alkanes and aromatic compounds can be found in nature due to the discharge of a broad variety of harmful chemicals resulting from many anthropogenic activities. Such xenobiotics prevail in various contaminated environments and can cause harmful disturbances to human health (Dawson *et al.*, 2007). With the development of

modern civilization, humanity is undoubtedly faced with the problem of environmental protection (Kumar and Kuma, 2004). Pollution due to phenol and petroleum oil, with alkanes being the major fraction, is a prevalent ecological hazard (Singh *et al.*, 2009). One of the most reliable approaches to solving these problems is to use the natural abilities of different types of microorganisms to break down

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and detoxify xenobiotic substances (Abbasian *et al.*, 2015; Lockington *et al.*, 2015; Khraisheh *et al.*, 2020; Al-Saadi and Mohammad Ali, 2020). The simultaneous biodegradation of these pollutants has become a very important challenge. The application of physicochemical processes for bioremediation of contaminated environments can cause secondary problems in the resultant effluents, therefore, biological treatments are preferred. Some microorganisms possess the catabolic capability of utilizing xenobiotic compounds, producing innocuous end products. Reports exist in literature that *Pseudomonas stutzeri* (Tambekar *et al.*, 2012), *Nocardia hydrocarbonoxydans* NCIM 2386 (Shetty *et al.*, 2008), *Micrococcus varians* EPRIS14, *Bacillus subtilis*-EPRS12, *Pseudomonas alcaligenes* (Safari *et al.*, 2019), *Bacillus licheniformis*-EPRIS21, *Bacillus laterosporus*-EPRIS41, *Pseudomonas putida*-DAF1, *Bacillus firmus*-EPRIS22 and *Acinetobacter* sp. EPRIS32 (Shourian *et al.*, 2009, Bayoumi and Abul-Hamd, 2010, Saraireh *et al.*, 2020), are able to perform phenol degradation. n-Hexadecane can be utilized by *P. putida*, *Rhodococcus erythropolis* and *Bacillus thermoleovorans* isolated from contaminated soils (Abdel-Megeed *et al.*, 2010), as well as by members of *Achromobacter*, *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Flavobacterium*, *Nocardia*, *Corynebacterium*, *Gordonia*, etc. (Pozdniakova *et al.*, 2008; Binazadeh *et al.*, 2009; Silva *et al.*, 2019). Therefore, many bacterial strains have been isolated with the ability to degrade n-hexadecane and phenol separately, which are often used to represent aliphatic and aromatic pollutants. However, few strains have been reported to have the dual ability (Colombo *et al.*, 1996; Sun *et al.*, 2012).

Employment of immobilization procedures in biotechnological processes raises biotechnology to a qualitatively new level. It should be noted that in the case of immobilized cells, all the advantages arise not as a result of changes in the properties of microorganisms, but as a result of significant changes in the conditions for biotechnological processes and proper selection of suitable immobilization matrices (Baron and Willaert, 2004). In this way performing bioremediation processes by immobilized cells favors the realization of faster, more stable, more effective processes, not disturbed by the toxicity of xenobiotics, that can be repeated many times for a long period of time (Hutchinson *et al.*, 2020).

The aim of the present work was to achieve higher simultaneous biodegradation of aromatic

and aliphatic xenobiotics by *Micrococcus luteus* cells, applying the immobilization technique.

Materials and Methods

Microorganism, media and cultivation

The strain employed in this study was isolated from hydrocarbon-contaminated soil, followed by adaptive selection on a nutrient medium with the following mineral composition (g/l): $K_2HPO_4 \cdot 3H_2O$, 4.8; KH_2PO_4 , 1.5; $(NH_4)_2SO_4$, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; trace elements (mg/l): $CaCl_2 \cdot 2H_2O$, 2.0; $MnCl_2 \cdot 4H_2O$, 0.4; $NiCl_2 \cdot 6H_2O$, 0.4; $ZnSO_4 \cdot 7H_2O$, 0.4; $FeCl_3 \cdot 6H_2O$, 0.2; $Na_2MoO_4 \cdot 2H_2O$, 0.2 and by standard enrichment techniques. In the course of the experiments aliphatic and aromatic hydrocarbons were used as sole carbon sources. The pH of the medium was adjusted to 7.0. The minimal salt medium (MSM) was solidified as MSM phenol agar by addition of 1.8% agar. Cultures grown on MSM agar supplemented with 500 mg/l phenol were used to inoculate 500 ml Erlenmeyer flasks containing 100 ml liquid MSM. Cultures were incubated during long-term semi-continuous biodegradation processes with shaking at 120 rpm at 28°C. Adaptation procedures were carried out by multiple passages on MSM agar with phenol.

Assays

Bacterial growth was assessed by determination of the optical density (OD_{610} nm) of the culture.

Analytical methods included assay of phenol concentrations (in mg/l) by colorimetric determination with 4-aminoantipyrine according to APHA (American Public Health Association, 1999).

Biodegradation of hexadecane was measured by the substrate depletion. Whole cultures were extracted with equal volumes of n-hexane and residual n-hexadecane was quantified by gas chromatography using a Hewlett-Packard model 5859 instrument equipped with a flame ionization detector.

Immobilization

Immobilization of the bacterial cells of the investigated strain was carried out to select the most appropriate carrier. Two types of matrices were tested. *In situ* immobilization into polymer cryogels was accomplished. Cells were harvested by centrifugation at 8000 x g and afterwards were re-suspended in a phosphate buffer solution (0.06 M, pH 7.0 at 20°C). Cell density of 60×10^9 g⁻¹ carrier material was applied. Then, 0.12 g of 10^6 g mol⁻¹ poly (ethylene oxide) (PEO), Union Carbide Corporation, Danbury, USA) was added to 5 ml of the cell suspension thus obtained under stirring

at 20°C. It was kept overnight to ensure complete dissolution of the polymer. Then, 0.006 g of cross-linking agent (N, N'-methylene bisacrylamide; Merck, Darmstadt, Germany) and 0.006 g photoinitiator [(4-benzoylbenzyl) trimethylammonium chloride; Sigma-Aldrich, Schnelldorf, Germany], dissolved in 1 ml water were added. The resulting mixture was poured into Teflon dishes (each portion consisted of 1 ml), in 5 dishes with a diameter of 20 mm, forming a 4 mm thick layer. The samples were frozen at -20°C for 2 h and irradiated with the full spectrum of UV-Vis light from a 400-W metal halide flood lamp (Dymax 5000-EC; Dymax Corporation, USA) for 5 min at a dose of $2.85 \times 10^5 \text{ J m}^{-2}$ and an input power of 930 W m^{-2} .

The cells were immobilized in polyacrylamide (PAAm) cryogels following the same procedure, except that 0.3 g of acrylamide (Merck) and 0.03 g of N, N'-methylene bisacrylamide were dissolved in 1 ml of water and mixed with 5 ml of cell suspension. Finally, 0.075 ml initiator (30 vol. % H_2O_2 ; Merck), were added prior to freezing. The diameter of each matrix disc was 1.6 – 1.7 cm and its weight was 1.0 – 1.1 g for the PAAm and PEO carriers, respectively.

Scanning Electron Microscopy (SEM) observations

SEM observations were carried out to evaluate the immobilization processes. Preparation was performed using cryogel disks with and without cells which were frozen in a freezer at -20°C, then fractured and freeze dried in an Alpha 1-2 Freeze Drier (Martin Christ) at -55°C and 0.02 mbar for 24 h. Afterwards, the gel specimens were fixed on a glass substrate and coated with a thin layer of gold for 60 sec. The interior and surface morphology of the gels and immobilized bacteria were studied using a JEOL JSM-5300 SEM operating at 10 kV. Magnification varied from 150 to 1000.

Results and Discussion

The adaptation capabilities of the bacterium in a semi-continuous process with repeated additions of xenobiotic were studied. The preliminary contact of the microorganism with the toxic compound is the way to increase its biodegradation potential. The catabolic ability of the investigated strain was followed to prove its potential for bioremediation. Multiple passages on solid media (10 passages) with phenol as a sole carbon and energy source were carried out. It could be assumed that the prolonged exposure to phenol probably led to catabolic changes in the cells, leading to better adaptation and survival (Baptiste *et al.*, 2019). The adaptability of cells

of *M. luteus* BN 56 was clearly demonstrated by the increased number of active cycles of operation for xenobiotic degradation leading to full depletion. Studies of the biodegradation processes in the presence of aromatic and aliphatic toxic substances in the medium were further conducted in a semi-continuous mode of cultivation. The hydrophilic (phenol) and hydrophobic (n-hexadecane) xenobiotic substances were supplied in equal concentrations. Each new cycle started after the depletion of xenobiotics and with the addition of fresh quantities of phenol and n-hexadecane (500 mg/l each). Substrate concentration of 500 was fully degraded in both cases so it was further used in our investigations. The curves describing the biodegradation process followed by measurement of the residual phenol and n-hexadecane concentration revealed that the process rate was constant for the first 10 cycles. A 12-28-hour period was required for complete degradation of the xenobiotics. A certain increase in the time needed in the next 10 cycles was observed when the biodegradation of n-hexadecane biodegradation was complete in 14 h and of phenol in 36 h. These 20 cycles with a total of 10g/l phenol quantity added and another 10g/l of n-hexadecane demonstrated not only a high rate of degradation, but also good resistance to the xenobiotics (Figure 1). The results presented in Fig. 1 reveal the strong catabolic potential of *M. luteus* BN 56. The twenty active cycles of biodegradation took place at a high rate and after 12-36 h residual quantities of phenol and n-hexadecane were not found. The total amount of phenol and n-hexadecane degraded was 10 g/l each. An important condition proved was that the addition of the second xenobiotic did not inhibit simultaneous biodegradation.

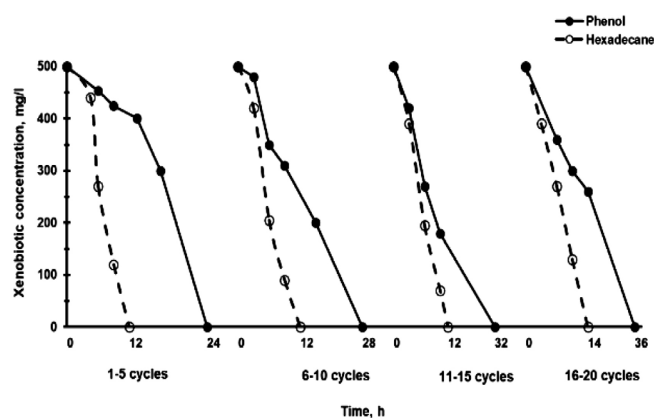


Fig. 1. Simultaneous biodegradation of phenol and n-hexadecane from the adapted strain of *M. luteus* BN56 under conditions of semi-continuous cultivation, presented as arithmetic mean values ($\bar{x}_{av} \pm \sigma$)

Having followed the growth of the culture before and after adaptation, it was evident that after a short lag-period the logarithmic phase began, which lasted about five days for the adapted cells. In the fifth supply of xenobiotic in a concentration of 500 mg/l, i.e. the fifth cycle, the stationary phase of growth was reached, which lasted until a slight delay in the catabolic processes (Fig. 2).

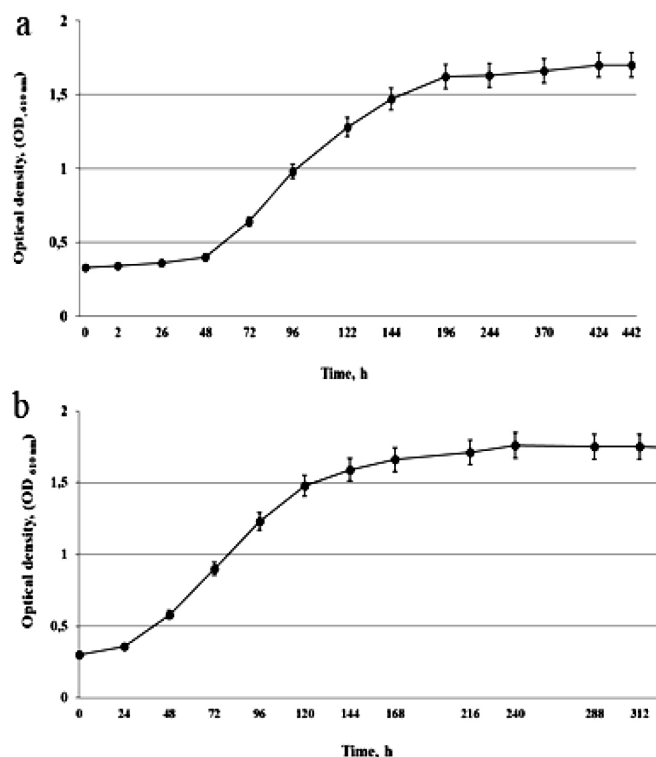


Fig. 2. Growth curve of non-adapted (a) and adapted cells of *M. luteus* BN56 strain (b) under conditions of semi-continuous cultivation, presented as arithmetic mean values ($\bar{x}_{av} \pm \sigma$).

In both cases the curves describing the growth of *M. luteus* BN56 cells were similar. It is essential that the cycles of biodegradation took place also during the stationary phase of growth. The growth of the adapted cells included a shorter lag-period and a logarithmic phase lasting until the fifth supply of both xenobiotics.

Investigating the catabolic activity of some microbial strains during the simultaneous biodegradation of several xenobiotics represents a new approach, as such a model is very close to the parameters found in different wastewaters or soils, where toxic compounds do not appear separately but in mixtures. For example, Gallego *et al.* (2003), presented their investigations on the simultaneous biodegradation of 2-chlor-phenol and m-cresol under continuous process conditions. Degradation of petroleum hydrocarbons (both aliphatic and aromatic) by thermophilic microorganisms (*R. rhodochrous* DSM6263) has been found (Nzila, 2018), together

with biodegradation of phenol and n-hexadecane by an *Acinetobacter* strain isolated from river waters during a single active cycle (Sun *et al.*, 2012).

In the present study, an attempt was made at simultaneous biodegradation of aliphatic and aromatic xenobiotics, i. e. hydrophobic and hydrophilic toxic substances, which are known to be degraded by various catabolic pathways (Kolvenbach *et al.*, 2014). The active simultaneous biodegradation could be explained by the presence of two membrane-bound enzymes (phenol hydroxylase and alkane monooxygenase) located at different sites in the cells, and the use of different channels for the transfer of the hydrophilic and hydrophobic xenobiotic into the cells (van Beilen and Funhoff, 2007, Ullrich and Hofrichter, 2007).

In our next experiments successful simultaneous biodegradation was accomplished of the aromatic xenobiotic phenol and the aliphatic xenobiotic n-hexadecane by applying the immobilization approach. Cells of the investigated strain were immobilized *in situ* in the biocompatible PEO and PAAm cryogels. First, the cells were mixed with the reagents in aqueous media and then the polymer network was formed by UV light induced crosslinking in the frozen state, according to the established mechanism of Lozinsky (2002). During freezing, the major portion of water formed large polycrystals. Cells, polymer (monomer), photoinitiator, crosslinking agent, and bound water (non-freezable solvent) accumulated in a non-frozen liquid microphase, where the formation of a network took place. After thawing, the cryogels consisted of smooth polymer walls resulting from the microphase, which were surrounded by interconnected pores filled mainly with free water from the melted ice crystals (Fig. 4a and 5a). Probably this water is one of the reasons why cells do not lose their catalytic activity and survive being entrapped. During repeated batch experiments with free cells, a decrease in the biodegradation capability was recorded after 10 cycles.

When comparing the two types of matrices, the PEO matrix appeared to be more suitable in terms of number of active cycles of simultaneous biodegradation of phenol and hexadecane (40). It should be noted that the rate of n-hexadecane biodegradation was higher (12 h) compared to phenol (18-28 h) (Fig. 3).

However, when immobilization was performed with the use of PEO cryogel matrices, very active biodegradation of n-hexadecane and phenol was observed after the first addition (first cycle). For forty cycles, the catabolic potential of the cells remained very active (Fig. 3). The selected method

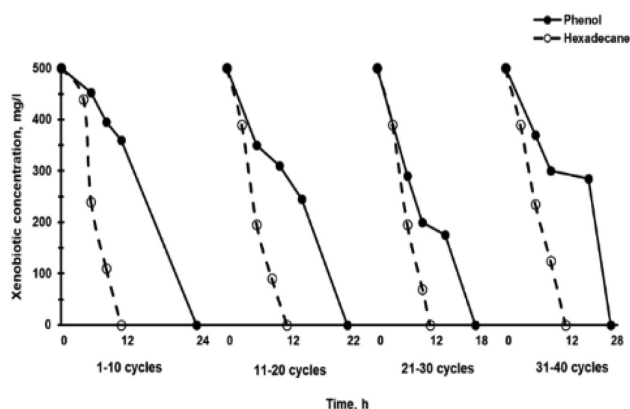


Fig. 3. Processes of semi-continuous biodegradation of phenol and n-hexadecane from PEO immobilized cells of *M. luteus* BN56, presented as arithmetic mean values (xav. $\pm \sigma$) of residual phenol and n-hexadecane for every ten cycles.

of immobilization proved to be successful and efficient for a long period of time. At the end of each of the first twenty cycles, the measured optical density ($OD_{610\text{ nm}}$) was 0.1. Over the next twenty cycles, the amount of desorbed cells increased slightly to 0.2 ($OD_{610\text{ nm}}$) compared to the optical density of free cells 1.6 ($OD_{610\text{ nm}}$). This fact clearly indicates a negligible leakage of cells and, accordingly, their good retention in the volume of the gel. The walls of the cryogel are partially closed and with interconnected pores containing the cells. This is how they prevent cells from leaking out.

Scanning electron microscopy observations confirmed that the cells preserved their shape and their regular distribution beneath the matrix surface along the formed channels and appeared partly embedded in the matrix pores after many cycles of use.

Immobilization of living cells into the super-macro porous supports offers enormous advantages for their reusability and stability in continuous operation. Several attempts have been made with this purpose in mind. Thus, Wilson and Bradley (1996) used a suspension of free cells and immobilized cells of *P. fluorescence* on a commercial bio-support as bioremediation agents in an aqueous

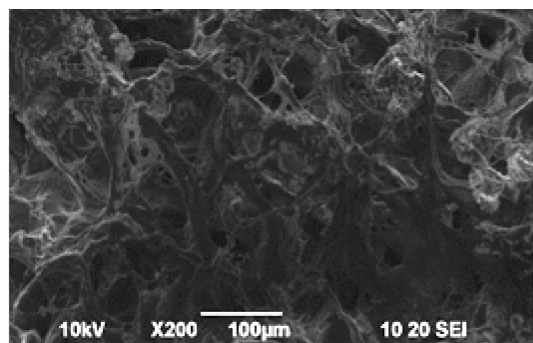
system with petrol as the carbon source. Quek *et al.* (2006) reported immobilization of *Rhodococcus* sp. F92 on polyurethane foam in the bioremediation of petroleum hydrocarbons, as immobilized cells can degrade a variety of petroleum products. In another study, *R. corynebacterioides* QBT immobilized on chitin and chitosan flakes significantly increased the biodegradation of crude oil (Gentili *et al.*, 2006). The application of immobilization techniques can protect cells from phenolic toxicity and increase their ability to degrade (Gouda, 2007). Immobilization not only simplifies the separation and recovery of used cells, but also makes their application multiple, which reduces the total cost (Cunningham *et al.*, 2004).

Conclusions

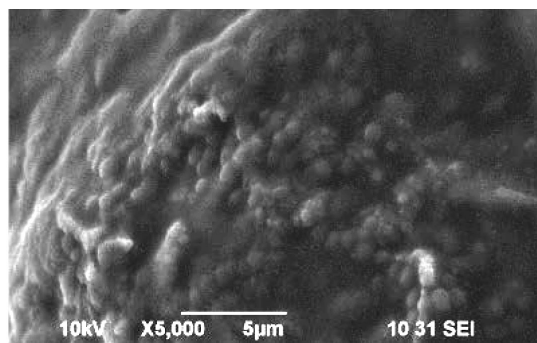
With the help of some adaptation and optimization procedures, full depletion of both aromatic and aliphatic xenobiotics tested was successfully achieved. In the course of the experiments, 10 g/l (100%) phenol and 10 g/l (100%) n-hexadecane were degraded for 20 active cycles of operation (duration of 12 - 36 h) by the free cells of *M. luteus* BN56. By means of an *in situ* immobilization procedure using PEO cryogel matrices, these processes were intensified, stabilized and reached 40 cycles of operation of successful simultaneous biodegradation of aromatic and aliphatic xenobiotics (20 g/l + 20 g/l). The catabolic ability of the investigated strain immobilized in PEO cryogel matrices can be applied in the bioremediation of wastewaters and soils containing such pollutants.

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a



b

Fig. 4. Scanning electron micrographs: PEO matrix only- a); cells entrapped in PEO- b)

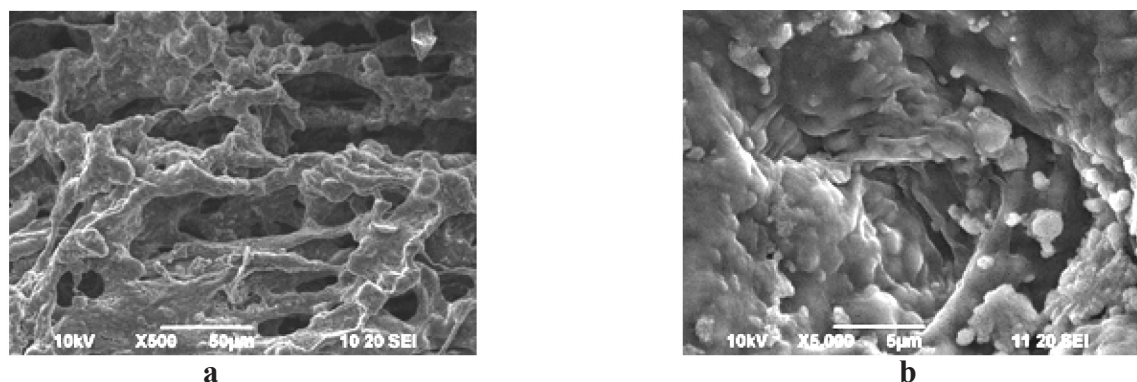


Fig. 5. Scanning electron micrographs: PAAm matrix only- a); cells entrapped in PAAm- b)

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Production, Purification and Characterization of Extracellular α -Amylase from Mutant Strain *Aspergillus Oryzae* MSPP

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Abstract

Aspergillus oryzae is well known species and it has an important role in the fermentation of oriental food products and in industrial application as a producer of hydrolytic enzymes. An extracellular α -amylase enzyme from mutant strain *A. oryzae* MSPP was purified to electrophoretic homogeneity by a simple procedure including ammonium sulfate precipitation, anion-exchange chromatography and Sephadex gel filtration. All purification steps were checked by PAGE. The molecular mass of the purified α -amylase was estimated to be 52 kDa. The enzyme had a pH optimum at 4.7 and was stable in the range of pH 4.7 to 10. The optimal temperature for the highest α -amylase activity was measured at 50°C. Half-life of the enzyme was 30 min at 50°C. Stability of the purified enzyme mainly depended on the presence or absence of soluble starch in the reaction mixture. Michaelis constant (K_m) for soluble starch as a substrate was estimated to be 5 mg/ml.

Keywords: α -amylase, *Aspergillus oryzae*, gel filtration, ion exchange chromatography

Резюме

Aspergillus oryzae е добре известен вид и има важна роля в получаването на азиатски ферментационни хранителни продукти и намира приложение в индустрията като продуцент на хидролитични ензими. Извънклетъчният ензим от мутантен щам *A. oryzae* MSPP е пречистен до електрофоретична хомогенност чрез опростена процедура, включваща преципитация с амониев сулфат, анионна обменна хроматография и Sephadex гел филтрация. Всички пречистващи стъпки са проверявани с полиакриламид гел електрофореза. Молекулната маса на пречистеният ензим е 52 кДа, рН оптимумът е 4.7, а рН стабилността е в обхват от 4.7 до 10. Оптималната температура, при която α -амилазата проявява най-висока активност е 50°C. Полуживотът на ензима е 30 минути при 50°C. Стабилността на пречистения ензим главно зависи от присъствието или отсъствието на разтворимо нишесте в реакционната смес. Изчислена е и константата на Михаелис (K_m) за разтворимо нишесте като субстрат в концентрация 5 мг/мл.

Introduction

Amylases are among the most important and widely used enzymes which spectrum of application have widened in many sectors such as clinical, medicinal and analytical chemistry (Kumari *et al.*, 2012). Besides their used in starch saccharification they also find application in food, baking, brewing, detergent, textile, paper and distilling industry (Pandey *et al.*, 2000; Singh *et al.*, 2014).

α -Amylases (1,4- α -D-glucan-glucohydrolase; E.C. 3.2.1.1.) are endo-amylases catalyzing the hydrolysis of internal α -1,4-D-glucosidic link

ages in polysaccharides (as starch, glycogen, dextrans, etc.). They can be derived from several sources, but the enzymes produced by microorganisms generally meet industrial demands (Fogarty, 1983; El-Fallal *et al.*, 2012; Elmansy *et al.*, 2018). Studies in enzyme production are pointed to optimize the fermentation processes, to obtain high active producers by mutagenesis, genetic engineering and to receive enzymes with specific properties as cold resistance, thermostability, etc. (Doyle *et al.*, 1989; Shaw *et al.*, 1995; Kirimura *et al.*, 1997; Petrova *et al.*, 2000).

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Lots of industrial enzymes are derived from filamentous fungi belonging to the genus *Aspergillus*. The fungi and their products are used in many fields, especially in food and food processing industry for many decades, which resulted in a so-called GRAS status (generally regarded as safe) for some of the strains such as *Aspergillus oryzae*.

In this study α -amylase enzyme from a highly active mutant strain *A. oryzae* MSPP was purified and some properties were determined.

Materials and Methods

Strains, media and culture conditions:

The strain used in this study is *A. oryzae* MSPP obtained by chemical mutagenesis of parental strain *A. oryzae* PP from Microbial collection of the Department of Biotechnology, Faculty of Biology, Sofia University. The parental strain was used in patent industrial technology BG 61573 B1 for production of fungal α -amylase enzyme (Peev *et al.*, 1998).

A. oryzae MSPP, a hyper-producer of α -amylase was maintained on Sabouraud agar. The strain was incubated at 28°C for 7 days and stored at 4°C. Inoculum for the fermentation was obtained on medium with the following composition (g/L): Czapek mineral salt medium with glucose – 20, insoluble starch – 10, soybean flour- 30 and maize extract – 10 ml. Initial pH was 6.3. The composition of fermentation medium for production of α -amylase enzyme was optimized by mathematical modeling (Box-Wilson method – data not shown) and contained (g/L): insoluble starch – 60, soybean flour- 30, wheat bran – 10, NaNO₃ – 3, KCl – 0.5, MgSO₄ x 5H₂O – 0.5, K₂HPO₄ – 1, FeSO₄ – 0.01 and pH of the medium was 6.3.

The cultivation process was done in a 5 L bioreactor with 3 L medium (work volume) at 28°C for 120 h. The bioreactor was inoculated with 10% (v/v) 24 hours inoculum. The cultivation was carried out at the following conditions: 1.5 L/L/min aeration for 72 hours and until 120 hour at 1 L/L/min, stirring at 600 rpm at 28°C. Mycelial mass was removed by filtration and the resulting cell free filtrate was used as starting material for enzyme purification.

Assay methods

Enzyme activity assay

α -Amylase activity was determined by measuring a decrease in the amount of soluble starch (1%) used as a substrate in the reaction mixture by starch – iodine method (Ruchlyadeva and Goryacheva, 1966). That assay method has

a correlation with SKB method. One unit (U) of enzyme activity was defined as the amount of enzyme that catalyzes the hydrolysis of 1 mg starch per minute under assay optimum conditions. Protein assay was determined by method of Lowry (Lowry *et al.*, 1951).

Electrophoretic methods:

PAGE and SDS-PAGE were performed on 12% polyacrylamide gel according to Laemmli (1950) to estimate the molecular mass of the purified amylase using standard protein kit from Pharmacia.

Purification of α -amylase

Fractional precipitation with ammonium sulphate: Culture filtrate from 120 h with highest α -amylase activity was saturated with solid ammonium sulphate at two steps to take the concentration from 40-80%. Two hours after adding first dose of ammonium sulphate at 4°C under constant stirring protein mixture was clarified by centrifugation at 7000 x g for 20 min at 10°C. The supernatant was further saturated to 80% with ammonium sulphate and the same procedure was repeated. The precipitate containing α -amylase was dissolved with 10 mM potassium phosphate buffer, pH 6.6 and subjected to ultrafiltration on Amicon (PM 10 membrane) until residual amounts of ammonium sulphate were washed and used for chromatographic purification. Amylolytic activity and protein content were determined in the saturated fractions.

Ion-exchange chromatography: Anion-exchange chromatography on Fractogel TSK DEAE-650 (Merck) column (26x60mm) was used for further purification. The ultrafiltrate from step 1 was put on the column, equilibrated with 10 mM potassium phosphate buffer, pH 6.6 and was washed with the same buffer until unbound material eluted out. Then three step gradient elution with phosphate buffer pH 6.6 (100 mM, 500 mM and 500 mM plus 1 M NaCl) was performed at a flow rate of 50 ml/h. Fractions of 10 ml were collected from the start of the gradient. All received fractions were checked for α -amylase according to assay and active fractions were collected and again concentrated by ultrafiltrated apparatus (Amicon, PM 10 membrane).

A sample of the concentrated enzyme was applied to a Sephadex G-100 (fine) column (16x750mm) which had previously equilibrated with 0.05 M phosphate buffer (pH 6.6) containing 0.05 M NaCl. The flow rate was 12 mL/h and the volume of each fraction was adjusted to 2 mL. All

active fractions were collected and concentrated by ultrafiltration.

Re-chromatography was carried out at the same conditions. The resulting purified enzyme was stored at 4°C.

Effects of temperature and pH on enzyme activity

To investigate the effect of temperature on enzyme activity, an enzyme analysis was performed at 10, 20, 30, 35, 40, 45, 50, 55, 60, 70 and 80°C. The effect of pH was studied at pH 3 - 6 with acetate buffer, at pH 6 - 8 with potassium phosphate buffer and at pH 8 - 10 with glycine-sodium hydroxide. Each of the buffers had final concentration 100mM.

Effects of temperature and pH on stability of the enzyme

To measure the effect of temperature, the enzyme solution was incubated at 30 - 80°C for different time intervals: 10, 20, 30, 60, 90, 120 min and then the remaining activity was determined according the α -amylase assay conditions. The effect of different pH buffers (described above) on enzyme stability was investigated for two hours. After incubation with each buffer the relative activity was assayed.

Effect of substrate concentration on enzyme activity

Soluble starch in different concentrations from 0.4 to 1.5% (in 0.1 step) was used to analysis the effect of substrate concentration on the enzyme activity.

Effect of metal ions.

Metal ions as K⁺, Ca²⁺, Mg²⁺, Zn²⁺, Cd²⁺, Ni²⁺, Cu²⁺, Mn²⁺, Al³⁺ were added to the substrate solution to a final concentration of 10 mM. The enzyme reaction was performed at 30°C for 10 min and relative activity was assayed.

Results

A. oryzae MSPP produced high levels of an extracellular α -amylase (Fig. 1). Maximum production of enzyme in bioreactor was observed at 120 h – 536 U/mL. After the fifth day of fermentation α -amylase activity was decreased. Then the culture broth was filtrated to remove cells and debris and the supernatant was collected. Only 150 mL of the filtrate was used for further purification of the enzyme.

The results of the supernatant partial precipitation with ammonium sulfate were summarized in Table 1.

The amylolytic activity in the fraction 0-40% was low and insignificant. A major part of amylase enzyme was determined in the supernatant. Second

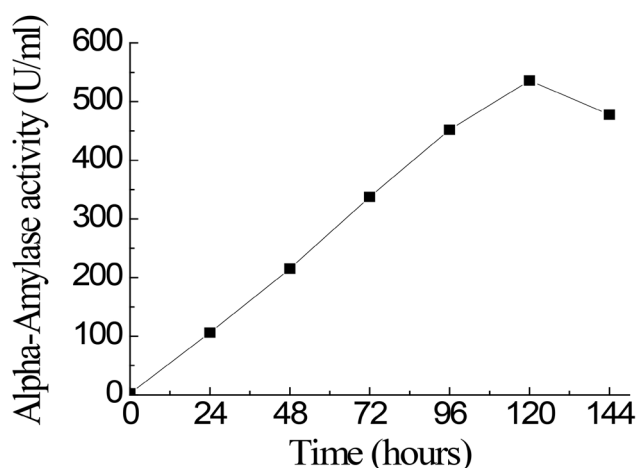


Fig. 1. Dynamic of α -amylase activity during cultivation of *A. oryzae* MSPP in bioreactor at 28°C and aeration from 0-72 h 1.5 L/L/min and 1 L/L/min from 72 h onward.

Table 1. Partial purification of the starch-hydrolyzing enzyme from culture of *Aspergillus oryzae* MSPP by ammonium sulphate precipitation.

Percent of saturation (fractions)	Protein (mg/ml)	Amylolytic activity (U/ml)
0-40	1.9	10.2
40-80	12	900

precipitation to 80% showed increased both in protein concentration and amylolytic activity.

A sample from this fraction was used for anion-exchange chromatography on DEAE Fractogel column. To remove ammonium salts, the probe was ultrafiltrated and concentrated on Amicon with 10 mM potassium phosphate buffer and then was applied to the column. α -Amylase was eluted as two peaks with 0.1 M and 0.5 M phosphate buffer, pH 6.6. The second peak had lower enzyme activity – 10% of the total amylase activity (Fig. 2). Similar results have been detected by Chung during the purification of α -amylase by *Thermococcus profundus* DT5432 (Chung *et al.*, 1995). According their experimental data the authors considered that these α -amylases were isoenzymes.

DEAE-650 column equilibrated with 10 mM potassium phosphate buffer (pH 6.6) - flow rate of 50 mL/h, fractions volume – 8 mL. The protein fractions were eluted with the same buffer by step-wise gradient (0.1 M, 0.5 M phosphate buffer and 0.5 M phosphate buffer plus 1M NaCl). The fractions between 10 and 18, containing α -amylase activity, eluted with 100 mM buffer, were combined, concentrated and applied to Sephadex G-100 column. It was observed two main fractions separated at the conditions of gel filtration – one, containing

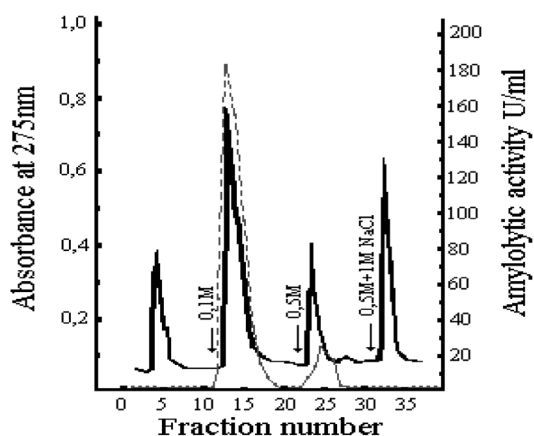


Fig. 2. Elution profiles of α -amylase activity (---) and an absorbance (—) of a Fractogel TSK

α -amylase activity (fractions 35-55) and another with larger molecular mass, a low protein content and a presence of carbohydrates (determined by Molisch test- data not shown).

Re-chromatography was carried out at the same conditions (Fig. 3B).

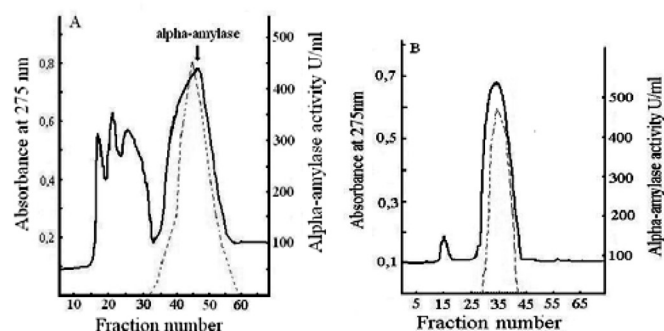


Fig. 3. Gel filtration chromatography of the concentrated active fractions after DEAE –Fractogel on Sephadex G 100; A: — absorbance, --- amylase eluted profile; B: re-chromatography: flow rate of 12 mL/h and the fractions volume – 2 mL.

Table 2. Purification steps of α -amylase from mutant strain *A. oryzae* MSPP

Steps	Volume (mL)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Fold of purification	Yield %
Crude enzyme	150	60674	2160	28.3	1.0	100
(NH ₄) ₂ SO ₄ precipitation	85	54740	1411	38.8	1.4	90.2
DEAE Fractogel	6	36252	751.2	48.3	1.7	78
Sephadex G-100	2.7	35961	625.6	57.5	2	71

The results of purification procedures were summarized in Table 2. The final preparation had 71% yield and 2.0 fold of purification.

The purified enzyme had a single band on SDS-PAGE with molecular mass approximately 52 kDa (Fig. 4A). Native PAGE was performed with properly diluted culture broth. Several proteins in

negligible quantities were observed on the polyacrilamide gel (Fig. 4B).

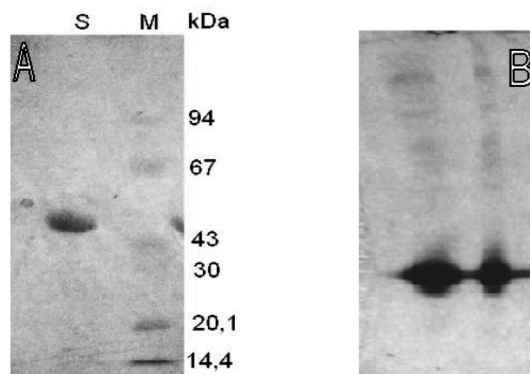


Fig. 4. Polyacrylamide gel electrophoresis: A: SDS-PAGE of the purified α -amylase: M – test kit (14.4 kDa – α -lactalbumin, 20.1 kDa – trypsin inhibitor, 30 kDa – carboxyanhydrase, 43 kDa – ovalbumin, 67 kDa - albumin, 94 kDa – phosphorylase); S – purified enzyme; B: Native PAGE of the culture filtrate.

These results demonstrated that *A. oryzae* MSPP synthesized high levels of the extracellular α -amylase, which is the main protein in the culture filtrate. This explains the low fold of purification which is enough to obtain electrophoretic homogeneity of the enzyme. The high yield allowed easily and widely application of the α -amylase in industry.

Effect of temperature and pH on α -amylase activity

Maximum activity was registered at 50°C (Fig. 5A). That temperature was an optimum for growing of the mesophylic strain *A. oryzae* MSPP. A temperature above 60°C a rapid decrease of activity was demonstrated.

The optimum pH of α -amylase was found to be at 4.7 (Fig. 5B). The enzyme activity was higher

than 80% in a range of 3.7 to 5.7 and was stable for 2 hours at 30°C. The incubation of the enzyme at higher pH values (over 7.8) led to quick loss of relative amylase activity – 10% at pH 8.6.

Effect of temperature and pH on α -amylase stability

Thermal inactivation of the purified α -amyl-

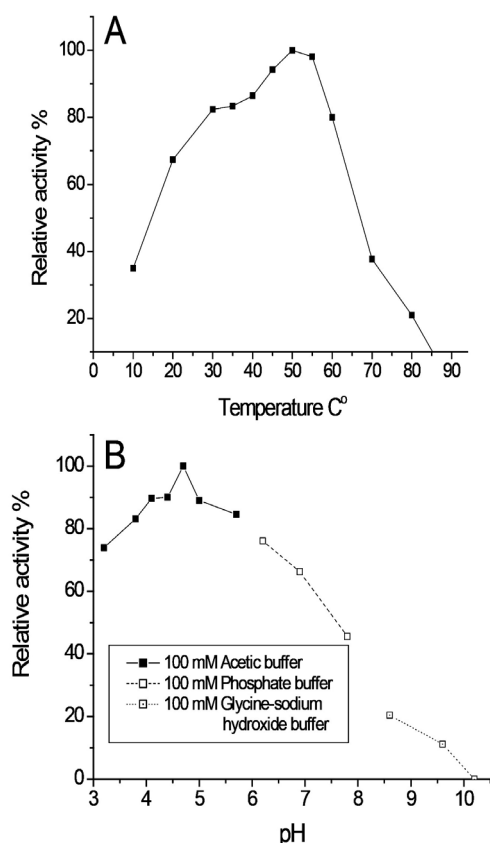


Fig. 5. Effects of temperature (A) and pH (B) on α -amylase activity.

ase was investigated by incubating the enzyme in the absence of substrate at various temperatures (Fig. 6) in 100 mM phosphate buffer pH 6.6. The enzyme activity was preserved 100% for two hours at temperature 30°C.

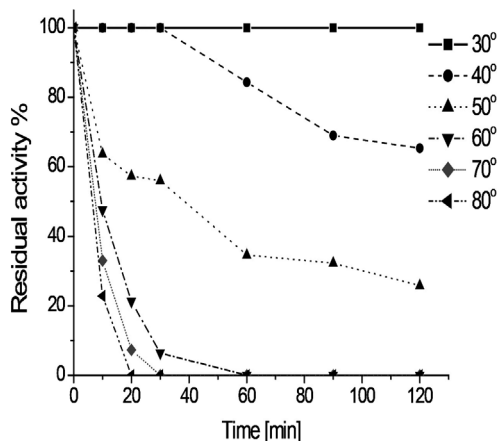


Fig. 6. Influence of temperature on α -amylase stability

The α -amylase activity was 100% for 30 min at 40°C, but at the end of the temperature treatment enzyme showed 30% decrease of the activity. Half-life of the enzyme at 50°C was 30 min. Temperatures higher than 50°C caused a quick decline of the amylase activity.

The effect of pH was also investigated. The enzyme was more stable at pH 4.7-10.1 – residual activity was more than 50% for two hours and lower at pH 3.2 (residual activity 32 %) (Table 3).

Table 3. Influence of pH on α -amylase stability

pH	Residual activity %
3.2	32.36
4.1	38.49
4.7	68.67
5.6	66.12
7.8	67.84
8.6	64.21
10.1	60.14

Effect of substrate concentration on enzyme activity.

The substrate concentration in the range from 0.4 % to 0.6 % caused exponential increase on amylase activity (Fig. 7).

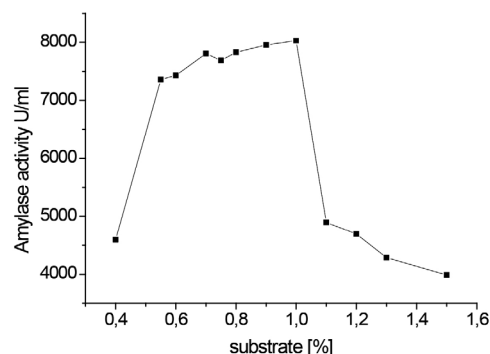


Fig. 7. Influence of the substrate concentration on the purified enzyme

Further increasing of starch concentration showed a slight grow of the amylase activity and after 1% substrate concentration the activity sharply decreased. The linear rate of the absorbance change was measured to give the initial velocity (v). The K_m was calculated by Lineweaver-Burk plotting, with at least six initial substrate concentrations. The K_m value for soluble starch as a substrate was estimated to be 5 mg/mL (date not shown).

Effect of metal ions on α -amylase activity

Cations, each at a level of 10 mM, had various effects on the purified amylase (Table 4). The metal ions, except Ca^{2+} decreased amylase activity during the incubation at 30°C for 10min. Mg^{2+} , Mn^{2+} and Zn^{2+} caused 80% restoration of amylase activity. Al^{3+} ions had 57.5% inhibition effect on catalytic activity of α -amylase.

Table 4. Effect of metal ions on α -amylase activity

Metal ions (10 mM)	Relative activity %
Control	100
Ca ²⁺	104.3
Mg ²⁺	85.6
K ⁺	71.4
Cd ²⁺	71.5
Zn ²⁺	81.7
Ni ²⁺	69
Al ³⁺	42.5
Cu ²⁺	61.7
Mn ²⁺	81.3

Discussion

Extracellular α -amylase from mutant strains *A. oryzae* MSPP was purified to electrophoretic homogeneity and partially characterized. In comparison with other fungal amylases, the properties of *A. oryzae* MSPP α -amylase were in agreement with the reported data. The purified α -amylase had molecular weight of 52 kDa, optimum temperature at 50°C, optimum pH 4.7, pH stability was observed in the range of pH 4 to pH 8 for 2 hours (over 80% activity). The half-life of the enzyme at 50°C was 40 min (mesophilic enzyme) and Km estimated for soluble starch was 5 mg/ml. The effect of metal ions showed no requirements of cations.

At this stage of investigation, α -amylase appeared to have common characteristic of α -amylases (Kariya *et al.*, 2003, Nagamine *et al.*, 2003). The α -amylase from *A. oryzae* MSPP was synthesized in high quantities in the culture broth (Fig. 4B – native PAGE). A pure α -amylase enzyme with electrophoretic homogeneity was obtained only after two-fold of purification with high yield 71%. Further studies and with the help of protein engineering and immobilization techniques can improve the enzyme activity and stability (Kumari *et al.*, 2012, Tonova, 2018, Al-Najada *et al.*, 2019) in higher range.

The purified enzyme is a potential candidate for application in the industry – food, pharmaceutical and clinical sectors where high purity amylases are required.

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Induction of Ecological Resistance to Potato Virus Y in Potato Varieties through Silencing of Main Viral Genes

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Abstract

Outbreaks of potato virus Y (PVY) have a serious negative impact on agricultural production, leading to significant losses. The need for an effective PVY control technique is essential to reduce yield losses in agriculture. Plants use a defense mechanism called RNA silencing, which was used to control PVY. Post-transcriptional gene silencing (PTGS) of major viral genes was induced by specific siRNAs that trigger specific degradation of viral RNA by forming a multi-component RNA-induced silencing complex (RISC). By using PTGS, resistance of potato varieties to PVY was achieved.

Keywords: PVY, PTGS, RNA silencing, siRNAs, potatoes, resistance

Резюме

Епидемиите от картофения вирус Y (PVY) имат сериозно отрицателно въздействие върху селскостопанската продукция, което води до значителни загуби. Необходимостта от ефективна техника за контрол на PVY е от съществено значение за намаляване на загубата на добив в селското стопанство. Растенията използват защитен механизъм, наречен РНК заглушаване, който ние използвахме за контрол на PVY. Ние индуцирахме посттранскрипционно генно заглушаване (PTGS) на основни вирусни гени чрез специфични миРНКи, които индуцират специфична деградация на вирусната РНК чрез формиране на многокомпонентен РНК-индуциран заглушаващ комплекс (RISC). Чрез използването на PTGS постигнахме устойчивост на картофени сортове спрямо PVY.

Introduction

Potviruses are the largest group of plant viral pathogens that are constantly expanding their territories and causing damage to their cultural hosts around the world. Outbreaks of PVY have a serious negative impact on agricultural production, leading to significant losses. The need for an effective PVY control technique is essential to reduce yield losses in agriculture.

Plants use a protective mechanism called RNA silencing, which can be used to target potviruses. RNA gene silencing, of which post-transcriptional gene silencing (PTGS) is a part, is a general term describing the related gene regulatory mechanisms driven by RNA in plants (Vance and Vaucheret, 2001). The first cases of gene silencing were reported with transgenic plants carrying artificially introduced transgenes (Napoli *et al.*, 1990).

The process is initiated by double-stranded RNAs (dsRNAs) - molecules that are produced during viral replication. The double-stranded RNAs are recognized by the plant as „non-self“ and subsequently cleaved by cellular Dicer-like enzymes, forming small interfering RNAs (siRNAs) with a length of 21-25 bp (Hammond *et al.*, 2000). These siRNAs are a major component of RNA gene silencing (Denli and Hannon, 2003). They initiate complement-specific RNA degradation by forming a multi-component cell complex (RISC), inducing RNA gene silencing, which destroys related mRNAs (Martinez *et al.*, 2002).

A remarkable feature of RNA gene silencing is its ability to propagate both from cell to cell and over long distances by inducing systemic RNA silencing throughout the body. This happens through

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complement-specific signal silencing obtained after induction of RNA gene silencing in single cells. In response, plant viruses encode proteins capable of suppressing RNA gene silencing (Mlotshwa *et al.*, 2002).

Material and Methods

Virus for plant inoculation: PVY^{N/NTN} virus strain

Host plants: 15 pots with potatoes cv. Marabel

Inoculation of plants with virus

One gram of leaf mass of a plant with clear symptoms was homogenized in 1 ml, cooled to 4°C 0.1M, potassium-sodium phosphate buffer, pH 8.0, containing 0.2% Na₂SO₃ and 0.2% ascorbic acid. Inoculations were performed by lightly rubbing the leaves with this homogenate. After 3-5 minutes the plants were washed with water. The next day they were imported into the phytostat. Symptoms were reported 7-25 days after inoculation, depending on the species (Petrov, 2015).

Total RNA extraction

RNEasy Plant Mini Kit (Qiagen, Germany) was used. Extraction was performed according to the instructions of the manufacturer.

In vitro system for production of double-stranded RNA (dsRNA)

dsRNA was synthesized by a combination of in vitro transcription and DNA template replication (according to the instructions of the Replicator RNAi Kit, Finnzymes, Finland). The DNA template for dsRNA synthesis was obtained by PCR reaction using Phusion High-Fidelity DNA polymerase. The PCR primers were designed so that the resulting PCR product contained our target sequence (PVY HC-Pro) surrounded by a T7 promoter sequence at its 5' end and a phy6 qRdRP promoter sequence at its 3' end. The PCR product was purified and transcribed into single-stranded RNA (ssRNA) by T7 viral RNA polymerase. This ssRNA was replicated in dsRNA from phi6 qRdRP viral replicase. The sequences of the constructed primers were: HC-Pro dsRNA 1 (5'-TAA TAC GAC TCA CTA TAG GG TAG GAT TCT GTC GAA TGC CGA CAA TTT T-3'), HC-Pro dsRNA 2 (5'-GGA AAA AAA TAC TGC AGA CCA ACT CTA TAA TGT TT-3') (Petrov *et al.*, 2015).

Detection of viral infection by DAS-ELISA

A kit from LOEWE Biochemica GmbH, Sauerlach, Germany, was used. ELISA plates were loaded with antiserum (IgG) for PVY, previously diluted according to the manufacturer's instructions

in 0.05M carbonate buffer. They were incubated for 4 hours at 37°C and the unbound components were washed three times with PBS-T buffer for 5 minutes. All samples were ground in extraction buffer containing 1% PVP (polyvinyl pyrrolidone) in a ratio of 1:10. The plaques were left for 16 hours at 4°C. After triple washing, alkaline phosphatase conjugate for PVY was added and the plates were incubated for 4 hours at 37°C. The substrate used was para-nitrophenyl phosphate (p-nitrophenyl phosphate, Sigma) in diethanolamine buffer (pH 9.8) at a ratio of 1 mg/1 ml. The reaction was carried out in the light and at room temperature, 3N NaOH was used to stop the reaction. Color adsorption was measured on a Multifunction Detector (DTX 880) at a wavelength of 405 nm. The samples for which the optical density (OD, absorption) was more than twice the value of the negative control (called cut-off value or Cut Off) were accepted as positive (Petrov *et al.*, 2015).

Results and Discussion

In the present study, post-transcriptional gene silencing (PTGS) of a major multifunctional viral gene such as HC-Pro was induced, using specific dsRNAs that initiate specific degradation of viral RNA by forming a multicomponent RNA-induced attenuation complex (RISC). By using PTGS, potato resistance to PVY was achieved (Fig. 1).

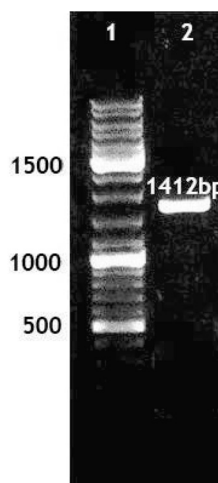


Fig. 1. PVY HC-Pro dsRNA

None of the potato plants treated with HC-Pro-dsRNAs and inoculated with PVY^{N/NTN} developed visual symptoms of disease. The optical density (OD) values of the plant samples from these pots remained below the limit value and were close to healthy control plants. The DAS-ELISA value (0.4) of one of the treated plants was close to the limit value, but was negative. Symptoms of infection in this pot were not observed either.

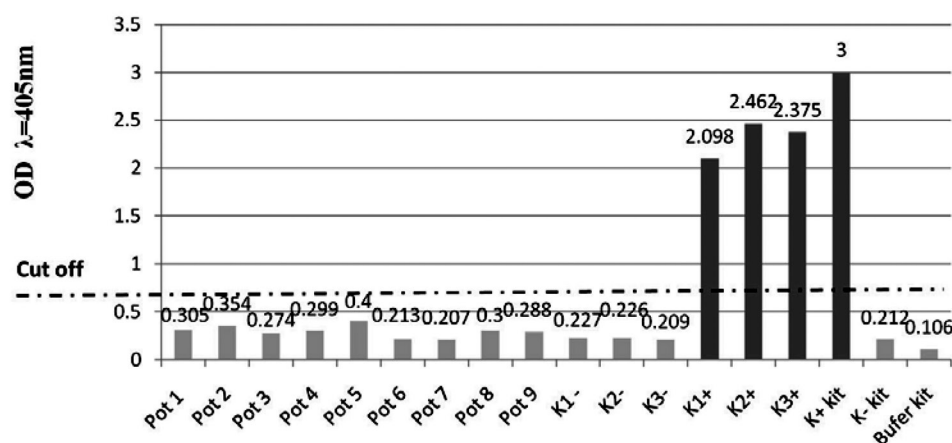


Fig. 2. DAS-ELISA assay of potato plants after treatment with dsRNA for HC-Pro of PVY^{N/NTN} and after inoculation with PVY^{N/NTN}. Samples 1-9: pots of potato plants treated with HC Pro-dsRNAs and inoculated with PVY^{N/NTN}; K1-, K2-, K3-: healthy potato plants not treated and not inoculated with virus; K1+, K2+, K3+: potato plants inoculated with PVY^{N/NTN} and not treated with HC-Pro-dsRNAs; K+ kit: positive control by ELISA kit; K- kit: negative control by ELISA kit)

High OD values were obtained for potato leaf samples inoculated with PVY^{N/NTN} (K +) only, indicating successful viral infection with PVY^{N/NTN} (Fig. 2).

Treatment of plants with dsRNAs specific for the HC-Pro gene region of PVY^{N/NTN} resulted in the growth of healthy plants that were not prone to developing disease symptoms of PVY^{N/NTN} (Fig. 2, pots 1-9). The strategy applied in this study to control viral infection was based on knowledge of the mechanisms of PVY replication and the protective responses of plants against pathogens, assuming that blocking a key viral protein for virus replication and suppressing PTGS will lead to blocking the development of the infection process itself. Gene silencing has been successfully induced against PVY also in tobacco plants and in potato plants of Arinda variety through the use of specific dsRNAs and siRNAs for the HC-Pro region of PVY, which effectively reduced the systemic spread of the virus (Petrov and Stoyanova, 2011; Petrov, 2012). Decreased expression of the PVY^N HC-Pro gene was found in newly grown potato leaves and therefore, decreased viral replication in all virus-inoculated plants. Old PVY leaves inoculated with potato plants remained infected and later defoliated. All new leaves of potato plants inoculated with PVY of Arinda variety that sprouted after treatment with dsRNA and siRNA remained healthy and virus-free (Petrov *et al.*, 2015).

Conclusion

Epigenetic control of viral infection was achieved by induction of post-transcriptional gene attenuation in Marabel variety potato plants by spe-

cific siRNAs targeting the HC-Pro gene region of PVY^{N/NTN}. This strategy effectively blocks the systemic spread of the virus and inhibits viral replication in host plants.

Acknowledgements

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Short Communication

Reduction of Potato Virus Y Damage on Pepper Plants through Systemic Acquired Resistance

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Abstract

Systemic Acquired Resistance (SAR) against Potato Virus Y (PVY) was induced in pepper plants using natural elicitors based on salicylic acid. Treatment of pepper plants with elicitors induced SAR against PVY infection, which resulted in a reduction in DAS-ELISA values and viral symptoms in plants caused by PVY. Treatment of pepper plants with a combination of two elicitors 3 days before virus inoculation induced strong PVY resistance in the experimental pepper plants.

Keywords: PVY, SAR, pepper, salicylic acid, treatment, resistance

Резюме

Индуцирахме Системна Придобита Устойчивост (SAR) срещу картофения вирус Y (PVY) в пиперни растения, използвайки естествени елиситори на базата на салицилова киселина. Третирането на пиперните растения с елиситори, индуцира SAR срещу PVY инфекция, което доведе до намаляване на стойностите на DAS ELISA и вирусните симптоми в растенията, причинени от PVY. Третирането на растения от пипер с комбинирана схема от два елиситора 3 дни преди инокулирането на вируса предизвика силна устойчивост към PVY в експерименталните пиперни растения.

Introduction

Potato virus Y (PVY) was first reported in 1931 as an aphid-borne virus belonging to a group of viruses causing damage to potatoes (Smith, 1931). PVY is the typical representative of the genus *Potyvirus* of the family *Potyviridae* (Shukla *et al.*, 1994). PVY was first reported in 1942 in Bulgaria by Kovachevski (Kovachevski, 1951), who found isolated cases of symptoms in pepper caused by this virus. Later, the author proved that the same disease is widespread in tobacco and it is also the cause of curling of leaves in potatoes. He was the first to report that PVY induced necrotic symptoms in tobacco and experimentally transmitted the virus by aphids *Myzus persicae* (Kovachevski, 1951).

Pepper isolates of PVY were classified into three pathotypes: PVY-0, PVY-1 and PVY-1.2, according to their ability to overcome the resistance genes (*vy1*, *vy2*) present in pepper cultivars (Selasie *et al.*, 1985). Within these three groups, pepper isolates were further defined as „common“ (PVY^C) or „necrotic“ (PVY^N) (D'Aquino *et al.*, 1995).

Several recessive genes for resistance to *Potyvirus* such as *pvr1* have been identified in pepper. The different alleles of these genes encode the translation initiation factor eIF4E. Homozygous potyvirus resistance genes have point mutations that interfere with the interaction of plant initiation factor eIF4E with the viral protein VPg. In this way, stable phenotypes are obtained at different levels that prevent viral accumulation, movement of the virus from cell to cell or over long distances (Kang *et al.*, 2005).

To date, no effective means to control PVY virus infection have been identified. However, plants have built a kind of protection against phytopathogens. Systemic acquired resistance (SAR) is characterized by broad-spectrum resistance to disease and is activated by local damages caused by necrosis-inducing pathogens, such as viruses, bacteria or fungi (Kuc, 1982; Kessmann *et al.*, 1994). Chemicals capable of activating SAR in very low concentrations in various plant species (Metraux *et*

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al., 1991; Kessmann *et al.*, 1994) served as a basis for the discovery of local and systemic defense mechanisms against diseases (Ryals *et al.*, 1996; Sticher *et al.*, 1997). In dicotyledonous plants, the biological induction of SAR by local infections caused by necrogenic pathogens is associated with systemic accumulation of salicylic acid (SA) and pathogenicity-related proteins (PR). SA levels increase in both infected tissues and adjacent uninfected tissues (Kessmann *et al.*, 1994; Sticher *et al.*, 1997).

Material and Methods

Introduction of elicitors

Plants were divided into groups of 15 plants each and were treated according to several different schemes:

- 1) with 3 mM aqueous BION solution, pH = 7 only
- 2) with EXIN only
- 3) with EXIN + BION

For each treatment scheme, treatment group and cultivar, the following controls were used: K- (healthy plant treated with distilled water only), K+ (PVY infected plant treated with distilled water only), K- BION (healthy plant treated with BION for phytotoxicity), K- EXIN (healthy plant treated with EXIN for phytotoxicity), K- BION+EXIN (healthy plants treated with this combination).

Pepper plants of the cv. Kurtovska Kapiya were treated 3 days before inoculation with PVY virus in three groups:

Group I - with EXIN 4.5 HP (Phytoxin VS, with the active ingredient 4.5% salicylic acid) at a concentration of 1 packet (10 ml) in 1 L of water.

Group II – with BION (cytokinin derived from salicylic acid, BTH) at a concentration of 3 mM.

Group III was combination with EXIN and 2.35 mM BION.

Spraying was carried out in a greenhouse at a temperature range of 21-24°C and a relative humidity of 45% at a dose of 5-15 ml of solution of the compounds (depending on the size of the plant) in a 3-day interval and 6 treatments.

Inoculation of plants with virus

One gram of leaf mass of a plant with pronounced symptoms was homogenized in 1 ml of cooled to 4°C 0.1 M potassium-sodium phosphate buffer, pH 8.0, containing 0.2% Na₂SO₃ and 0.2% ascorbic acid. Inoculations were performed by lightly rubbing the leaves with this homogenate. After 3-5 minutes the plants were washed with water. The next day they were imported into the

phytoestat. Symptoms were reported 7-25 days after inoculation depending on the species (Petrov *et al.*, 2015).

Detection of viral infection by DAS-ELISA

A kit from LOEWE Biochemica GmbH, Sauerlach, Germany was used. ELISA plates were loaded with antiserum (IgG) for PVY, diluted according to the manufacturer's instructions in 0.05M carbonate buffer. They were incubated for 4 hours at 37 °C and the unbound components were triple washed with PBS-T buffer for 5 minutes. All samples were ground in extraction buffer containing 1% PVP (polyvinyl pyrrolidone) in a ratio of 1:10. The plaques were left for 16 hours at 4°C. After triple washing, alkaline phosphatase conjugate for PVY was added and the plates were incubated for 4 hours at 37°C. The substrate used was para-nitrophenyl phosphate (p-nitrophenyl phosphate, Sigma) in diethanolamine buffer (pH 9.8) at a ratio of 1 mg/1 ml. The reaction was carried out in the light and at room temperature, 3N NaOH was used to stop the reaction. Color adsorption was measured on a Multifunction Detector (DTX 880) at a wavelength of 405 nm. The samples for which the optical density (OD, absorption) was more than twice the value of the negative control (called cut-off value or Cut Off) were accepted as positive (Petrov *et al.*, 2015).

Results and Discussion

Benzothiadiazole (benzo-1,2,3-thiadiazole-7-carbothionic acid S-methyl ester) is a functional analogue of SA and has the trade name BION. Its resistance-inducing effect against many fungal plant pathogens has been shown (Hukkanen, 2008). In plant samples treated with BION (1-15, Fig. 1), half reduction of DAS-ELISA values for PVY was obtained and in plants treated with EXIN (19-33, Fig. 1) - only slight reduction of DAS-ELISA values. Complete reduction of PVY symptoms and DAS-ELISA values to the level of healthy plants was achieved in the plants treated with the combination BION+EXIN (37-51, Fig. 1).

BION, applied alone or in combination with potassium phosphonate as a foliar spray, reduces the spread of root rot caused by *Phytophthora* in coniferous *Pinus radiata* and other plants. This shows the future potential of these resistance inducers to displace toxic and dangerous fungicides. Commonly used chemical inducers are salicylic acid, methyl salicylate and benzothiadiazole, which affect the production of phenolic compounds in plants. Inducers of systemic resistance have a negative effect on insect pests, fungal and viral diseases

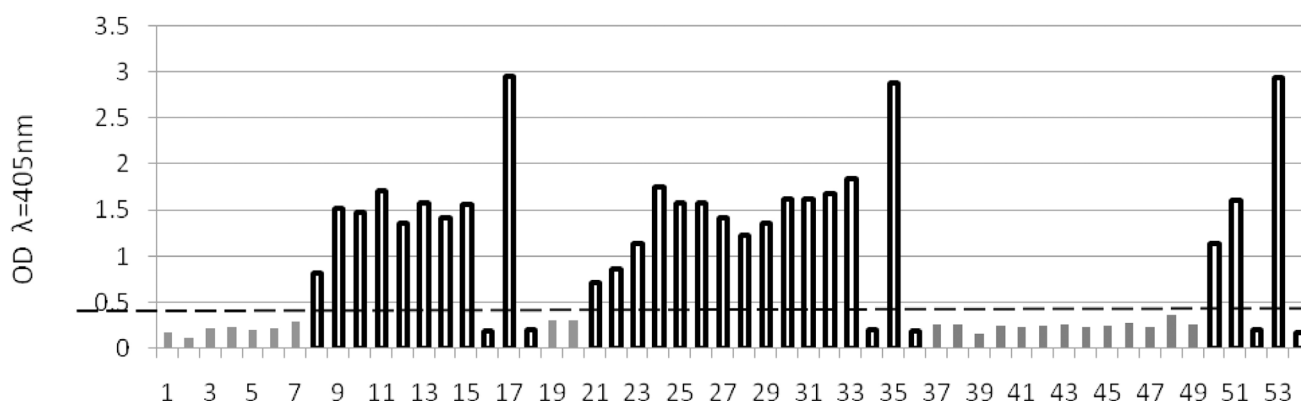


Fig. 1. Results from DAS -ELISA after treatment of pepper plants of cv. Kurtovska Kapiya with BION and EXIN and before inoculation with PVY. 1-15 - plants treated with BION and inoculated with PVY; 16 - plants treated with BION only (for toxicity); 17 - plants infected with PVY only (K+); 18 - untreated and non-inoculated plants (K-); 19-33 - plants treated with EXIN and inoculated with PVY; 34 - plants treated with EXIN only (for toxicity); 35 - plants infected with PVY only (K+); 36 - untreated and non-inoculated plants (K-); 37-51 - plants treated with BION + EXIN and inoculated with PVY; 52 - plants treated with BION + EXIN only (for toxicity); 53 - plants infected with PVY only (K+); 54 - untreated and non-inoculated plants (K-)

(Holopainen *et al.*, 2009). Salicylic acid is involved in the response to pathogenic attack in various plant species. Induction of salicylic acid synthesis is required in viral, fungal and bacterial resistance reactions (Murphy and Carr, 2002; Shah, 2003). When treating sensitive tobacco or tissue plants with SA or its derivatives such as aspirin, a reduction in Tobacco mosaic virus (TMV) accumulation is observed (White *et al.*, 1983).

Conclusion

The best result was obtained after treatment of plants with the combination BION + EXIN. Healthy plants were 86% (13 of 15 plants) 21 days after inoculation with PVY.

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Chronicle

Professor Budin Michov, MD, PhD, DSc



Prof. Budin Michov studied medicine in the High Medical Institute in Sofia. As a student he published his first book *Molecular mechanisms of bacterial inflammation*. After the medical education prof. Michov began working as assistant professor in the Department of Medical Biochemistry at the same high school. There he published many articles in international journals, specialized in biochemistry and after years in clinical laboratory, and published the book *Molecular Mechanisms of Inflammation and Allergy* (1977). Simultaneously he defended dissertations and received the scientific degrees PhD and DSc.

The articles of prof. Michov are devoted to different items: a new complex compound in TRIS-borate buffers, mobility calculation of composed ions, calculating the TRIS-borate ion mobility, ionic mobility parameter, simplifying the Henry function, electrophoresis in one buffer at two pH values, electrophoresis in expanded stationary boundary, TRIS-formate-taurinate buffer system for SDS electrophoresis in homogeneous polyacrylamide gels, electrophoresis of cerebrospinal-fluid proteins in agarose gels, geometric and electrokinetic radii and electric potentials of charged and dissolved particles, and more. He invented a new device for concentration gradient system, and buffer recirculator for electrophoresis.

Because of these publications he was invited by the editor-in-chief of the international journal *Electrophoresis* to work as a scientist in his laboratory at the Technical University of Munich in Germany.

Months after the start of his work in Germany, prof. Michov made a discovery (the most sensitivity analytical method for separating proteins in SDS gels), which he patented in Germany and EU. On the basis of this patent he founded own company in Germany. Later the largest biochemical company in Germany and Europe, SERVA in Heidelberg, employed him as a scientific advisor. Meanwhile the editor-in-chief of the 300-years old scientific publishing house Walter de Gruyter came to him from Berlin to Munich and asked him to write a book on electrophoresis in German. As a result, the book *Elektrophorese. Theorie und Praxis* (1996) in about 430 pages was published in Berlin and New York.

After nearly ten years work in Germany, Prof. Michov decided to return to Bulgaria to help his country. He took part in a competition for an associate professor at the Department of chemistry and biochemistry in the Medical University of Sofia and won it. Starting his habilitation, prof. Michov decided to publish a textbook for medical students to explain them why they should study chemistry. So, the book *Chemistry in Medicine* in nearly 800 pages appeared. Shortly after the book was published, a competition for a professor place was announced, and he submitted his documents. The leadership of Medical University, however, decided to expel him from the competition procedure and to give the professor place a person of the “internal” circle. Therefore, some colleagues sued prof. Michov for “plagiarism”. The trial lasted about 6 years. All experts in court proved that no plagiarism existed, however, the competition procedure for the professor place failed and the health of prof. Michov was severely hurt.

After the court trial, prof. Michov realized that he could not continue his activity at the Medical University of Sofia, and took part in another competition procedure for a professor of medical biochemistry and head of the Department of Biochemistry in the newly established Second Medical Faculty at Sofia University St. Kliment

Ohridski. He won the competition and began working to develop a perfect biochemical department on the world level. However the chief of the Government hospital Lyubomir Spasov expelled him from the Medical Faculty making so that prof. Michov got only a half of the professor pension – 350 EURO. This is his pension until now!

After he was expelled from Sofia University St. Kliment Ohridski, prof. Michov gave lectures and examined students for a short time in the Medical colleges in Vratsa and Sofia (Yordanka Filaretova), but the government of Medical University of Sofia expelled him, too.

After his dismissing, without any material support, prof. Michov published his books *Encyclopedia of Molecular Medicine* and the historical book *Red Water*. In the last book he described the struggle of his ancestors against the Turkish slavery. Later the publishing house Walter de Gruyter in Boston and Berlin published the worldwide largest monography *Electrophoresis. Theory and Practice* by prof. Michov.

Encyclopedia of Molecular Medicine, Sofia (2018)

The book *Encyclopedia of Molecular Medicine* is dedicated to the molecular bases of medicine. There is no analogue of it in the world literature. On 930 pages in two columns (all about 2000 A4 pages) *Encyclopedia of Molecular Medicine* represents the latest data and theories of medical biochemistry, molecular pathology and molecular pharmacology. In its beginning, as a helping part, the fundamentals of chemistry are described, too.

The book contains a rich illustrative material, represented by hundreds of chemical formulas and figures in color or in black and white. The *Encyclopedia of Molecular Medicine* contains also a glossary of terms, a subject index, *etc.* It analyzes the molecular mechanisms of inflammatory and allergic diseases, genetic and tumor diseases, of the diseases of nervous system, liver, kidneys, lungs, immune system, blood, and circulatory system.

The publishing house Walter de Gruyter in Boston and Berlin has proposed prof. Michov and signed with him a contract for publishing the same book under the title *Molecular Medicine*. It should appear in three years. The advertising of this book has begun worldwide.

Electrophoresis. Theory and Practice, Walter de Gruyter, Berlin – Boston (2020)

It was a challenge and a hard work by prof. Michov to write the book *Electrophoresis. Theory and Practice* using own works and the great progress in protein and nucleate electrophoresis in the last years. On about 900 pages this book is the largest book on electrophoretic methods all over the world, which serves as a guide for studying DNA, RNA and proteins. It is offered everywhere in the world, by Amazon, too.

Meanwhile Cambridge Scholars Publishing invited and signed with prof. Michov a contract obliged him to write a textbook for the students in Cambridge University. This book, titled *Electrophoresis Fundamentals*, is in press now and will appear soon in Great Britain, USA, Australia, Canada, India, China and other countries to help students in their modern education.

Angel S. Galabov



Chronicle

Awards of Foundation “Acad. Prof. Dr. Stephan Angeloff” for 2020

At its meeting on 15 March 2021, the Stephan Angeloff Foundation considered the nominations for the “Outstanding published paper by a young scientist in 2020” Competition Award of the Foundation. The jury, including the Chairman of the Foundation, Acad. Angel S. Galabov, Corr. Mem. Hristo Naidenski, Prof. Lilia Vassileva (with a written statement, not being present for health reasons), Prof. Maria Angelova, and Prof. Penka Petrova (two absentees: Prof. Nadia Cherepova was abroad, and Prof. Chavdar Vassilev), named two winners: Assist. Prof. **Adelina Stoyanova** PhD, for the article “Effect of Consecutive Alternating Administration of a Triple Combination of Anti-Enteroviral Compounds in Mice Infected with Coxsackievirus B3” (published in *Pathogens and Disease* vol. 78, IF =2.166) and Assist. Prof. **Lyudmila Dimitrova** PhD, for the article “In Vivo Assessment of Acute and Subacute Toxicity of Ethyl Acetate Extract from Aerial Parts of *Geum urbanum* L.” (published in *Biotechnology & Biotechnological Equipment* vol. 35, IF = 1.186).

The jury also awarded prizes to schoolboys Viktor Sotirov (16 years old) and Krassimir Sotirov (13 years old), nominated by Avangard Association, Kyustendil, for their scientific paper “Characteristics of cold-pressed apple juices” presented in the framework of *European Researchers’ Night*, and at the National Youth Forum *Science, Technology, Innovation, Business*.

In memoriam



Milton da Costa (1948-2020)

Milton Simões da Costa was Professor of Microbiology, Department of Life Sciences, University of Coimbra, Portugal. He obtained B.S. at University of Arizona, USA (1968), M.S. in Northern Arizona University, USA (1972) and Ph.D. in Indiana University, USA (1977). Milton was President of the Portuguese Society of Microbiology/Sociedade Portuguesa de Microbiologia (SPM) within 1996-2002. In FEMS, he acted initially as the Delegate for SPM, and in 2003 was elected Vice-President of FEMS. He spent three years as Vice-President, and in 2007 took office as President of FEMS, a position he held until 2010. During his Presidency, Milton worked energetically and enthusiastically to revise and improve the organization of FEMS. After the FEMS period Milton chaired the Microbiology and Biotechnology of the Mediterranean Science Commission (2010-2013) and then became member of the International Committee on Systematics of Prokaryotes (2014-2017).

He was a well-known, much respected and hugely popular figure among European microbiologists for his teaching, research and contribution to the microbiology community. His interest lay in extremophiles, but he was a microbiologist who loved diversity and had almost limitless interests. He published more than 100 papers, contributed to several significant books, and presented at many major conferences. Milton recognized the beauty of studying microbes, whatever they may be and enjoyed both teaching and research.

His work continued until recently, including a Horizon 2020 project working together with industrial and international partners to accelerate the identification of proteins with biotechnological potential from metagenomes. Milton brought interest, wonder and humour to the classroom and was able to engage with all levels of colleagues, students and politicians.

In addition to the above words expressed by FEMS eminent and leading colleagues about Milton's professional path and contribution to microbiology, I would like to say that he was always a very good friend of Bulgarian microbiologists and of Bulgaria. Milton was an active participant in our Congresses of Bulgarian Microbiologists with International Participation, organized by the Bulgarian Society for Microbiology, held in Yundola (2010) and in Tryavna (2014), and in the Balkan Congresses of Microbiology/Microbiologia Balkanica – the Eight Balkan Congress of Microbiology in Veliko Tarnovo, Bulgaria (2013), and the Tenth Balkan Congress of Microbiology in Sofia, Bulgaria (2017).

We will never forget our meetings and discussions with Milton, his wise and fruitful ideas and concrete proposals on a series of important topics in our wonderful science. *Acta Microbiologica Bulgarica* lost a prominent and remarkable member of its Editorial Board.

Angel S. Galabov

In memoriam



Academician Evgeni Golovinski was a world-famous Bulgarian biochemist who made an outstanding contribution to the advancement of bioorganic chemistry. He was born on 18 March 1934 in Burgas, where he graduated from First Men's High School. Then he graduated with an honours degree in Chemistry from the Faculty of Natural Sciences and Mathematics of Sofia University St. Kliment Ohridski, and started working at the Higher Medical Institute in Sofia, where he was hired as a doctoral student and defended his doctoral dissertation. His scientific career was connected with the Institute of Molecular Biology at the Bulgarian Academy of Sciences, where Golovinski headed the Molecular Design and Biochemical Pharmacology Section for more than 20 years, occupied the position of scientific secretary and Deputy Director of the Institute. He specialized at the Pasteur Institute, Paris, at the universities of Münster, Jena and Essen in Germany, as well as at the International Centre for Cancer Research in Lyon, France. He was Doctor of Biological Sciences since 1977 and Professor of Bioorganic Chemistry since 1979, Corresponding Member of the Bulgarian Academy of Sciences since 1989, and Academician since 1997. E. Golovinski was the author of more than 250 research articles, 43 author's certificates, 4 patents in Germany, and numerous monographs and widely popular science books. His research interests were in the field of medical chemistry and pharmacobiochemistry. He introduced a number of new methods in the synthesis of drugs, studied the pharmacobiochemistry of new antitumor drugs and revealed the mechanism of degradation of xenobiotics in the human body, his works being

cited many times at home and abroad. In the field of microbiology, he worked on the synthesis of new substances with antibacterial, antiviral and antifungal activities.

Academician Golovinski was member of the editorial boards of numerous foreign scientific journals and editor-in-chief of the Proceedings of the Bulgarian Academy of Sciences, Nature Journal of the Bulgarian Academy of Sciences, and Chemistry Journal of the Ministry of Education and Science. He was scientific supervisor in the compilation process of the widely popular Encyclopedia Bulgaria, and co-author of five encyclopedias. With his work as Chairman of the Humboldt Union in Bulgaria, Academician Golovinski contributed enormously to the popularization of this world-renowned scientific foundation in Bulgaria, and many Bulgarian scientists were granted Humboldt Research Fellowships for specialization in leading scientific institutes and universities in Germany. Acad. Golovinski was Doctor Honoris Causa of the Southwest University Neofit Rilski, Burgas University Prof. Asen Zlatarov, and the Sofia University St. Kliment Ohridski.

Evgeni Golovinski was bestowed with the First Class State Order of St. St. Cyril and Methodius, the Federal Cross of Merit on Ribbon of the Federal Republic of Germany, St. Kliment Ohridski Medal, Prof. Marin Drinov Medal on Ribbon of the Bulgarian Academy of Sciences in recognition of his substantial scientific and public work. He was awarded honorary citizenship of the cities of Burgas and Blagoevgrad.

A brilliant lecturer and undisputed polymath, Acad. Golovinski was passionate about his work with young scientists and supervised nearly 30 doctoral students. His high school and university textbooks, including the co-authored textbook Experimental Microbiology are still in use by students to this day. For many years, Acad. Golovinski was a lecturer at Sofia University St. Kliment Ohridski and New Bulgarian University, and visiting lecturer at a number of universities abroad. He created a school of scientists in the field of pharmacobiochemistry, many of whom have already become associate professors, professors and corresponding members.

Galina Sachanska

Acta Microbiologica Bulgarica

GUIDE FOR AUTHORS

Types of articles

The journal publishes reviews, original research works, short communications, and letters to the editor from all areas of microbiology. The manuscripts should not represent research results which the authors have already published or submitted to other journals or books. The papers submitted for publication in *Acta Microbiologica Bulgarica* are peer-reviewed by two experts from the respective scientific field who remain anonymous to the authors.

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All papers are published in English, accompanied by a bilingual summary (English and Bulgarian). Manuscripts should be submitted in Times New Roman, size 12, with double-spacing, on a white paper in A4 format, with margins of 3 cm. The length of short communications should not exceed 1800 words. The manuscripts should be submitted in doc or docx format.

1. **Title.** The title of the manuscript should be as short and accurate as possible.

2. **Authors' information.** The submitted manuscript should contain the names and surnames of the authors. Please ensure to include the full names of the authors' affiliations, each on a separate line. The corresponding author should be marked by an asterix (*) and the e-mail address of this author should be included on a separate line.

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Additional guidelines. Letters to the editor are very short unstructured reports, without abstracts and preferably without any figures or tables.

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The references used are cited **in the main text** as follows:

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Weinedborner M. (2001) *Encyclopedia of Food Mycotoxins*. Springer Verlag, pp. 294.

Matthews, K., K. Kniel, Montville, T. (2017). *Food microbiology: An introduction*. 4th Edition, ASM Press.

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- Reference to a dissertation:

Barišić, Z. (2011). Uropatogena *Escherichia coli*: povezanost otpornosti na kinolone s prisutnošću činitelja virulencije [dissertation]. Zagreb: Medicinski Fakultet Sveučilišta u Zagrebu.

• For citing patents, reports, fact sheets, regulations or other official documents, please provide all available information like document official abbreviation or number, year, title, link (if available), etc. A possible example is:

CR 517 (2011). Commission Regulation (EU) No 517/2011 of 25 May 2011 on the reduction of the prevalence of certain salmonella serotypes in laying hens.

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