Sandra de Angelis

EXOPOLYSACCHARIDE PRODUCTION AND ANTIOXIDANT ACTIVITY BY SUBMERGED CO-CULTURE OF MACROMYCETES AND MICROALGAE

CURITIBA – BRAZIL

Sandra de Angelis

EXOPOLYSACCHARIDE PRODUCTION AND ANTIOXIDANT ACTIVITY BY SUBMERGED CO-CULTURE OF MACROMYCETES AND MICROALGAE

Dissertation presented in partial fulfillment of the requirements for the Degree Master of Sciences: Mention Microbiology, Plant Biology and Biotechnologies in the University of Provence, University of the Mediterranean Sea and Federal University of Parana.

Carlos Ricardo Soccol, Ph.D., HDR
Advisor

Miguel Daniel Noseda, Ph.D.
Co-advisor

CURITIBA – BRAZIL

“There is a time for everything,
and a season for every activity under heaven:

a time to be born and a time to die,
a time to plant and a time to uproot,

a time to kill and a time to heal,
a time to tear down and a time to build,

a time to weep and a time to laugh,
a time to mourn and a time to dance,

a time to scatter stones and a time to gather them,
a time to embrace and a time to refrain,

a time to search and a time to give up,
a time to keep and a time to throw away,

a time to tear and a time to mend,
a time to be silent and a time to speak,

a time to love and a time to hate,
a time for war and a time for peace”.

Ecclesiastes 3, 1-8

ACKNOWLEDGEMENTS

This dissertation is the result of two years of work during which I have been accompanied and supported by many people. It is now my great pleasure to take this opportunity to thank them.

First, I would like to express my sincere gratitude to Professor Carlos Ricardo Soccol, who has been my advisor since the beginning of my study. He provided me with many helpful suggestions, important advice and constant encouragement during the course of this work.

I would like to sincerely thank Professor Miguel Noseda on behalf of devoting their precious time and made many valuable suggestions and gave constructive advice during my research work.

I also wish to express my appreciation to Rodiney de Arruda Mauro, for his very kind and generous support, as well as his patience, understanding during the work and for his expertise and criticism in the preparation of this manuscript.

Sincere thanks to Professors Luciana, Maria Eugênia and Julio de Carvalho for their positive encouragement during the experiments and for taking intense academic interest in this study as well as providing valuable suggestions that improved the quality of this study.

Thank Professor Jean Luc Tholozan for the opportunity to participate in this international cooperation program.

I am very grateful for the precious collaboration with microscopy to Milton from the State University of Ponta Grossa and Ismael from the Positivo University.

I would also to thank The Brazilian Agricultural Research Corporation's – Embrapa - for their financial support and specially the Embrapa Forestry for the use of the facilities during my program.

I am also indebted to Gabriela Della Giustina and Edson Kim, my trainees, for their valuable help. Without their help, the field work would not have been accomplished in time.

I am grateful to all my friends from the Laboratory of Bioprocess and from the Laboratory of Biochemistry, for their friendship and support during the time I stayed

there. From the staff, Andréa, Chico, Gabriele, Gerson, Gisele, Joana, Juliane and Luiz Gustavo are especially thanked for their care and attention.

I also thank my parents for their support and understanding. My mother always kept me away from family responsibilities and encouraged me to concentrate on my study.

A special thank you goes to my loving son, Gustavo and for my sister Andrea and niece Camila, for their help, encouragement, and patience during my program.

I would like to express my heartiest thanks and gratitude to all of my friends, Flá, Juli, Maga, Clau, for their kindness, affection and mental support.

Finally, and most importantly, I would like to thank the almighty God for sustaining me with abundant health, for the wisdom and perseverance that he has been bestowed upon me during this research project, and indeed, throughout my life. Thank you my God for being the voice in my head when it was easier to give up. Thank you for the comfort I found in your presence. Thank you for your Love, better and greater than anything else in my life.

ABSTRACT

Microalgal and fungal biomass and algae and fungi derived compounds have a very wide range of potential applications, from animal feed and aquaculture to human nutrition and health products. Some of these microorganisms are considered as rich sources of natural antioxidants and the polysaccharides from them have antitumor and immunomodulatory activity. Microalgae and macromycete may serve as a continuous and reliable source of natural products because they can be cultivated in bioreactors on a large scale. Furthermore, the qualities of the microorganism's cells can be controlled, so that they contain no herbicides and pesticides, or any other toxic substances, by using clean nutrient media for growing.

Agaricus blazei and *Trametes versicolor* are well known polysaccharides productors with many medicinal properties. In the same way, *Spirulina platensis* and *Chlorella vulgaris* have been studied for their biological properties since a long time.

There is a growing interest in finding alternative antioxidant food preservatives. These include natural products that have the potential to provide health benefits such as the reduction of tumor growth and the lowering of serum cholesterol levels. Antioxidant food preservatives such as BHT are commonly used to minimize autoxidation of oil-based food products. Some concerns regarding potential liver toxicity from chronic use of these compounds have been expressed. Microorganisms' metabolites have potential advantages over BHT in that they are natural antioxidants with no known toxicity and thus may also have potential health benefits associated with their consumption.

EPS are characterized as secondary metabolites being produced predominantly during the stationary growth phase of the microorganism. The capacity of basidiomycetes and microalgae to produce extracellular polysaccharides has been related since the beginning of the 1950s. In stress conditions, the microorganisms produce a great amount of EPS, being in that way a metabolic strategy of them for growth and development in unfavorable conditions. They have the purpose of protection against tensions of extreme habitats and harmful conditions.

The aims of this study were to identify new sources of exopolysaccarides and safe antioxidants and to investigate the relationship between the monoculture and

the co-culture under submerged fermentation by basidiomycetes, *A. blazei* and *T. versicolor*, and microalgae, S. *platensis* and *C. vulgaris*.

Keywords: microalgae; macromycetes; exopolysaccharide; antioxidant activity; co-culture.

RESUMO

Biomassa de fungos e algas e seus compostos derivados têm uma gama muito ampla de potenciais aplicações, desde alimentos para animais e aquicultura até alimentação humana e produtos de saúde. Alguns destes micro-organismos são considerados ricas fontes de antioxidantes naturais e seus polissacarídeos extracelulares têm atividade antitumoral e imunomoduladora. Microalgas e macromicetos podem servir como uma fonte contínua e confiável de produtos naturais, pois podem ser cultivados em biorreatores em grande escala. Além disso, a qualidade das células dos micro-organismos pode ser controlada, de modo que eles não contenham herbicidas e pesticidas ou outras substâncias tóxicas, usando nutrientes limpos para o crescimento.

Agaricus blazei e *Trametes versicolor* são conhecidos produtores de polissacarídeos com muitas propriedades medicinais. Da mesma forma, *Spirulina platensis* e *Chlorella vulgaris* tem sido estudadas por suas propriedades biológicas há muito tempo.

Há um crescente interesse em se encontrar alternativas antioxidantes para conservação de alimentos. Incluindo os produtos naturais, que têm o potencial de fornecer benefícios de saúde, como a redução de crescimento de tumores e redução dos níveis de colesterol sérico. Conservantes de alimentos antioxidantes, como o BHT, são comumente usados para minimizar a auto-oxidação de produtos alimentares a base de óleo. Algumas preocupações relativas à toxicidade hepática potencial do uso crônico destes compostos têm sido expressas. Metabolitos de micro-organismos têm vantagens potenciais sobre o BHT, pois são antioxidantes naturais sem nenhuma toxicidade conhecida além de terem os benefícios potenciais à saúde associados ao seu consumo.

EPS são caracterizados como metabólitos secundários produzidos principalmente durante a fase estacionária de crescimento do microorganismo. A capacidade dos basidiomicetos e microalgas produzirem polissacarídeos extracelulares tem sido demonstrada desde o início da década de 1950. Em condições de estresse, os microrganismos produzem uma grande quantidade de EPS, sendo esta uma estratégia metabólica para o crescimento e desenvolvimento em condições desfavoráveis. Eles têm a finalidade de proteção contra tensões de habitats extremos e condições prejudiciais.

Os objetivos deste estudo foram identificar novas fontes seguras de antioxidantes e exopolissacarídeos e investigar a relação entre a monocultura e co-cultura em fermentação submersa pelos macromicetos, *A. blazei e T. versicolor,* e microalgas, *S. platensis* e *C. vulgaris*.

Palavras-chaves: microalgas; macromicetos; exopolissacarídeo; atividade antioxidante; co-cultura.

LIST FIGURE

LIST TABLE

TABLE OF CONTENTS

1 GENERAL INTRODUCTION

1.1 Chapters outline

This study was planned to identify new sources of exopolysaccharides and safe antioxidants and to investigate the relationship between the monoculture and the co-culture under submerged fermentation by macromycetes, *Agaricus blazei* and *Trametes versicolor*, and microalgae, S*pirulina platensis* and *Chlorella vulgaris*. Chapter 1 gives an introduction into the current knowledge with a literature review. Chapter 2 to 5 consists of the parts of our work, which is going to be prepared for submission to be published. Chapter 6 is the conclusion of these studies.

Chapter 2 represents a screening of macromycetes and microalgae looking for strains that can produce a great amount of exopolysaccharide and biomass in submerged culture. The data obtained from this screening were used for selecting strains for further investigations on these studies.

Chapter 3 deals with the production and partial characterization of exopolisaccharides extracted from submerged fermentation by co-culture of basidiomycetes, *A. blazei* and *T. versicolor*, and microalgae, *S. platensis* and *C. vulgaris.* The study focuses the physicochemical properties, composition, and structure of the exopolysaccharides synthesized.

Chapter 4 establishes the submerged co-culture conditions driving to maximal productivities of exopolysaccharides and biomass. Many culture parameters, including source of nitrogen, source of carbon, irradiance, light cycle, temperature, culture age, salinity, airflow and the concentrations of sulfur, phosphorous, potassium were tested for the production of EPS and biomass. In the present study, a two-step optimization strategy of statistical experimental design was employed to identify the culture parameters and find the optimal culture conditions for mycelial biomass and EPS production in submerged co-culture.

Chapter 5 “Antioxidant activities of ethanol extracts from submerged co-culture by basidiomycetes, *A. blazei* and *T. versicolor*, and microalgae, *S. platensis* and *C. vulgaris”.* This study was aimed at determining the antioxidant activity (DPPH free radical-scavenging, β-carotene/linoleic acid systems, reducing power, radical scavenging capacities and inhibition of lipid peroxidation) and total phenolic content.

Chapter 6 discusses the results from chapter 2 to 5 and provide summary conclusions.

1.2 Literature Review

This following literature review provides an overview on the knowledge on the macromycetes, microalgae, their production and consumption relevant to this study. It is important to understand the sinks and source of fungal and algal exopolysaccharides and antioxidant compounds production process. This literature review is used as background information for this entire work.

1.3 Macromycetes

Fungi have played an important role as food, medicine, poison and for religious and other purposes in the life of man since remote times. For centuries, mushrooms have been used as food and for medicinal or functional purposes by various ethnic groups throughout the world, especially in Japan, China and Mexico (MOLITORI, 1994).

Filamentous fungi or mushrooms, especially basidiomycetous fungi, are a popular and valuable food. The crude protein content of cultivated mushrooms is generally high, about 20–44% of dry matter, but the fat content is low, 3–7% of dry matter, (DIKEMAN et al., 2005). Mushrooms are also a good source of minerals, vitamin B2, niacin and folates (MATTILA et al., 2002). Some of them produce compounds with potential pharmacological effects and are called medicinal mushrooms (MAU; LIN; SONG, 2002) and have long been attracting a great deal of interest in many areas of foods and biopharmaceuticals, and are regarded as popular or effective medicines used to treat various human

diseases. The consumption of mushrooms or compounds present in mushroom extracts is suggested to have several health benefits.

Studies have demonstrated that the regular consumption of mushrooms or consumption of isolated bioactive constituents present in mushrooms is benefit to health. Mushrooms may thus be considered as functional food (CHANG, 1989). The list of possible effects of mushrooms which promote good health is long and includes immunity improvement (INOUE; KODOMA; NAMBA, 2002), blood levels cholesterol and lipid reduction (FUKUSHIMA et al., 2002), blood pressure reduction (KABIR; YAMAGUCHI; KIMURA, 1987), blood glucose attenuation (KONNO et al., 2001), among several other actions (LEE et al. 1994; LEE et al. 1996; SONG et al. 1998; KIM et al. 1999; KIM et al. 2001; MAU et al. 2002; KIM et al., 2005). Recent literatures report that more than 270 medicinal fungi are used in traditional chinese medicine for their preventive and/or curative effects (CHENG; LEUNG, 2008).

Mushrooms accumulate a variety of secondary metabolites such as phenolic compounds, polyketides, terpenes and steroids possibly involved in their medicinal effects and functional values (TURKOGLU et al., 2007). It is important to note that the accumulation of these compounds is dependent of management, processing and stage of fruiting body maturity (BARROS et al., 2008, BRAUER; KIMOMONS; PHILLIPS, 2002).

Mushroom cultivation and processing are well established industrial branches, especially in Asiatic and European countries. More than 10 species are cultivated at very large scales there. Mushroom products include fresh or dried fruiting bodies, besides food additives, pharmaceuticals and nutraceuticals, or functional foods (SOCCOL et al., 2006).

Mushrooms or mushroom fibers have hypocholesterolemic effects on rodents and rabbits (BOBEK et al., 1994; BOBEK et al., 1995; BOBEK et al., 1998; BOBEK; GALBAVÝ, 1999; FUKUSHIMA et al., 2000; FUKUSHIMA et al., 2001). In vitro, *Agaricus bisporus* extract can suppress aromatase activity and prevent breast cancer cell proliferation (GRUBE et al., 2001) and lentin present in *Lentinus edodes* decreases the activity of HIV-1 reverse transcriptase and leukemia cell proliferation (NGA, 2003) *Pleurotus ostreatus* has also potential as an antioxidant after liver injury (JAYAKUMAR; RAMESH; GERALDINE, 2006).

1.3.1 Agaricus blazei Murill

Classification: Fungi, Basidiomycota, Agaricomycotina, Basidiomycetes, Agaricomycetes, Agaricales, Agaricaceae, *Agaricus*.

Figure 1 - Photo of *Agaricus blazei* fruiting bodies (A) in natural environment and (B) cultivated

Agaricus blazei Murill, macrofungi belonging to the Basidiomycetes class, is an edible, medicinal mushroom originating from Brazilian subtropical regions. This mushroom was identified as *A. blazei*, known by common names royal sun mushroom, god mushroom, himematsutake, kawarihaaratake, or almond-flavored portobello. It is widely used today in several oriental countries both as an edible mushroom, considered as functional food, and natural therapy in the form of a medicinal extract used mostly for prevention and treatment of cancer (CALPIS, 2001; RIBEIRO; SALVADORI, 2003; AMERICAN DIETETIC ASSOCIATION, 2004). It is used traditionally against a range of diseases. In Brazil it has been cultivated commercially for the health food market, consumed as concentrated extract or tea and popularly used against diabetes, atherosclerosis, hypercholesterolemia, heart disease, cancer and chronic hepatitis (MIZUNO, 1995; MENOLI et al., 2001; BELLINI et al., 2003; GUTERREZ et al., 2004; FIRENZUOLI et al. 2007; HETLAND et al., 2008).

Experiments conducted in Japan with mice verified that *A. blazei* significantly activates the immune system (MIZUNO, 1999). A number of immunity-enhancing, anticancer, and antitumor fractions were isolated from *A.*

blazei. This species was shown to be the most effective anticancer mushroom in a study comparing its effects with shiitake, maitake (*Grifola frondosa)*, reishi, and other medicinal mushrooms (WASSER, 2002).

A. blazei has been demonstrated to be a good source of antioxidant compounds (SOARES, 2009). Barros and co-workers (2008) investigated the antioxidant activity of five *Agaricus sp.* and showed that *A. silvaticus* was the most efficient species presenting the highest ‘‘antioxidant power” in the assays.

Although some works have described that *A. blazei* isolated β-glucan could stimulate the proliferation of lymphocyte T-cells in mice (MIZUNO et al., 1998) and exhibit antitumor and antimicrobial activities in animals (YAN et al., 1999), *A. blazei* medical effects are intensively promoted without any real scientific evidence of clinical benefit for the patients (MENOLI et al., 2001; FIRENZUOLI et al., 2007).

A protein-polysaccharide complex (1→ 6) β-D-glucan has been isolated from crude aqueous extracts of the fruiting body of the Basidiomycete *A. blazei*. This complex was characterized by a growth inhibitory effect on sarcoma-180 implanted in mice, showing immunomodulatory and immuno-stimulating properties (ITOH et al., 1994), possibly due to immunological mechanisms involving the action of various immuno-competent cells. A selective tumoricidal effect of soluble proteoglucan extracted from *A. blazei* Murrill, mediated via natural killer-cell activation and by the induction of apoptosis, was demonstrated by FUJIMIYA and collaborators (1999).

1.3.2 Trametes versicolor Pilát

Classification: Fungi, Basidiomycota, Agaricomycotina, Basidiomycetes, Agaricomycetes, Polyporales, Polyporaceae, *Trametes*.

Figure 2 - Photo of *Trametes versicolor i*n natural environment

The mushroom *Trametes versicolor* is a basidiomycete commonly found year-round on dead logs, stumps, tree trunks, and branches. This macrofungi occurs throughout the wooded temperate zones of Asia, Europe, and America and may be the most common shelf fungus in the Northern Hemisphere (GREGORY; HIRST, 1957; HYDE; ADAMS, 1960).

Many different synonymies have been used in the literature for *T. versicolor*, including *Agaricus versicolor*, *Boletus versicolor*, *Polyporus versicolor*, *Polystictus versicolor*, *Poria versicolor* and *Coriolus versicolor*. *T. versicolor* is also commonly known as *Yun-Zhi* (Chinese), *Kawaratake* (Japanese) and “turkey tail” mushroom.

Researchers have found that this mushroom has antimicrobial, antiviral and anti-tumorproperties (JONG; BIRMINGHAM, 1993; ULRIKE et al., 2005; CHENG; LEUNG, 2008). Nowadays *T. versicolor* is mainly used as an adjuvant in the treatment of cancer (TSANG et al., 2003; HATTORI et al, 2004; LEE et al., 2006). Its extracts show stimulatory effects on the immune system and to inhibit the growth of cancer cells and is called a biological response modifier (LEUNG et al., 2006). In the clinical practice of traditional medicine, *C. versicolor* is recommended for various types of cancers, chronic hepatitis, and infections of the upper respiratory, urinary, and digestive tracts (JONG; YANG, 1999; LI, 2003). *C. versicolor* extract is available as a health supplement.

The protein-bound polysaccharides or polysaccharopeptides produced by *T. versicolor* are effective immunopotentiators, which are used to supplement

the chemotherapy and radiotherapy of cancers and various infectious diseases. Antitumor activity of polysaccharopeptides has been documented. *T. versicolor* polysaccharopeptides PSP/PSK posse anticancer activity (SAKAGAMI et al., 1991; DONG et al., 1996; MAO et al., 1996; ZHOU et al., 2007). Oral administration of PSK/PSP has controlled various carcinomas in experimental animals and humans, suppressing proliferation of some human cancer cell lines (NG, 1999).

Several kinds of protein-bound polysaccharides have been shown to be produced by the white rot fungus, *T. versicolor*. Although some of these polymers are structurally distinct, they are not distinguishable in terms of their physiological activity (RAU et al. 2008).

1.4 Microalgae

Microalgae are organisms containing chlorophyll, which make photosynthesis. A wide part of these organisms is freely found in fresh or marine water, making part of phytoplankton, and is the base of the feeding chain in water ecosystems, being responsible for up to 50% carbon fixing and oxygen production on the planet (OLIVEIRA, 1993).

Microalgae are very efficient solar energy converters and they can produce a great variety of metabolites. Man has always tried to take advantage of these properties through algal mass culture. Despite the fact that many applications for microalgae have been described in the literature, these micro-organisms are still of minor economic importance (RICHMOND, 1990).

Marine microalgae can be used as a source of products of high aggregated value such as pigments, exopolysaccharides, fatty acids and proteins. In addition, microalgae are used in aquaculture as protein and fatty acid source for shrimp culture (VOLKMAN et al. 1989; COUTTEAU et al.1994). However, each strain of microalgae and type of product requires processes designed to increase the production of enriched biomass (FÁBREGAS et al. 1995).

1.4.1 *Chlorella vulgaris* Beijerinck

Classification: Plantae, Chlorophyta, Trebouxiophyceae, Chlorellales, Chlorellaceae, *Chlorella.*

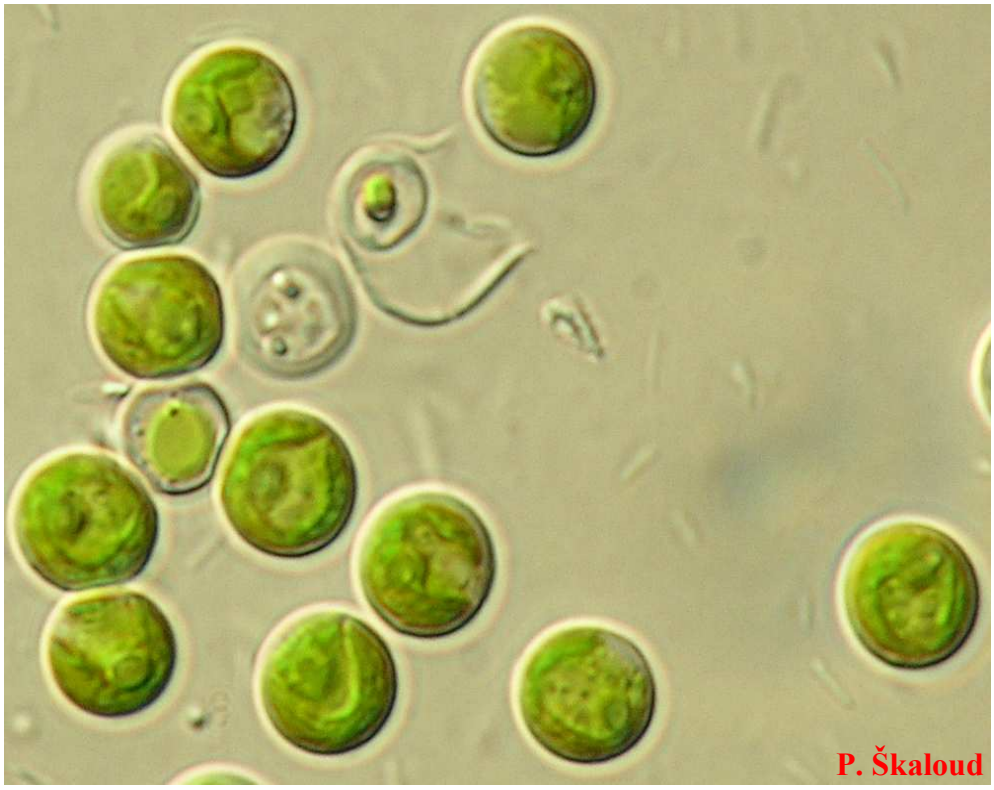


Figure 3 - Photo of *Chlorella vulgaris*

Chlorella is a microscopic single celled freshwater green algae that have the potential to subside the biochemical imbalances induced by various toxins associated with free radicals. The unicellular algae *Chlorella vulgaris* contains many bioactive substances with medical properties.

Experimental studies have demonstrated the medicinal properties of *Chlorella vulgaris* antitumor effect (MORIMOTO et al., 1995), hepatic protective properties, antioxidant properties (VIJAYAVEL et al., 2007) and antibacterial effects (TANAKA et al., 1986). Chemical analysis performed by Hasegawa et al. revealed that Chlorella extract contained 44.3 g protein, 39.5 g carbohydrates and 15.4 g nucleic acids in 100 g (dry weight) whole material. Biochemically, Chlorella contains many dietary antioxidants such as lutein, α-carotene, β-carotene, ascorbic acid and atocopherol (SHIBATA et al. 2003).

Chlorella vulgaris is considered to be a biological response modifier (DAVYDOV; KRIKORIAN, 2000), as demonstrated by its protective activities against viral and bacterial infections, in normal and immunosuppressed mice, (QUEIROZ et al., 2003) and against tumors (JUSTO et al., 2001). A novel glycoprotein obtained from *C. vulgaris* showed antimetastatic immunopotentiation (TANAKA et al., 1998). Of interest are the studies that demonstrate the prophylactic effect of this alga in the models of stress-induced peptic ulcer (TANAKA et al., 1997).

1.4.2 Spirulina platensis

Classification: Plantae, Cyanophyta, Cyanophyceae, Nostocales, Oscillatoriaceae, *Spirulina.*

Figure 4 - Photo of *Spirulina platensis* (10µm)

Spirulina platensis is a photosynthetic, filamentous, spiral-shaped, multicellular, green-blue microalga. For these microorganisms cell division occurs by binary fission. Since this material contains chlorophyll a, like higher

plants, botanists classify it as a microalga belonging to Chyanophyceae class; but according to bacteriologists it is a cyanobacteria due to its prokaryotic structure. It is a gram-negative, photolysis mediated oxygen evolving, cosmopolitan prokaryotes that have survived and flourished on the earth for over two billion years with the creation of oxygenic environment (SERGEEV et al., 2002).

This microalgae can survive in almost every habitat such as from oceans to fresh water, soil to bare rocks, deserts to ice shelves, hot springs to Arctic and Antarctic lakes as well as in the form of endosymbionts in plants, lichens and several protists (THAJUDDIN; SUBRAMANIAN, 2005). In some of these habitats they form dominant microflora in terms of total biomass and productivity. As a result of obstinate survival in assorted habitats the microalgae exhibit a range of secondary metabolites, each with specialized functions to compete successfully on the planet.

The ability of *S. platensis* to synthesize numerous complex secondary metabolites such as peptides, polyketides, polyssacharides and alkaloids etc. has fascinated the researchers for their pharmaceutical and biotechnological exploitations (SPOLAORE et al., 2006).

Owing to their ecological and biochemical diversity, *S. platensis* have been regarded as good candidates for various biotechnological applications and their potential in the conversion of light energy into renewable forms of useful chemicals for food, feed, pharmaceutical and other industries has often been claimed and assessed (DE PHILIPPIS; VINCENZINI, 1998). Extensive studies on the practical exploitation of these microorganisms have been carried out and reviewed. A relatively new field of possible exploitation of *S. platensis* has arisen in the last decade by the growing industrial interest towards polysaccharides of microbial origin, that often show advantages over the polysaccharides extracted from plants or marine macroalgae. As a result, a wide search for microalgal strains able to produce good yields of new polysaccharides with potentially useful properties has been undertaken, also involving *S. platensis* because of the well-known capability of some strains to excrete these polymers.

Before Columbus, Mexicans (Aztecs) exploited this microorganism as human food; presently, African tribes (Kanembu) use it for the same purpose. Its

chemical composition includes proteins (55%-70%), carbohydrates (15%-25%), essential fatty acids (18%) vitamins, minerals and pigments like carotenes, chlorophyll a and phycocyanin. The last one is used in food and cosmetic industries. *S. platensis* grows under highly selective conditions of high pH (about 9), high bicarbonate concentration and high irradiance (CLEMENT et al., 1980).

In 1958, Forrest et al. discovered a fluorescent substance in the cyanobacterium *Anacystis nidulans* and identified it as biopterin glucoside. MATSUNAGA et al. (1993) isolated the same fluorescent compound *from Oscillatoria sp.* and later showed that the cellular content of biopterin glucoside was increased by increasing ultraviolet irradiation (WACHI et al., 1995). In 1994, researchers reported a simple isolation of phycocyanin from *S. platensis* and the interaction of the phycocyanochromophore with protein to clarify the mechanism of color development (KAGEYAMA et al., 1994).

S. platensis has been recognized as producer of exopolysaccharides (MOUHIM et al., 1993) and therefore may take an important place in this field of increasing interest. Beside potential economic aspects, there is interest in the exopolysaccharide of *S. platensis* for its involvement in modifications of physical properties of the culture medium that may be of importance for modeling growth in photobioreactors.

Spirulina spp. is now a well known health food and is cultivated on a large scale appearing on the market in form of tablets.

1.5 Exopolysaccharides (EPS)

Polysaccharides are polymeric carbohydrate structures, formed of repeating units (either mono or di-saccharides) joined together by glycosidic bonds. These structures are often linear, but may contain various degrees of branching. Polysaccharides are often quite heterogeneous, containing slight modifications of the repeating unit. Depending on the structure, these macromolecules can have distinct properties from their monosaccharide building blocks.

Microorganisms synthesize a wide spectrum of multifunctional polysaccharides including intracellular polysaccharides, structural polysaccharides and extracellular polysaccharides or exopolysaccharides (EPS).

EPS are secreted by a microorganism into the surrounding environment. EPS are generally constituted of monosaccharides and some non-carbohydrate substituents (such as acetate, pyruvate, succinate, and phosphate).

EPS are characterized as secondary metabolites being produced predominantly during the stationary growth phase of the microorganism. The capacity of macromycetes and microalgae to produce extracellular polysaccharides has been related since the beginning of the 1950s. In stress conditions, the microorganisms produce a great amount of EPS, being in that way a metabolic strategy of them for growth and development in unfavorable conditions. They have the purpose of protection against tensions of extreme habitats and harmful conditions.

Microbial polysaccharides are attracting increasing interest for their potential applications in the food, cosmetic and pharmaceutical industries, competing with other natural polysaccharides obtained from plants and macroalgae (SANTA, 2006). Among these, micro algal and fungi extracellular polymeric substances (EPS) of polysaccharidic nature present unique biochemical properties that make them interesting from the biotechnological point of view. EPS are compounds whose chemical composition is complex and with peculiar properties. Besides the standard applications of microbial EPS as food coatings, emulsifying and gelling agents, flocculants, hydrating agents etc., the specially nature of micro algal and fungi polysaccharides show physical-chemical properties with advantages interesting for biomedical applications, since polysaccharides have been demonstrated to possess inhibitory properties against various types of viruses and tumors (ITOH et al., 1993).

A variety of compounds including polysaccharide (NARUSE; TAKEDA, 1974; UENO et al.,1980), Coriolan (NARUSE; TAKEDA, 1974), Krestin (PSK) (HIRASE et al., 1970) and polysaccharopeptide (PSP) (YANG et al., 1987) with antitumor activity (JONG; DONOVICK, 1989) have been reported to be present in mushrooms.

Basidiomycetes have been studied extensively for their capacity of degradation. The so-called white rot fungi, which degrade lignin, have this peculiar capacity that leads to research on degradation of xenobiotics. In addition to enzymes, there is evidence that the extracellular polysaccharides produced by these lignocellulolitic fungi play an important role in the process (GUTIÉRREZ, A., 1995). These EPS can immobilize the exocellular enzymes. According to CATLEY (1992), the gel formed by these biopolymers prevents the hyphal dehydration, permits cell adherence to other cells or to surfaces and could possibly select molecules from the environment.

Some EPS from microalgae and mushrooms are able to stimulate the non-specific immune system and to exert antitumor activity through the stimulation of the host's defense mechanism (CHIHARA et al., 1970; MIZUNO, 1999; WASSER; WEIS, 1999; LINDEQUIST et al., 2005; FAN et al., 2005; SANTA, 2006). The drugs activate effectors' cells like macrophages, T lymphocytes and NK cells to secrete cytokines, which are anti proliferative and induce apoptosis and differentiation in tumor cells. Molecular weight, degree of branching, number of substituent, as well as ultra structure, including the presence of single and triple helices, significantly affect the biological activities of EPS (ADACHI et al., 2001). Higher antitumor activity seems to be correlated with higher molecular weight, lower level of branching and greater water solubility of EPS (ZJAWIONY, 2003).

EPS can be a useful adjunct to conventional therapy for cancer and other diseases. They appear to be nontoxic in long term use and to benefit the health.

The majority of biologically active polysaccharides produced by fungi are linear or branched β -1,3 - D- glucans, heteroglycans, or the complexes between β -1,3 – D -glucan and proteins (KULICKE et al., 1997; WASSER; WEIS, 1999; BAO et al., 2002; PAULSEN et al., 2002) .

1.6 Antioxidant Activity

Oxidation is essential to most living organisms for the production of energy to fuel biological processes. However oxidative stress occurs in a biological system after an increased exposure to oxidants, a decrease in the antioxidant

capacity of the system, or both. It is often associated with or leads to the generation of reactive oxygen species (ROS), including free radicals, which are involved in the onset of many diseases such as cancer, rheumatoid arthritis, cirrhosis and arteriosclerosis as well as in degenerative processes associated with ageing. Reactive free radicals may come from endogenous sources through normal physiological and metabolic processes such as mitochondrial respiration. Alternatively, they could result from exogenous sources such as exposure to pollutants and ionizing irradiation, and particularly oxygen derived radicals are capable of oxidizing biomolecules, resulting in cell death and tissue damage (AMES, et al., 1993; CHEVION et al., 2000; HALLIWELL; GUTTERIDGE, 2003). Free radicals are produced in normal and pathological cell metabolism.

Almost all organisms are well protected against free radical damage by oxidative enzymes such as superoxide dismutase and catalase or chemical compounds such as S-tocopherol, ascorbic acid, carotenoids, poliphenol compounds and glutathione (NIKI et al., 1994). However, these systems are many times insufficient to totally prevent the damage, resulting in diseases and accelerated ageing.

Oxidation is also one of the most important processes of food deterioration since it may affect food safety, colour, flavour and texture. Synthetic antioxidants have been used in stabilization of foods. The most commonly used synthetic antioxidants are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tert-butylated hydroxyquinone (TBHQ), that are applied in fat and oily foods to prevent oxidative deterioration (Löliger, 1991). On the other hand, BHA and BHT were found to be anticarcinogenic as well as carcinogenic in experimental animals. Originally, BHA appeared to have tumor-initiating as well as tumor-promoting action. Recently, it has been established that tumor formation appears to involve only tumor promotion caused by BHA and BHT (BOTTERWECK et al., 2000).

The activities of antioxidants depend not only on their structural features (e.g. activities towards peroxyl and other active species), but also on many other factors, such as concentration, temperature, light, type of substrate, physical state of the system, as well as on microcomponents acting as pro-oxidants or synergists (YANISHLIEVA-MASLAROVA, 2001).

Several important factors that may affect the results of antioxidant studies in lipid systems are the initial concentration of primary autoxidation products, such as hydroperoxides of the lipid and free fatty acids. Primary oxidation products may reduce both the efficiency of the antioxidants and the time for radical-mediated consumption of antioxidant (DECKER et al., 2005). Free fatty acids may demonstrate a pro-oxidant effect and should be minimized. Other lipid microcomponents, such as metals, fatty alcohols and mono- and diacylglycerols, have been also shown to decrease the effectiveness of phenolic antioxidants (DECKER et al., 2005).

Natural products with antioxidant activity may be used to help the human body to reduce oxidative damage (HALLIWELL; GUTTERIDGE, 2003). Many fruits, vegetables, herbs, cereals, sprouts, seeds, microalgae and edible mushrooms have been investigated for antioxidant activity in the last years.

The antioxidant activity of plant materials was well correlated with the content of their phenolic compounds (VELIOGLU, et al., 1998). Phenolics are one of the major groups of nonessential dietary components that have been associated with the inhibition of atherosclerosis and cancer (WILLIAMS & IATROPOULOS, 1997). The bioactivity of phenolics may be related to their ability to chelate metals, inhibit lipoxygenase and scavenge free radicals (DECKER, 1997).

1.7 Submerged Fermentation

EPS are extracted from mushrooms or mycelia cultivated on solid substrates (PARK et al., 1997) and mycelial biomass produced in submerged fermentations (WANG et al., 1996b).

In submerged culture, *T. versicolor* and *A. blazei* can be grown as mycelial pellets or predominantly dispersed mycelium. Conditions favoring one morphology over the other are unclear, but hydrodynamic shear forces appear to play a role. Whether pelleted growth or dispersed mycelium is the preferred source of the bioactive polysaccharides has not been established. Mycelial

broths of these mushrooms tend to be highly viscous because of the suspended filamentous biomass and the extracellular dissolved polymers. Broths possess a yield stress (ZHOU et al., 2007).

In nature, the mushrooms grow on solid substrates and not in liquid media. A few reports of solid-state culture for producing the EPS exist. Although the technology for large-scale production of food mushrooms is well established, this type of culture is poorly controlled, labor-intensive, and prone to contamination. Methods for controlled solid-state fermentation have become available, but submerged culture appears to be the preferred option for producing fungal and algal polysaccharides (CUI et al., 2006).

1.8 Research questions and objectives

This study had six main objectives, whereof one represents the central part of this study and the others are considered as supplement. This first, central objective is produce exopolyssacharide and antioxidant compounds under submerged co-culture fermentation of macromycetes, *Agaricus blazei* and *Trametes versicolor*, and microalgae, *Spirulina platensis* and *Chlorella vulgaris*.

The supplementary and specific objectives were:

- Select among the microorganisms the highest EPS and biomass producers.
- Isolate and characterize the EPS by chromatographic and spectroscopic methods.
- Compare EPS produced in mono-cultures and co-culture.
- Optimize the conditions of submerged culture process for the production of EPS and biomass.
- Evaluate *in vitro* the antioxidant activities of filtrates and ethanol extracts.

Major questions of these investigations were:

- Is it viable to cultivate macromycetes and microalgae under submerged fermentation in co-culture?
- Is it viable to produce EPS under submerged fermentation by macromycetes and microalgae in co-culture?
- Are the EPS produced in co-culture different from those produced separately?
- Can the microorganisms produce more efficient antioxidant compounds when they are cultivated together?
- Which are the optimum conditions of submerged co-culture process for EPS production?

2 SCREENING OF MACROMYCETES AND MICROALGAE FOR THE PRODUCTION OF EXOPOLYSACCHARIDE AND BIOMASS IN SUBMERGED CULTURE

2.1 Abstract

Eight strains of macromycetes, isolated from different ecosystems, were screened for production of exopolysaccharides (EPS) and biomass in submerged culture. *Trametes versicolor* (CC 124) and *Agaricus blazei* (LBP 03) were the highest EPS producers (4.13 and 3.44 g dry w/L respectively) after 10 days of incubation. The best producer of biomass was *Trametes versicolor* (CC 124) with 11.66 g dry w/L in 10 days of incubation. When the *T. versicolor* culture filtrate was submitted to freezing prior to polysaccharide precipitation, a gelatinous fraction was formed. Five strains of microalgae were screened for the same purposes. *Spirulina platensis* (PARACAS) was the highest EPS producer, with 0.490 g dry w/L in 20 days of incubation.

Keywords: exopolysaccharide; screening; micromycetes; microalgae.

2.2 Introduction

Macromycetes have been studied extensively for their capacity of degradation. The so-called white rot fungi, which degrade lignin, have this peculiar capacity that leads to research on degradation of xenobiotics. In addition to enzymes, there is evidence that the extracellular polysaccharides produced by these lignocellulolitic fungi play an important role in the process (GUTIÉRREZ, 1995). These EPS can immobilize the exocellular enzymes. According to CATLEY (1992), the gel formed by these biopolymers prevents the hyphal dehydration, permits cell adherence to other cells or to surfaces and could possibly select molecules from the environment.

The capacity of microalgae to produce extracellular polysaccharides has been related since the beginning of the 1950s. In stress conditions, the

microorganisms produce a great amount of EPS, being in that way a metabolic strategy of them for growth and development in unfavorable conditions. They have the purpose of protection against tensions of extreme habitats and harmful conditions.

A practical aspect of the study and characterization of fungal and algal EPS is the availability of data for the investigation of its physiological and ecological importance. In addition, this biopolymer may have potential industrial applications. Another possible application of these biopolymers is in human health. There is intensive research on fungal polysaccharides as antitumor agents.

The fungal and algal biomass can have various uses, which is an advantage as far as the fermentation is concerned because the process residue is reduced. Possible uses for this biomass are food or feed in the form of protein supplement or source of lipids. It can also be used for the extraction of flavors and other metabolites, such as enzymes and polysaccharides.

The aim of this work was to screen the strains of macromycetes and microalgae for EPS and biomass production in submerged culture.

2.3 Materials and methods

Microorganisms. Eight strains of Basidiomycetes and 5 strains of microalgae were screened. The pure cultures came from the Embrapa Bank for Mushrooms - Brasília - Brazil, and from the collection of Bioprocesses and Biotechnology Laboratory (LPB)/Federal University of Parana – Paraná – Brazil, are shown in Table 1. The microalgae strains were obtained from the Biochemical Engineering Laboratory/Federal University Foundation of Rio Grande do Sul – Rio Grande do Sul – Brazil, are shown in Table 2.

Table 1 - Results of the fungi screening for the production of EPS (P_p) and Biomass: (P_x), with the conversion yield of glucose in polymer ($Y_{p/s}$) and in Biomass ($Y_{x/s}$); (P_x) = g dry weight biomass/L culture; (P_p) = g dry weight biopolymer/L culture; ($Y_{x/s}$) = g dry weight biomass/g consumed glucose; ($Y_{x/s}$) = g dry weight biopolymer/g consumed glucose

Code	Strain	(P_x) g dry w/L	($Y_{x/s}$)	(P_p) g dry w/L	($Y_{p/s}$)
CC126	*Ganoderma applanatum*	2.91	0.239	0.21	0.017
CC 57	*Lentinus edodes*	0.99	0.116	0.64	0.075
CC 299	*Lentinus edodes*	0.43	0.090	0.82	0.171
CC 18	*Lentinus edodes*	0.34	0.046	0.75	0.101
CC124	*Trametes versicolor*	11.66	0.798	4.13	0.283
LBP 09	*Pleurotus ostreatus*	4.06	0.366	0.57	0.051
LBP 03	*Agaricus blazei*	9.22	0.515	3.44	0.192
CC144	*Ganoderma lucidum*	0.92	0.137	1.39	0.207

Table 2 - Results of the microalgae screening for the production of EPS (P_p) and Biomass (P_x)

Code	Strain	(P_x) g dry w/L	(P_p) g dry w/L
LEB 106	*Chlorella vulgaris*	2.705	0.289
LEB 108	*Chlorela minutissima*	1.362	0.135
PARACAS	*Spirulina platensis*	1.490	0.490
LEB 52	*Spirulina platensis*	1.352	0.310

Liquid basidiomycetes culture medium. glucose 20 g/L, yeast extract 3 g/L, K2HPO4 0.6 g/L and MgSO4 0.3 g/L; pH 6.0. This medium was selected in preliminary studies as adequate for EPS production by macromycetes (GERN, 2005). Erlenmeyer flasks containing 250 ml of sterilized culture medium were inoculated with the suspension in sterile water of fungal mycelium grown on potato dextrose agar slants. It was the pre-inoculum. Incubation was done at 29°C on shaker at 150 rpm. It was made a scale-up transferring the pre-inoculum to 600mL of medium culture, prepared with the same components and incubated at same conditions.

Microalgae culture medium. The *Spirulina platensis* culture was grown in Zarrouk medium (ZARROUK, 1956) and the *C. vulgaris* culture was grown in modified Bristol's medium – MBM (WATANABE, 1960). Incubation was done in 250 ml Erlenmeyer flasks at 29°C under illumination of white fluorescent light (24 hrs light; 3000 lux). It was made a scale-up transferring the pre-inoculum to 3000mL of medium culture, prepared with the same components and incubated at same conditions.

Screening. For the screening, the incubation times were 10 days for basidiomycetes and 20 days for microalgae. The culture was filtered to separate biomass, which was washed twice with distilled water and quantified as dry weight (105ºC to constant weight). Ethanol was added to the culture filtrate (4:1 v/v) and after 24 h at 4°C the precipitated biopolymer was separated by centrifugation (10.000 rpm for 10 minutes) and also quantified as dry weight.

Glucose assay. The residual glucose content of the culture filtrate was determined with a colorimetric method, according SOMOGYI-NELSON (1954).

2.4 Results

Almost all the strains produced EPS in different quantities (Table 1 and 2). The best yield was produced by *Trametes versicolor*, with 4.13 g dry w/L (conversion yield, Yp/s= 0.283) and *Agaricus blazei* with 3.44 g dry w./L (Yp/s= 0.192) after 10 days of incubation.

There is no relation between biomass and EPS production. The conversion yield of glucose as polymer varied between 0.017 and 0.283 and the best yields were those of *Trametes versicolor* (0.283) and *Ganoderma lucidum* (0.207).

Different strains of *Lentinus edodes* showed different results not only for biomass, but also for polymer production. These data confirm the diversity of EPS production among different strains in submerged culture.

An observation was made concerning the formation of an insoluble gel when the *Trametes versicolor* culture filtrate was frozen prior to polysaccharide precipitation (Figure 5).

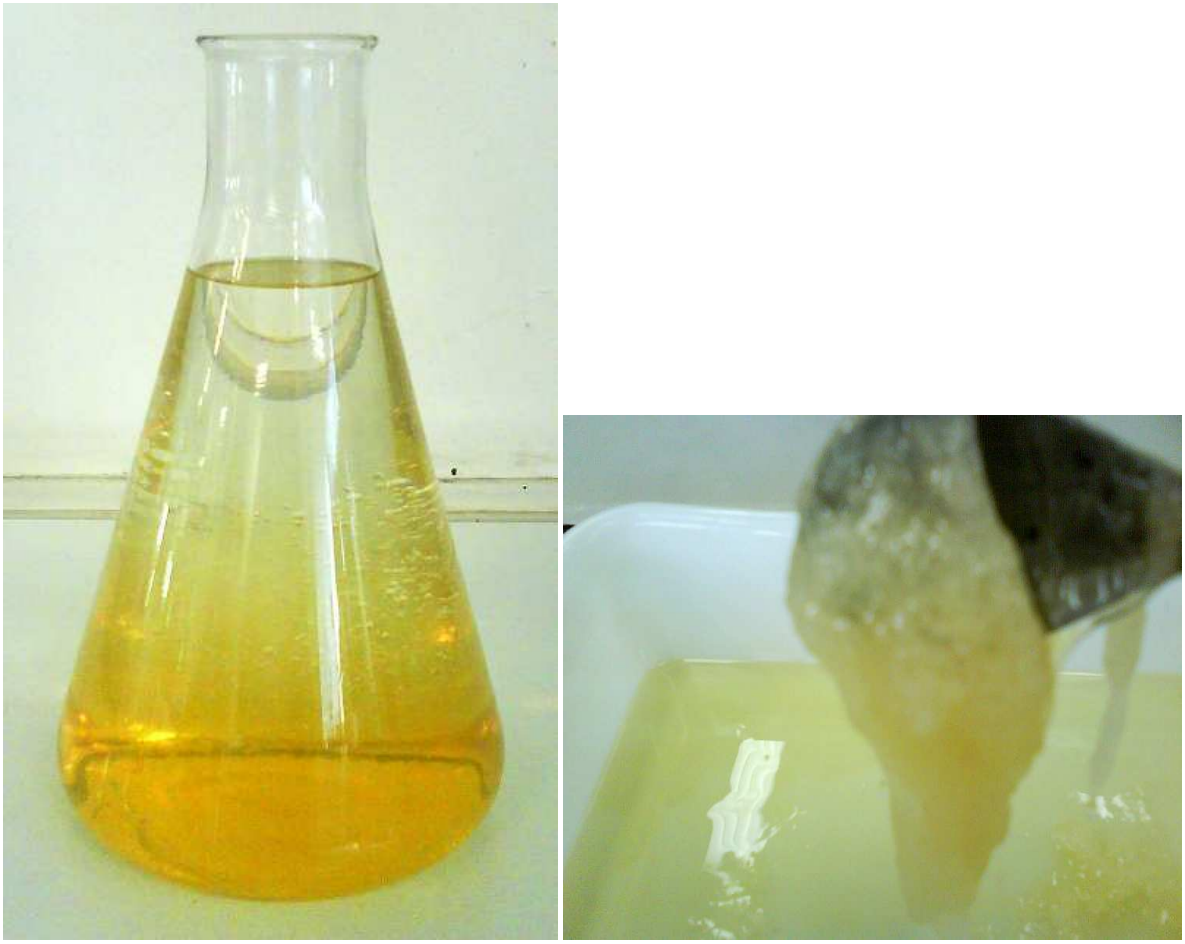


Figure 5 - *Trametes versicolor* EPS precipitation

Biomass production ranged from 0.34 to 11.66 g /L. Some strains, such as *L. edodes* and *G. lucidum*, had a slow growth rate in these culture conditions. Others, such as *Trametes versicolor* produced more than 10 g/L of biomass. The conditions used for the submerged culture could be considered adequate for biomass production.

During estimation of polymer and biomass produced it is important to consider that EPS adherent to the hyphae are also entrapped into the pellets

formed during the submerged culture, which means that the dry weight of biopolymer which precipitated from the culture filtrate does not correspond to the total EPS and that the biomass can be overestimated. To minimize this problem biomass was washed twice with distilled water.

During the screening it was observed that the submerged cultures showed different characteristics according to the fungal species. The pellets formed can be regular or irregular in form and size. The form varies from spherical to cylindrical and the size from 1 to 20 mm. In some cases the formation of pellets was not observed, but rather a mycelial agglomeration without a defined form.

The pellets were smooth, hairy (with looser outer zones) or with fringes of aggregated hyphae that give the pellet a star form. The color and consistency were also different, as well as the flavour. The culture filtrate was very clear for every strain, and in some cases, like for *A. blazei*, it was very viscous.

When there is a depletion of glucose in the medium it was observed that pellets and the medium begin to become darker. The dead hyphae are decomposed and the resulting substances are reabsorbed by the mycelium.

2.5 Conclusion

This work showed that the macromycetes and microalgae strains screened are EPS producers. The possibility of using these biopolymers for medical application promises a large opportunity to improve the study of such group of fungi.

3 OPTIMIZATION OF SUBMERGED CO-CULTURE PROCESS FOR THE PRODUCTION OF EXOPOLYSACCHARIDES BY MACROMYCETES, *Agaricus blazei* AND *Trametes versicolor*, AND MICROALGAE, *Spirulina platensis* AND *Chlorella vulgaris*

3.1 Abstract

The objective of the present study was to determine the submerged co-culture optimum conditions for extra cellular polysaccharides (EPS) and biomass production by basidiomycetes, *A. blazei* and *T. versicolor*, and microalgae, *S. platensis* and *C. vulgaris.* Culture temperature, agitation speed and initial pH were identified to have significant effects on exopolysaccharide (EPS) production by a Plackett–Burman design. The optimal glucose concentration for EPS production in the most experiments was 52 g/L, corresponding optimal initial pHs were found to be 5.0. For the fermentation with *A. blazei* and *C. vulgaris,* under the optimized culture conditions and without a pH controlled, the maximum concentration of biomass and EPS were 11.67 g/L and 5.4 g/L, respectively. The microorganisms themselves are kept apart, indeed, the contact with different microorganisms grown in a mixture was showed that is desired in this case and we verified that it can improve the EPS production and reduce the fermentation time.

Keywords: exopolysaccharide; co-culture; experimental designs; Plackett–Burman design; Box-Behnken design.

3.2 Introduction

Co-culture systems containing two microorganisms for the production of useful substances are described. We developed a co-culture system and established the parameters to take the co-culture viable. The proposed co-culture system allowed increasing the EPS production when we compare with the EPS produced by each microorganism isolated. The results obtained showed that under the co-culture conditions established, using a combination of macro fungus

and microalgae, the amount of EPS produced was enhanced, even when the microorganism alone was cultivated.

The co-culture put the microorganisms in stress conditions, and they produce a great amount of EPS, being in that way a metabolic strategy of them for growth and development in these system unfavorable conditions.

It is well-known that optimization of cultivation medium is an important approach for enhancement of the desired product; hence groups of statistical experimental designs were applied to the process of medium optimization.

Many culture parameters, including source of nitrogen, source of carbon, irradiance, light cycle, temperature, culture age, salinity, airflow and the concentrations of sulfur, phosphorous, potassium affect the production of EPS. In a co-culture we can observe the influence of many other parameters, beyond the individual culture conditions can affect or sometimes inhibit the growth of the other microorganism.

The development of biotechnological applications of EPS depends on the identification of culture parameters that control the synthesis of EPS and the establishment of culture conditions driving to maximal productivities.

Statistical experimental design has been widely used in many areas of science and industry. This methodology can be adopted on several stEPS of an optimization strategy such as for screening experiments or searching for the optimal conditions of a targeted response (ZHANG et al., 2006). Considering that the EPS produced can potentially be used commercially, we conducted the optimization procedure in flask cultures, using factorial design of experiments, for high EPS and biomass production.

In the present study, a initial optimization strategy of statistical experimental design was employed to identify the culture parameters and find the culture conditions for mycelial biomass and EPS production by basidiomycetes, *A. blazei* and *T. versicolor*, and microalgae, *S. platensis* and *C. vulgaris* in submerged co-culture and the critical effect of culture pH is described.

A screening test by Plackett–Burman design (PLACKETT; BURMAN, 1946) was used to evaluate the impact of seven fermentation factors: pH; aeration; illumination; fungal inoculums rate; microalgae inoculums rate. Because our previous assays, the seven factors that may affect the exopolysaccaride

production were selected. A two level full factorial design will require 27 = 128 runs. It will be time-consuming and costly. Therefore, an eight run Plackett-Burman experiment will be conducted. The results revealed which factors have a significant influence on biomass and exopolysaccarides production.

In recent years, tools like Box-Behnken design have been frequently applied to the optimization of analytical methods, considering its advantages such as a reduction in the number of experiments that need be executed resulting in lower reagent consumption and considerably less laboratory work. Furthermore this method allow the development of mathematical models that permit assessment of the relevance as well as statistical significance of the factor effects being studied as well as evaluate the interaction effects between the factors (BOX et al., 2005; BRUNS et al., 2006).

The first step of multivariate optimization is accomplished screening the factors studied (in this case, Plackett- Burman design), in order to obtain the significant effects of the analytical system. After determining the significant factors, the optimum operation conditions are attained by using more complex experimental designs such as the Box-Behnken design (BBD), a three-level design.

According FERREIRA and co-workers (2007), the Box-Behnken is a good design for response surface methodology because it permits: (i) estimation of the parameters of the quadratic model; (ii) building of sequential designs; (iii) detection of lack of fit of the model; and (iv) use of blocks. The authors showed a comparison between the Box-Behnken design and other response surface designs (central composite, Doehlert matrix and three-level full factorial design) and demonstrated that the Box-Behnken design are slightly more efficient than the central composite design but much more efficient than the three-level full factorial designs.

3.3 Materials and methods

3.3.1 Microorganism and media

The microorganisms used in this study were: the macrofungi: *Trametes versicolor* and *Agaricus blazei*; and the microalgae: *Spirulina platensis and Chlorella vulgaris.*

The co-culture systems studied were: *A. blazei* with *T. versicolor*, *C. vulgaris* with *S. platensis*, *A. blazei* with *C. vulgaris*, *A. blazei* with *S. platensis*, *T. versicolor* with *C. vulgaris* and *T. versicolor* with *S. platensis.*

Macro fungi culture – A culture of *A. blazei,* LPB 03 strain, was taken from the culture collection of Bioprocesses and Biotechnology Laboratory (LPB) at Federal University of Parana (UFPR), isolated by F. Leifa, , Ph.D. The strain of *T. versicolor*, CC 124 strain, from the collection of Embrapa Bank for Mushrooms, which was isolated by Embrapa's researcher, A. F. Urban, Ph.D. The stocks cultures were maintained on potato dextrose agar (PDA) slant. Slants were incubated at 25°C for 7 days and then stored at 4°C. Sub-cultures were made at each three months to maintain the strain active. Inoculum preparation and flask cultures was initially grown on PDA medium in a Petri dish, and then transferred to the seed culture medium by punching out 5 mm of the agar plate culture with a sterilized self-designed cutter (BAE et al. 2000). The seed culture was grown in a 250 ml flask containing 150 ml of medium (pH 7.0) prepared with glucose 20 g/L, yeast extract 3 g/L, K2HPO4 0.6 g/L and MgSO4 0.3 g/L at 29 ± 2 °C on a rotary shaker incubator at 150 rpm for 10d. The flask culture experiments were performed in a 250 mL flask containing 150 mL of the media after inoculating with 4% (v/v) of the seed culture (GERN, 2005).

Microalgae culture – The PARACAS strain of *Spirulina platensis* and LEB 12 strain of *Chlorella vulgaris* were obtained from the Biochemical Engineering Laboratory - Federal University Foundation of Rio Grande do Sul/FURG. The *S. platensis* culture was grown in Zarrouk medium (Zarrouk, 1956). *C. vulgaris* culture was grown in Modified Bristol Medium (MBM) (Watanabe, 1960). The strains were pre-cultivated for several days in 70 ml respective media in 250 ml Erlenmeyer flasks at 29 ± 2 °C with continuous aeration under illumination of

white fluorescent light (24 hrs light; 3000 lux), and then were harvested by centrifugation (2800 rpm). The cells were inoculated in a 5000 ml Erlenmeyer flask containing 2000 ml of medium and used for the studies.

3.3.2 Fermentations runs

All batch fermentations for Plackett-Burman design were carried out in Erlenmeyer flasks (250 ml) with different volumes of fermentation medium. The flasks, after autoclaving at 115 °C for 15 min and cooling to room temperature (25 °C), were inoculated and kept according to Table 3.

Bioreactor fermentations - Submerged cultivation will carry out. The fermentation medium will be inoculated with the seed culture and then cultivated in a 10 L stirred-tank fermenter (New Brunswick Scientific Bioreactor) equipped with a pH, temperature, agitation speed, aeration measure and control unit.

3.3.3 Analytical methods

Samples collected at various intervals from shake flask were filtered by filter-paper (Whatman 1). The resulting culture filtrate was concentrated to ¼ of the original volume by vacuum oven below 50 ºC. The filtrated was dialyzed with a using a 12–14 kDa cut-off Ester Membrane and mixed with four times its volume of absolute ethanol, stirred vigorously and left overnight at 4°C. The precipitated EPS was centrifuged at 10000 g for 10 min, discarding the supernatant. The precipitate of pure EPS was lyophilized and the weight of the polymer was estimated. Dry weight of mycelium was measured after repeated washing of the mycelial pellet with distilled water and drying overnight at 70°C to a constant weight.

Sugar content – The EPS quantification was done by phenol-sulfuric method (DUBOIS, 1956), and the quantification of residual sugar (glucose) was carried out according SOMOGYI-NELSON (1954). The reading of the absorbance for both methods was performed in spectrophotometer (Power Wave XS, BioTek).

Proteins content – The quantification of protein contents was performed according to LOWRY et al. (1951). The determination of absorbance was also carried out in spectrophotometer.

3.3.4 Experimental design and statistical analysis

The Plackett-Burman experimental design was used to evaluate the relative importance of the variables on EPS production by the strains in a co-culture.

Plackett–Burman design is a very useful tool for screening ‘n’ variables (with one dummy variable) in just ‘n + 1’ tests. This design is very practical, especially when the investigator is faced with a large number of factors and is unsure which settings are likely to be close to optimum responses. In this study, it was used to identify the major fermentation parameters that affect the biomass and EPS production.

The experimental design is shown in Table 3 and the design matrix is developed by using the software Statistica 7.0 (StatSoft Inc., Tulsa, OK, USA). Each variable is represented in two levels, i.e., a high level and a low level. All experiments were carried out in triplicate. The Pareto chart displaying the magnitude of each factor estimate was generated by using the same software.

Table 3 - Randomized Plackett–Burman experimental design for evaluating factors influencing biomass and EPS production by co-cultures

Run	**Variables/levels**						
	Culture temperature (°C)	**Agitation speed (rpm)**	**Fungal inoculation rate(v/v)(%)**	**Microalgae inoculation rate (v/v)(%)**	**Initial pH**	**Ilumination rate (lux)**	**Glucose concentration (g/L)**
1	25	120	15	50	4.5	50	50
2	30	120	5	10	7.0	50	50
3	25	180	15	50	7.0	50	10
4	30	180	5	10	4.5	50	10
5	25	120	15	50	7.0	1000	10
6	30	120	5	10	4.5	1000	10
7	25	180	15	50	4.5	1000	50
8	30	180	5	10	70	1000	50

Table 4 - Box–Behnken design matrix along with the experimental values for evaluating factors influencing biomass and polysaccharide production by *A. blazei* and *C. vulgaris*. *Data based on two trials, relative standard derivation of biomass is 0.7% and extracellular polysaccharide is 3,2%

Run	Variables/levels						Responses*	
	Glucose Concentration		Agitation speed		Initial pH		Biomass (g/L)	Extracellular Polysaccharide (mg/L)
	VAR_1(g/L)	Code var_1	VAR_2(RPM)	Code var_2	VAR_3	Code var_3		
1	45	0	140	-1	4.5	-1	6.81	2.09
2	55	0	140	0	4.5	0	11.45	4.18
3	45	1	220	1	4.5	0	9.00	3.65
4	55	1	220	-1	4.5	0	9.40	3.17
5	45	1	180	0	3.5	-1	7.33	2.13
6	55	-1	180	1	3.5	0	8.49	2.99
7	45	0	180	-1	5.5	1	8.69	2.15
8	55	0	180	1	5.5	-1	7.38	2.13
9	50	0	140	0	3.5	0	11.38	4.15
10	50	-1	220	0	3.5	1	9.52	3.53
11	50	-1	140	-1	5.5	0	9.03	3.17
12	50	0	220	0	5.5	0	11.60	4.11
13	50	0	180	1	4.5	1	11.13	4.08
14	50	-1	180	0	4.5	-1	7.12	2.26
15	50	1	180	0	4,5	1	10,71	4,01

A total of 15 runs were used to optimize the range and levels of chosen variables. The Box–Behnken design was carried out using the software Statistica 7.0 (StatSoft Inc., Tulsa, OK, USA), presented in Table 4. Each run was performed in duplicate and thus the values given in Table 5 were averages of two sets of experiments.

The software Statistica, version 7.0, was used for obtaining the response surface plots.

3.4 Results

3.4.1 Plackett-Burman Design

Considering the practicability and controllability, seven variables (culture temperature, agitation speed, fungal and microalgae inoculation density, initial pH

illumination rate and glucose concentration) were chosen to be optimized. In the first approach, the Plackett–Burman design was applied to reflect the relative importance of the above seven variables. The main effect of each variable upon response was estimated as the difference between both averages of measurements made at the high level and at the low level of that factor. The responses in Tables 5 - 9 show a wide variation, which reflects the importance of parameter optimization to reach higher productivity.

The Pareto chart displays the effect of each factor on the response and it is a convenient way to view the results of a Plackett–Burman design.

As can be seen, VAR1 (culture temperature) did not show a significant effect, just negatively influencing the biomass production by the *A. blazei* and *S. platensis* and EPS production by *T. versicolor* and *C. vulgaris.* VAR4 (microalgal inoculation density) exhibited negative influence, while VAR2 (agitation speed), VAR3 (fungal inoculation density) and VAR7 (glucose concentration) showed positive effect, on all responses. In addition, VAR6 (illumination rate) showed positive effect on all responses except for *A. blazei* and *T. versicolor* co-culture, while VAR5 (initial pH) showed negative effect on all responses except for *A. blazei* and *T. versicolor* co-culture too. (Fig. 6-10).

Table 5 - Randomized Plackett–Burman experimental design for evaluating factors influencing biomass and polysaccharide production by *A. blazei* and *C. vulgaris.* *Data based on three trials, relative standard derivation of biomass is 0,7% and extracellular polysaccharide is 3,2%.

Run	Variables/levels							Responses*	
	Culture temperature (°C)	Agitation speed (rpm)	Fungal inoculation rate(v/v)(%)	Microalgal inoculation rate (v/v)(%)	Initial pH	Ilumination rate (lux)	Glucose concentration (g/L)	Biomass (g/L)	Extracellular Polysaccharide (mg/L)
1	25	120	15	50	4.5	50	50	6.14	2.48
2	30	120	5	10	7.0	50	50	5.88	2.33
3	25	180	15	50	7.0	50	10	4.12	1.42
4	30	180	5	10	4.5	50	10	5.33	1.62
5	25	120	15	50	7.0	1000	10	6.68	2.08
6	30	120	5	10	4.5	1000	10	6.32	2.31
7	25	180	15	50	4.5	1000	50	9.43	4.00
8	30	180	5	10	7.0	1000	50	9.10	3.79

Table 6 - Randomized Plackett–Burman experimental design for evaluating factors influencing biomass and polysaccharide production by *A. blazei* and *S.platensis.* *Data based on three trials, relative standard derivation of biomass is 2.3% and extracellular polysaccharide is 7.3%

Run	Variables/levels							Responses*	
	Culture temperature (°C)	Agitation speed (rpm)	Fungal inoculation rate(v/v)(%)	Microalgal inoculation rate (v/v)(%)	Initial pH	Ilumination rate (lux)	Glucose concentration (g/L)	Biomass (g/L)	Extracellular Polysaccharide (mg/L)
	25	120	15	50	4.5	50	50	6.19	2.05
2	30	120	5	10	7.0	50	50	5.52	1.60
3	25	180	15	50	7.0	50	10	2.53	0.92
4	30	180	5	10	4.5	50	10	3.29	1.62
5	25	120	15	50	7.0	1000	10	4.20	1.66
6	30	120	5	10	4.5	1000	10	3.11	1.61
7	25	180	15	50	4.5	1000	50	9.28	3.73
8	30	180	5	10	7.0	1000	50	8.98	3.52

Table 7 - Randomized Plackett–Burman experimental design for evaluating factors influencing biomass and polysaccharide production by *T.versicolor* and *C. vulgaris*

*Data based on three trials, relative standard derivation of biomass is 1.9% and extracellular polysaccharide is 3.7%.

	Culture temperature (°C)	Agitation speed (rpm)	Fungal inoculation rate(v/v)(%)	Microalgal inoculation rate (v/v)(%)	Initial pH	Ilumination rate (lux)	Glucose concentration (g/L)	Biomass (g/L)	Extracellular Polysaccharide (mg/L)
	25	120	15	50	4.5	50	50	4.49	3.28
2	30	120	5	10	7.0	50	50	3.99	2.62
3	25	180	15	50	7.0	50	10	2.77	1.95
4	30	180	5	10	4.5	50	10	3.33	2.41
5	25	120	15	50	7.0	1000	10	3.61	2.98
6	30	120	5	10	4.5	1000	10	3.56	2.20
7	25	180	15	50	4.5	1000	50	5.19	4.25
8	30	180	5	10	7.0	1000	50	4.96	4.11

Table 8 - Randomized Plackett–Burman experimental design for evaluating factors influencing biomass and polysaccharide production by *T.versicolor* and *S.platensis.*
*Data based on three trials, relative standard derivation of biomass is 4.5% and extracellular polysaccharide is 5.7%

Run	Variables/levels							Responses*	
	Culture temperature (°C)	Agitation speed (rpm)	Fungal inoculation rate(v/v)(%)	Microalgae inoculation rate (v/v)(%)	Initial pH	Ilumination rate (lux)	Glucose concentration (g/L)	Biomass (g/L)	Extracellular Polysaccharide (mg/L)
1	25	120	15	50	4.5	50	50	6.74	2.62
2	30	120	5	10	7.0	50	50	6.54	2.20
3	25	180	15	50	7.0	50	10	3.83	1.67
4	30	180	5	10	4.5	50	10	4.80	1.92
5	25	120	15	50	7.0	1000	10	4.68	2.02
6	30	120	5	10	4.5	1000	10	4.32	1.99
7	25	180	15	50	4.5	1000	50	7.64	3.84
8	30	180	5	10	7.0	1000	50	7.28	3.31

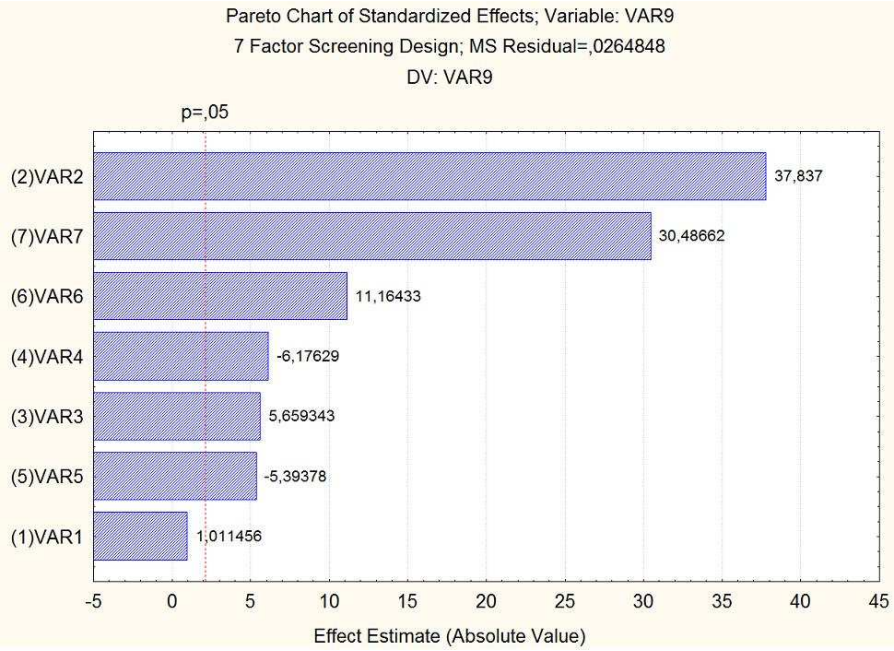
Table 9 . Randomized Plackett–Burman experimental design for evaluating factors influencing biomass and polysaccharide production by *A. blazei* and *T.versicolor*
*Data based on three trials, relative standard derivation of biomass is 4.5% and extracellular polysaccharide is 5.7%

Run	Variables/levels							Responses*	
	Culture temperature (°C)	Agitation speed (rpm)	Fungal A inoculation rate(v/v)(%)	Fungal B inoculation rate (v/v)(%)	Initial pH	Ilumination rate (lux)	Glucose concentration (g/L)	Biomass (g/L)	Extracellular Polysaccharide (g/L)
	25	120	15	50	4.5	50	50	8.51	4.32
2	30	120	5	10	7.0	50	50	8.94	4.69
3	25	180	15	50	7.0	50	10	8.20	3.62
4	30	180	5	10	4.5	50	10	7.74	3.55
5	25	120	15	50	7.0	1000	10	6.90	2.19
6	30	120	5	10	4.5	1000	10	6.18	1.86
7	25	180	15	50	4.5	1000	50	9.56	5.40
8	30	180	5	10	7.0	1000	50	10.04	5.73

Some researchers chose the most significant 3 or 5 factors affecting the response as major factors for further optimization (COCKSHOTT; SULLIVAN, 2001; CHEN, 2005 and YASSER, 2005). Others, for example, thought that the variables with confidence level above 80% or 85% (PUJARI; CHANDRA, 2000) were significant. In the present study the variables with confidence level greater

than 95% were considered as major factors. VAR1 (culture temperature), VAR4 (microalgal inoculation density) and VAR3 (fungal inoculation density) had confidence low levels and hence, were considered statistically insignificant on the most part of responses (Fig. 1-5). On the other hand, VAR 7(glucose concentration), VAR2 (agitation speed) and VAR6 (illumination rate) had confidence levels above 95% and were considered statistically significant in the production of biomass and EPS. Moreover, VAR7 and VAR2 were found to be most significant variables affecting biomass and EPS production when just macromycetes are cultivated together (Fig.5). Although VAR 7 (glucose concentration) hardly influenced EPS production and VAR 1 (culture temperature) affected EPS production insignificantly. We selected the co-culture of *Agaricus blazei* and *Chlorella vulgaris*, with VAR 7 (glucose concentration), VAR2 (agitation speed) and VAR5 (initial pH) for further optimization in the next stage considering their important effect on the other responses. VAR6 was fixed in 3000 lux, despite of the positive influence, because of the equipments limitation. Other variables with less significant effect were not included in the next optimization experiment, but instead were used in all trials at their low level in consideration of lowering cost.

A



B

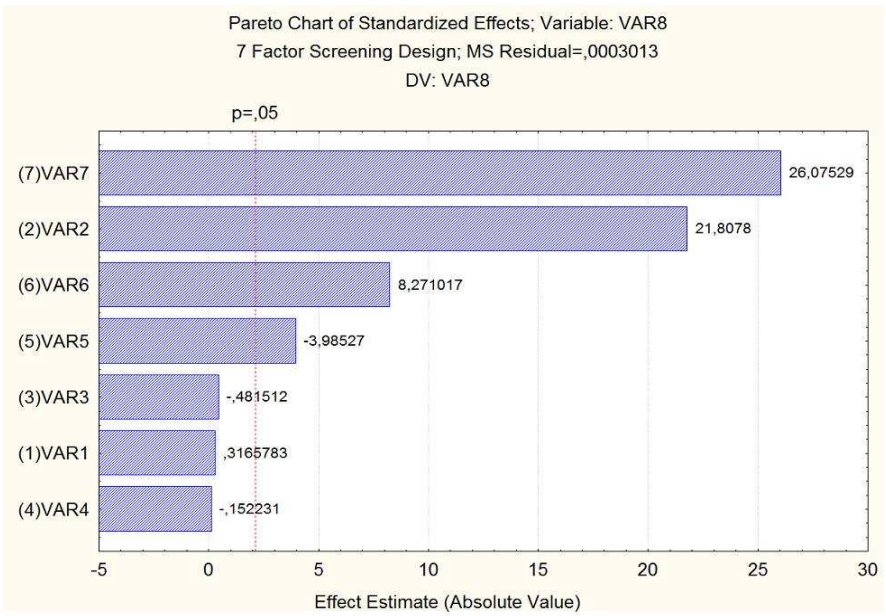
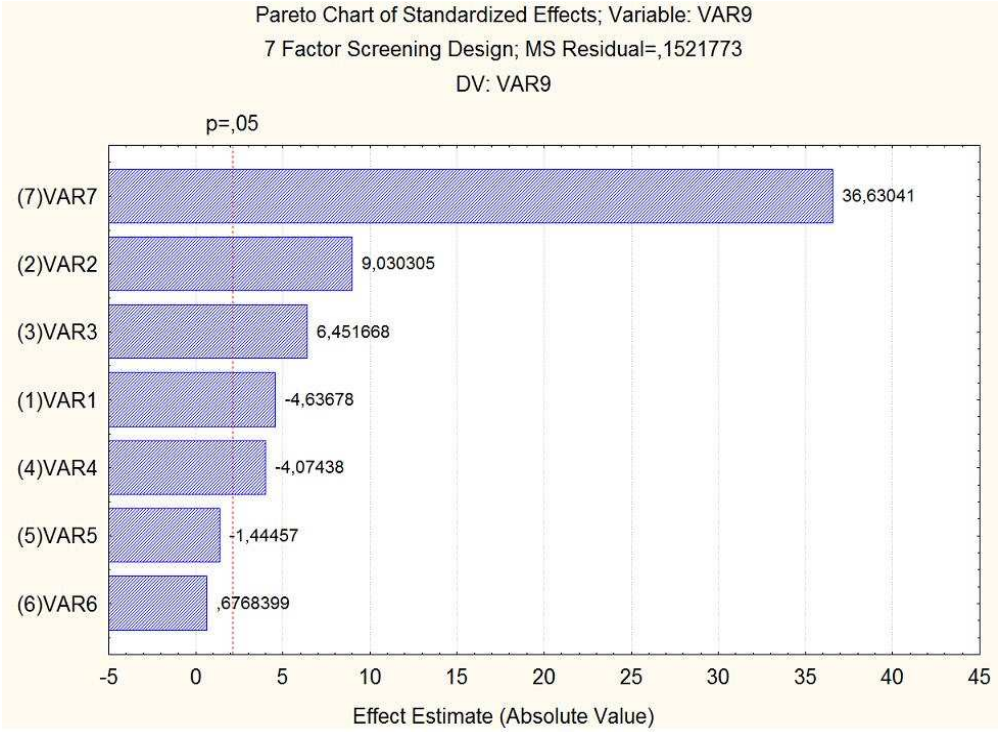


Figure 6 - Pareto chart rationalizing the effect of each variable on the production of biomass (A) and extracellular polysaccharide (B) by *Agaricus blazei* and *Chlorella vulgaris*. The vertical line indicates confidence level of 95% for the effects. VAR1–VAR7 indicates culture temperature, agitation speed, microalgal inoculation density, fungal inoculation density, initial pH, illumination, rate and glucose concentration, respectively

A



B

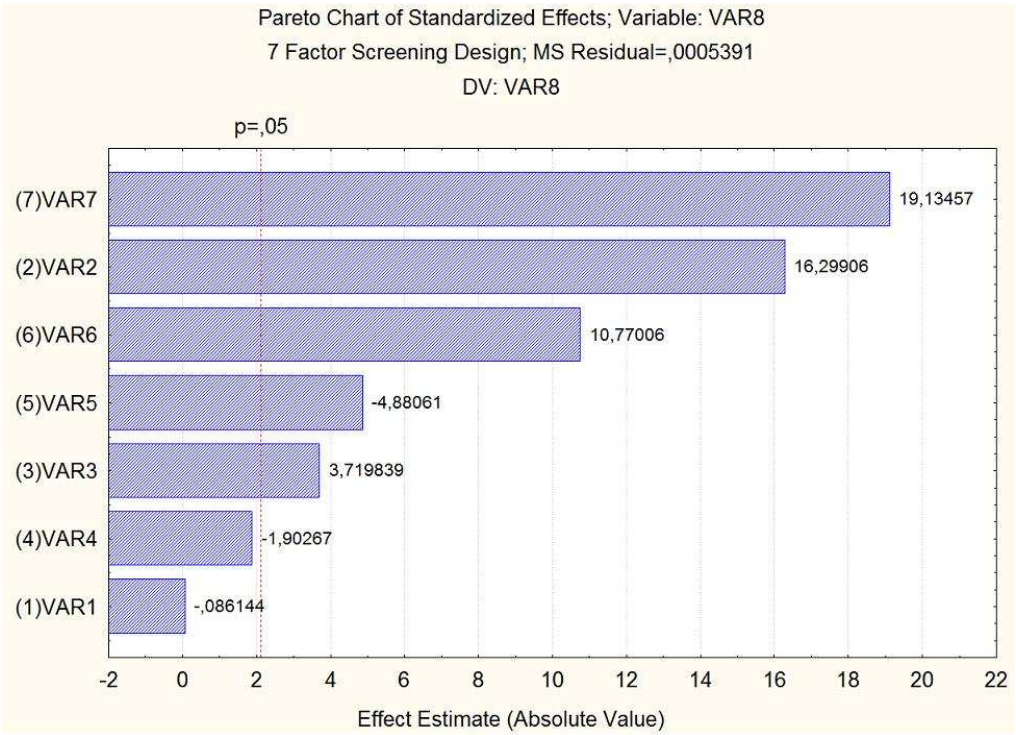
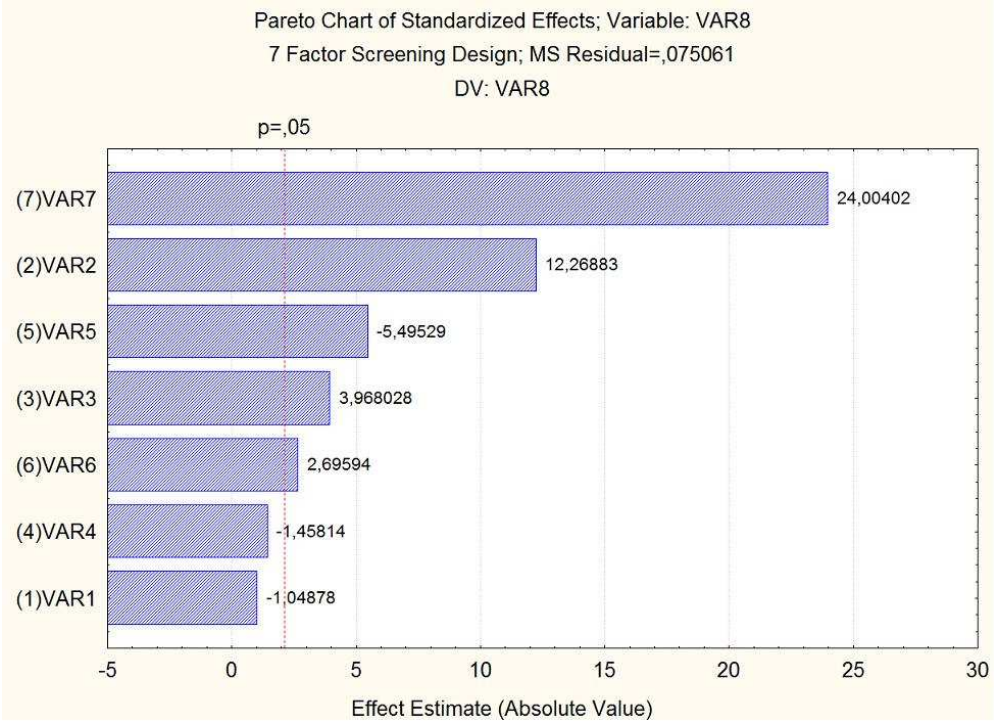


Figure 7 - Pareto chart rationalizing the effect of each variable on the production of biomass (A) and extracellular polysaccharide (B) by *Agaricus blazei* and *Spirulina platensis*. The vertical line indicates confidence level of 95% for the effects. VAR1–VAR7 indicates culture temperature, agitation speed, microalgal inoculation density, fungal inoculation density, initial pH, illumination rate and glucose concentration, respectively.

A



B

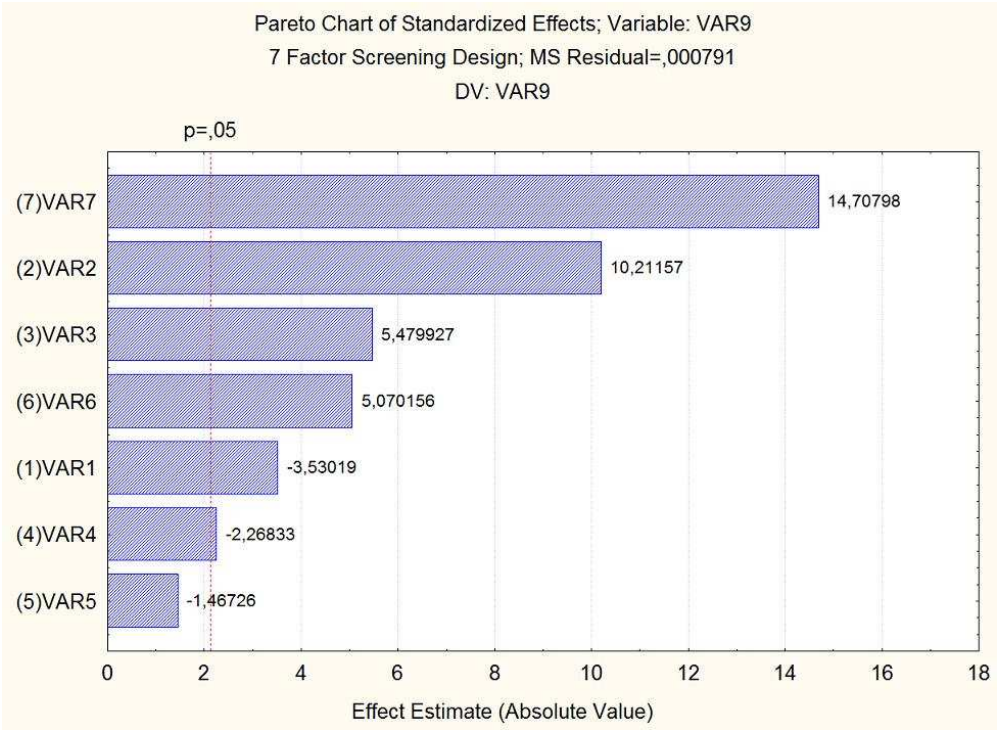
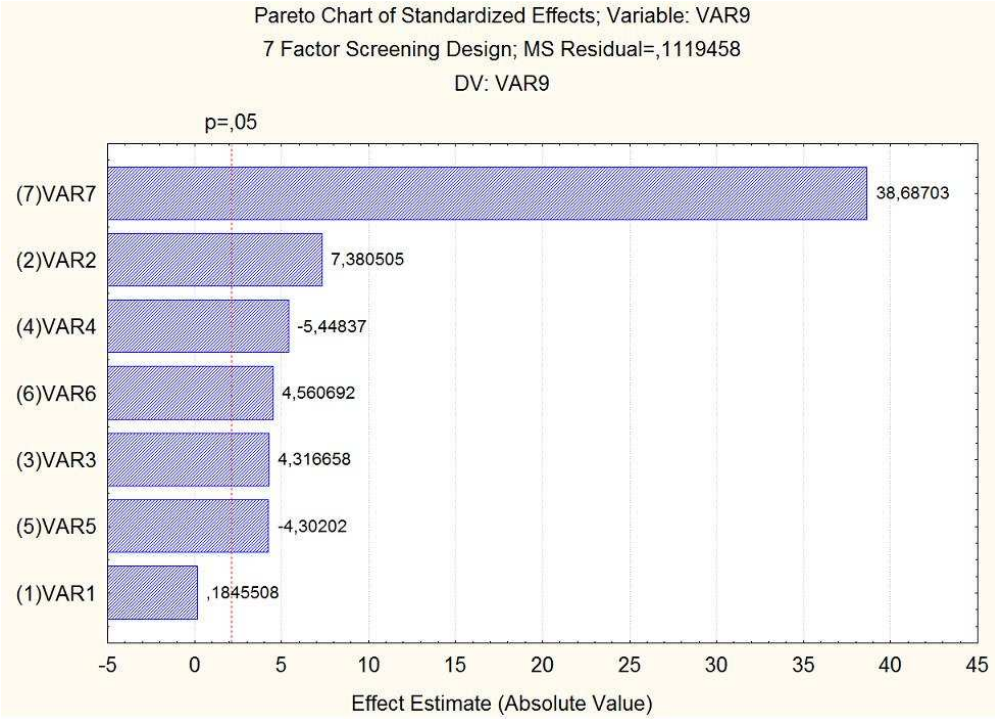


Figure 8 - Pareto chart rationalizing the effect of each variable on the production of biomass (A) and extracellular polysaccharide (B) by *Trametes versicolor* and *Chlorella vulgaris*. The vertical line indicates confidence level of 95% for the effects. VAR1–VAR7 indicates culture temperature, agitation speed, microalgal inoculation density, fungal inoculation density, initial pH, illumination rate and glucose concentration, respectively

A



B

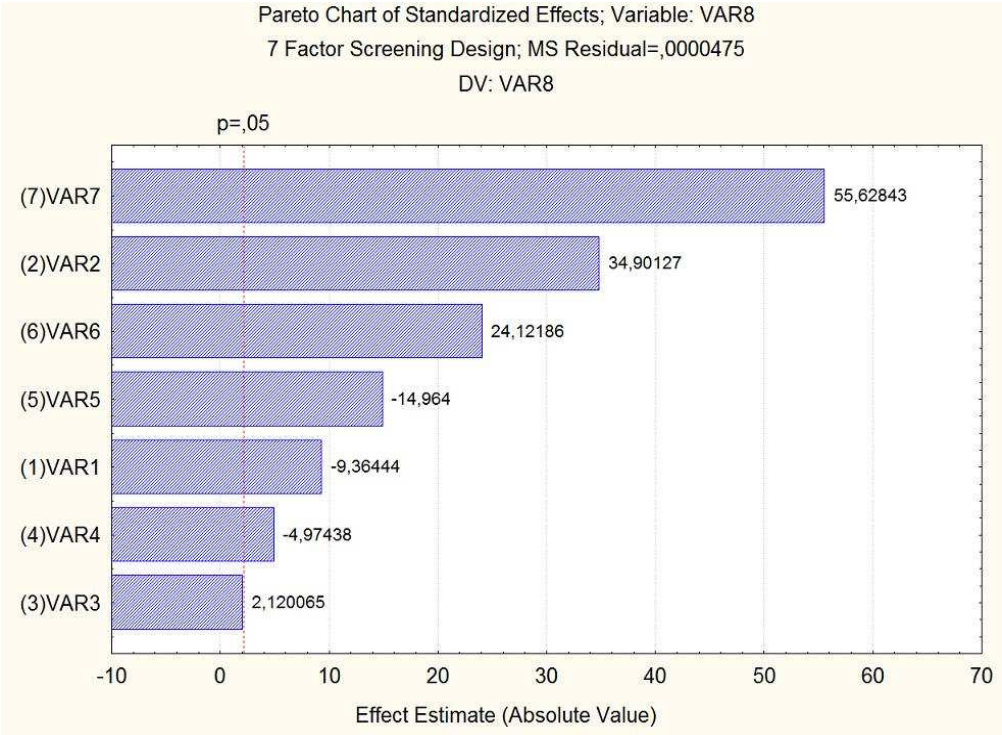
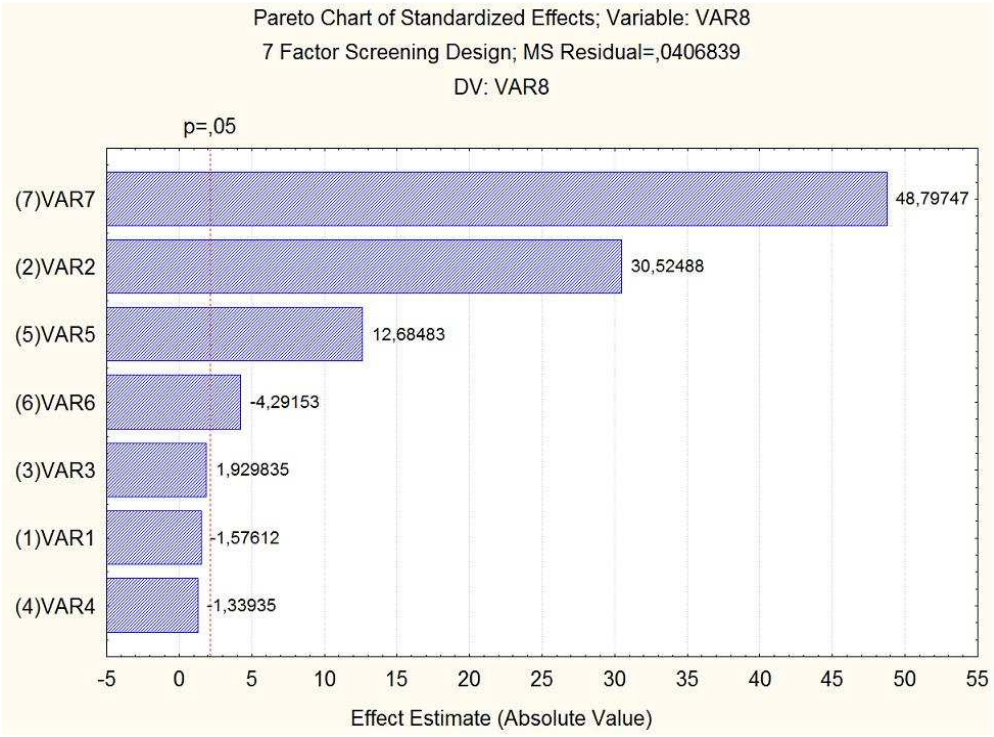


Figure 9 - Pareto chart rationalizing the effect of each variable on the production of biomass (A) and extracellular polysaccharide (B) by *Trametes versicolor* and *Spirulina platensis*. The vertical line indicates confidence level of 95% for the effects. VAR1–VAR7 indicates culture temperature, agitation speed, microalgal inoculation density, fungal inoculation density, initial pH, illumination rate and glucose concentration, respectively

A



B

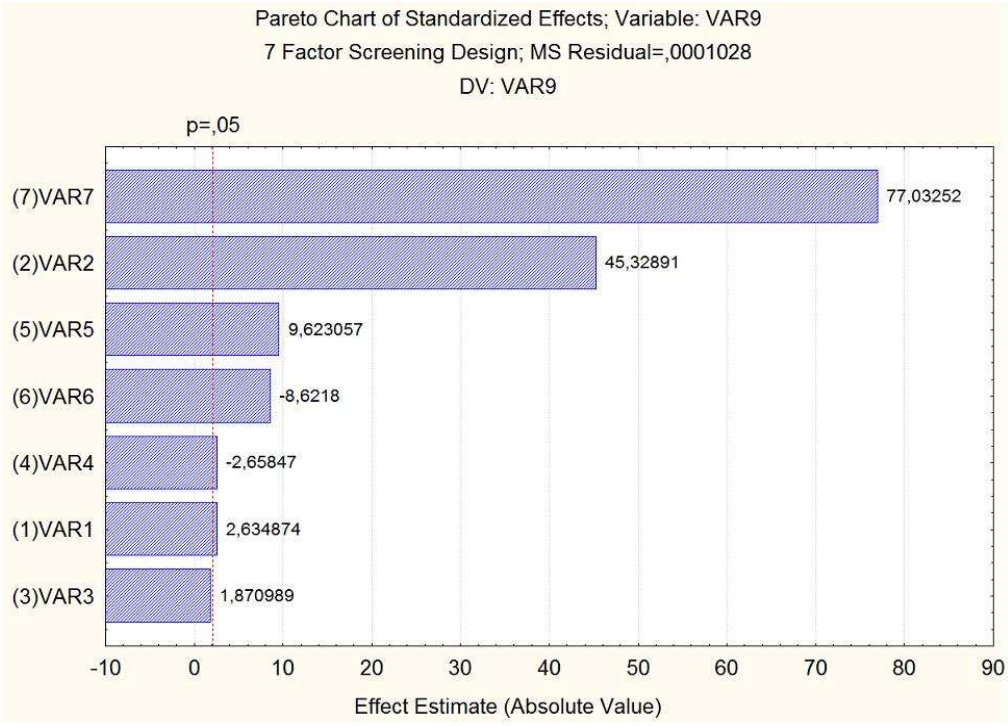


Figure 10 - Pareto chart rationalizing the effect of each variable on the production of biomass (A) and extracellular polysaccharide (B) by *Agaricus blazei* and *Trametes versicolor*. The vertical line indicates confidence level of 95% for the effects. VAR1–VAR7 indicates culture temperature, agitation speed, microalgal inoculation density, fungal inoculation density, initial pH, illumination rate and glucose concentration, respectively

We can note that microalgal inoculation density (VAR4) showed a negative effect on the most part of responses, which was expected because a fungal monoculture can product more EPS and biomass than an algal monoculture. The responses seem insensitive to the variance of fungal inoculation density or demonstrated a positive effect.

Culture temperature was not an important factor in submerged fermentation. As demonstrated in this study, culture temperature showed an insignificantly effect on responses.

Initial pH demonstrated a negative effect and its due to the influence of microalgae that increase the initial pH. In a bioreactor with controlled-pH conditions we could increase the product yield. Initial pH was proven to be important for the production in uncontrolled-pH batch culture. The results of Plackett–Burman design also show that the initial pH value is important in the biomass accumulation and EPS production. Lower initial pH demonstrated favorable effects on biomass and EPS production.

3.4.2 Box–Behnken Design

Using the Box–Behnken method, 15 sets of experiments with appropriate combinations of glucose concentration, agitation speed and initial pH for the co-culture of *A. blazei* and C*. vulgaris* were conducted.

The response surface and the contour surface plots representing the EPS and biomass yields over changes in independent variables VAR1, VAR2 and VAR3 showed clearly that the optimum conditions for obtaining maximum responses lie in the current experimental region (Figs. 6-11).

The response surface and the contour surface plots representing the EPS and biomass yields over changes in independent variables VAR1, VAR2 and VAR3 showed clearly that the optimum conditions for obtaining maximum responses lie in the current experimental region (Figs. 6-11).

As can be seen, the EPS and biomass yields steadily increased with increasing glucose concentration till up to approximately 52 g/L, but decreased slowly beyond the range. High glucose concentration is beneficial for fungal growth. The agitation speed, in the same way what happens with glucose concentration, increased the EPS and Biomass yields with increasing the speed till up to approximately 200rpm, but decreased slowly beyond the range. The agitation speed is beneficial for good mixing throughout the fermenter, which ensures sufficient air transfer in culture, and consequently improves the cell growth and metabolite synthesis. But too high agitation speed results in damage to cell structure and decrease in the yield of secondary metabolite. Similarly, moderate initial pH (approximately 5.0) appears to be the most favorable for EPS and biomass production according to these plots.

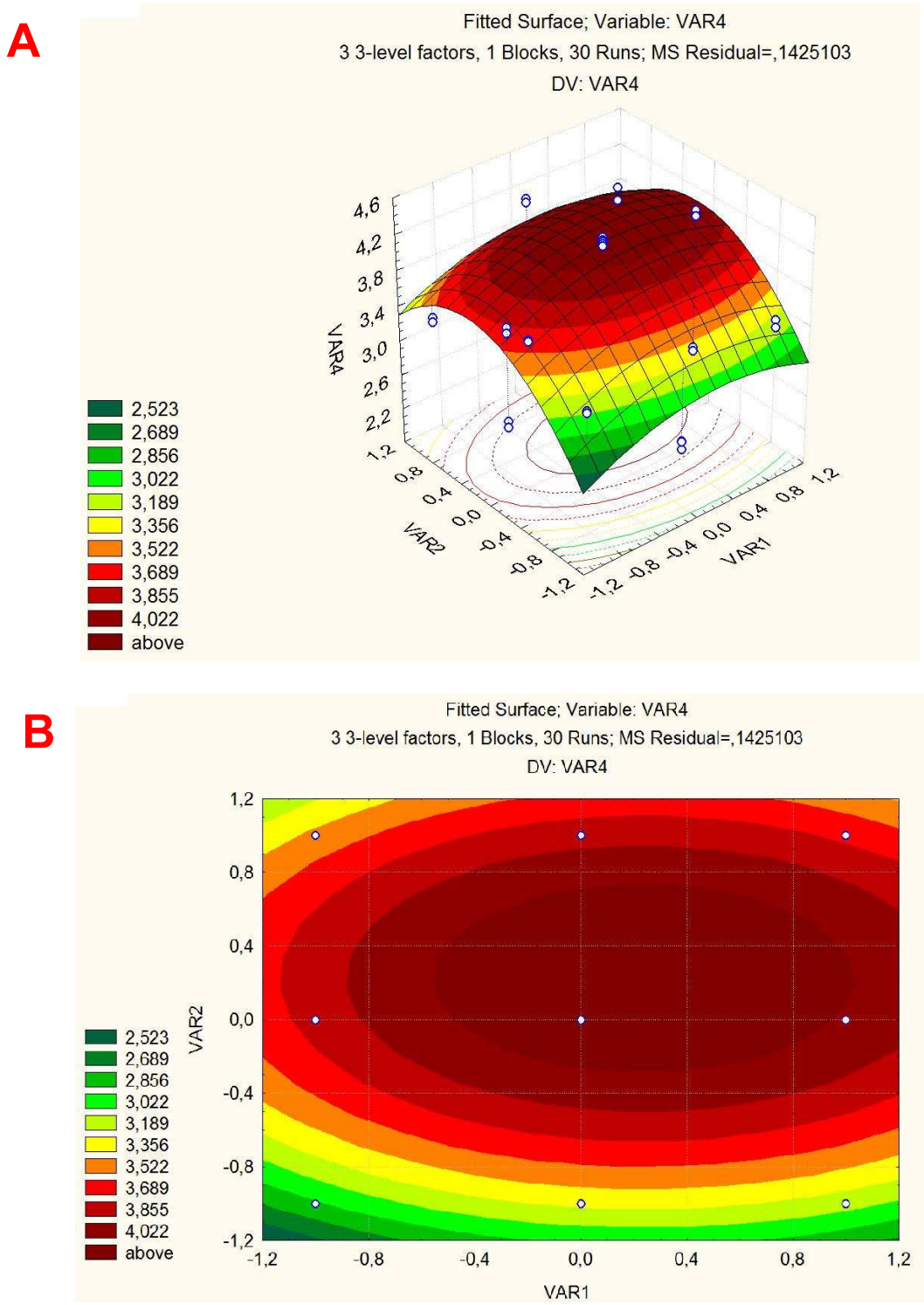


Figure 11 - Response surface (A) and contour surface (B) plots of the EPS yield: effects of glucose concentration (VAR1) vs. agitation speed (VAR2) and their interactive effect with initial pH (VAR3) set at centre level

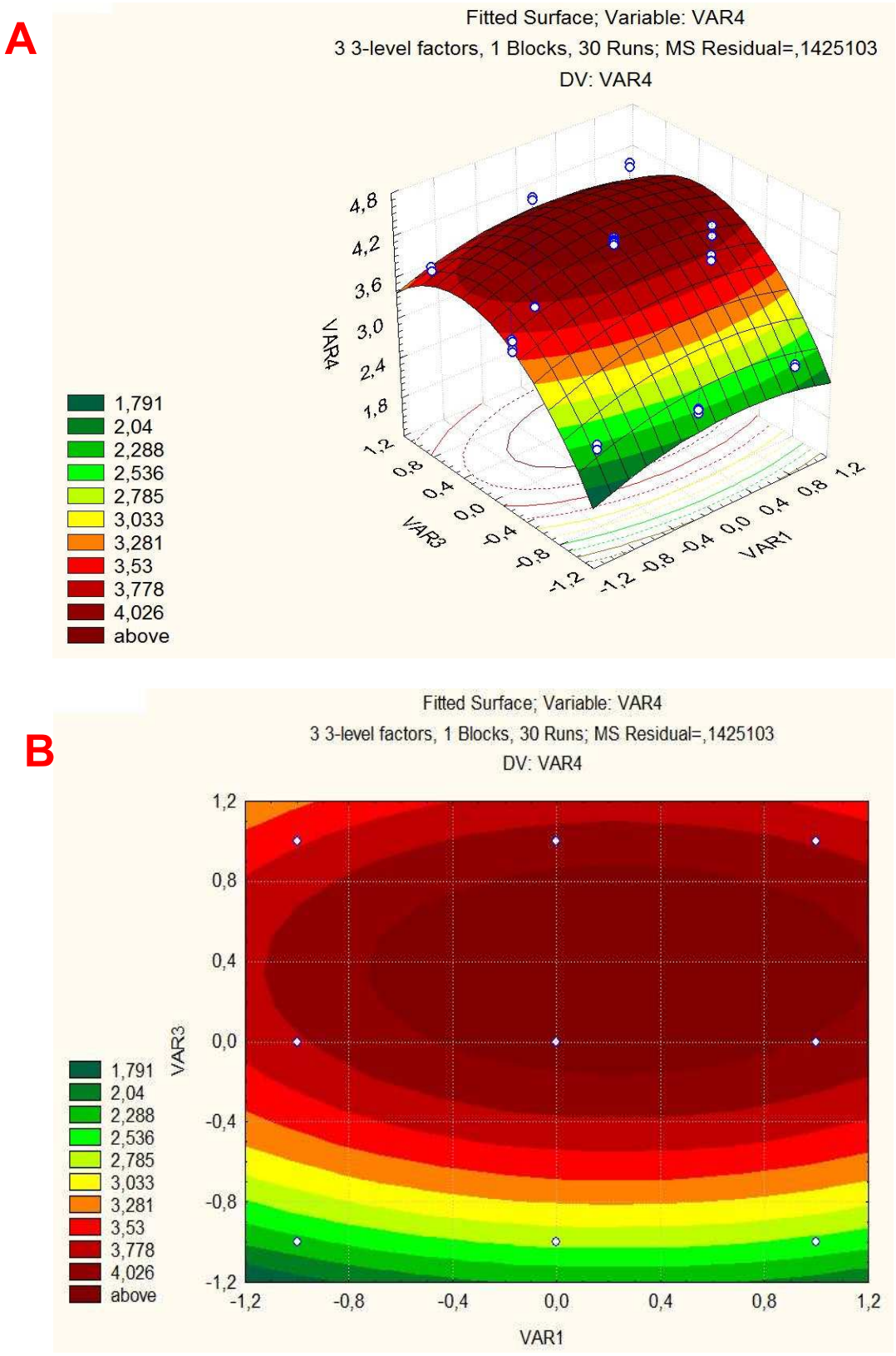


Figure 12 - Response surface (A) and contour surface (B) plots of the EPS yield: effects of glucose concentration (VAR1) vs. initial pH (VAR3) and their interactive effect with agitation speed (VAR2) set at centre level

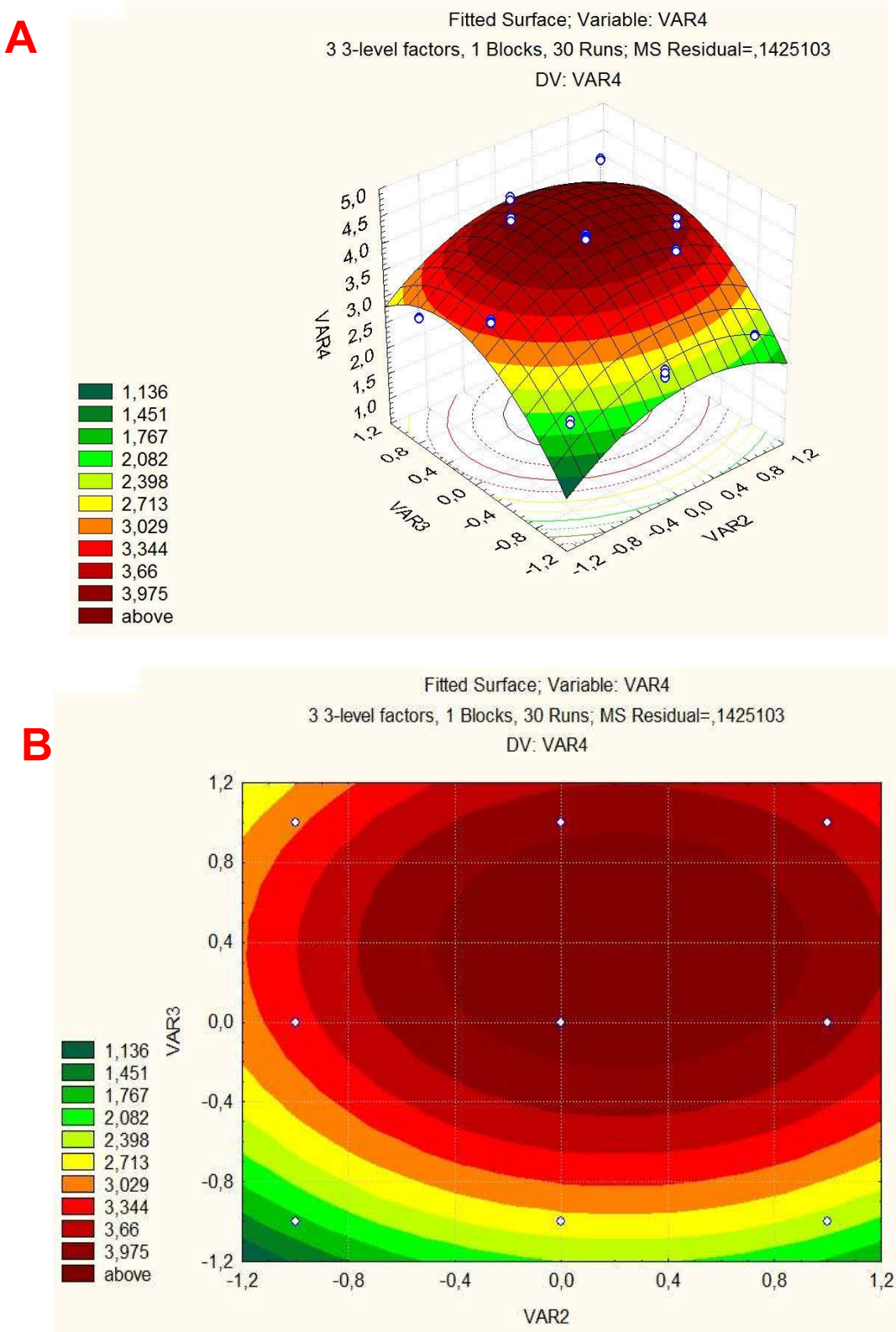


Figure 13 - Response surface (A) and contour surface (B) plots of the EPS yield: effects of agitation speed (VAR2) vs. initial pH (VAR3) and their interactive effect with glucose concentration (VAR1) set at centre level

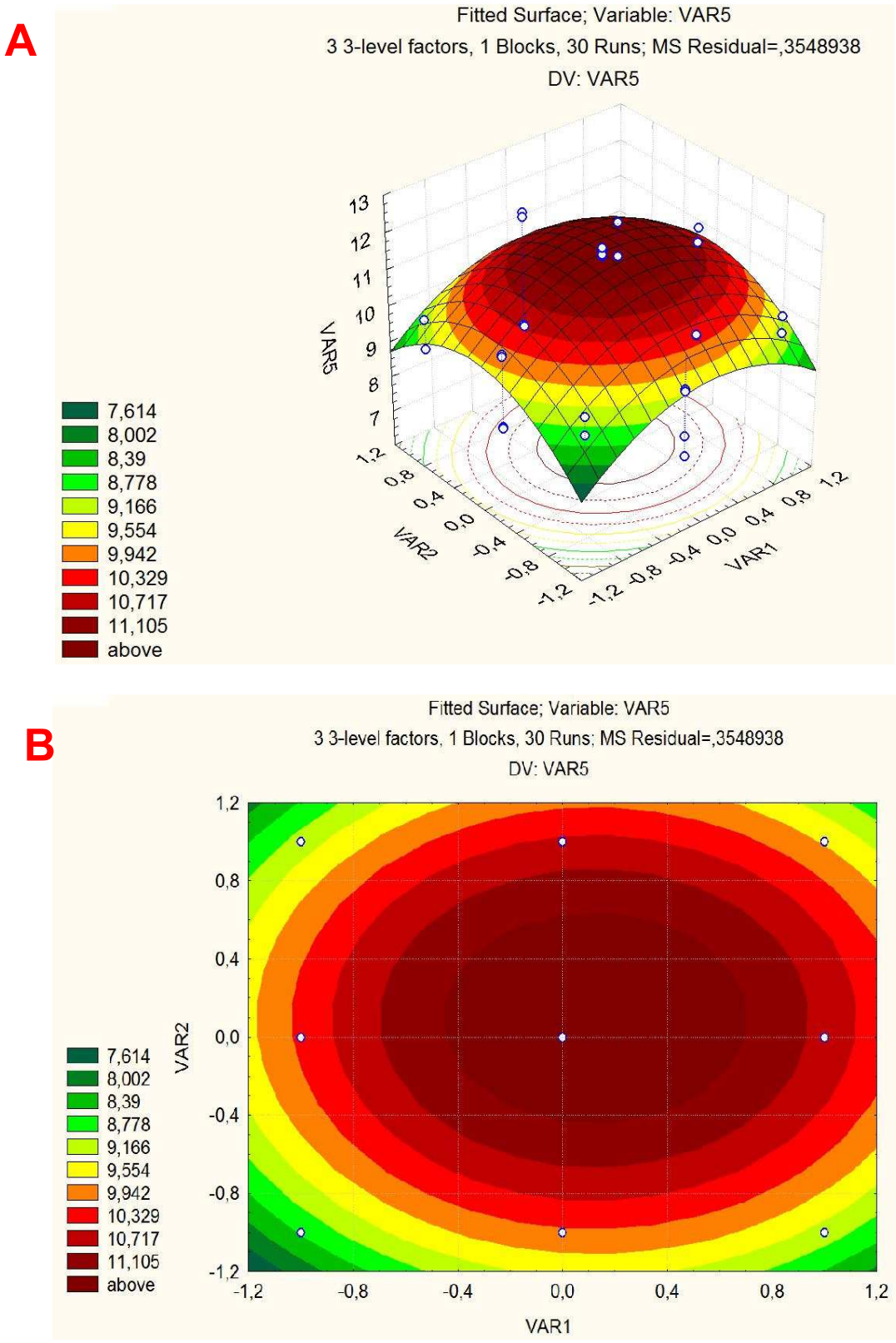


Figure 14 - Response surface (A) and contour surface (B) plots of the biomass yield: effects of glucose concentration (VAR1) vs. agitation speed (VAR2) and their interactive effect with initial pH (VAR3) set at centre level

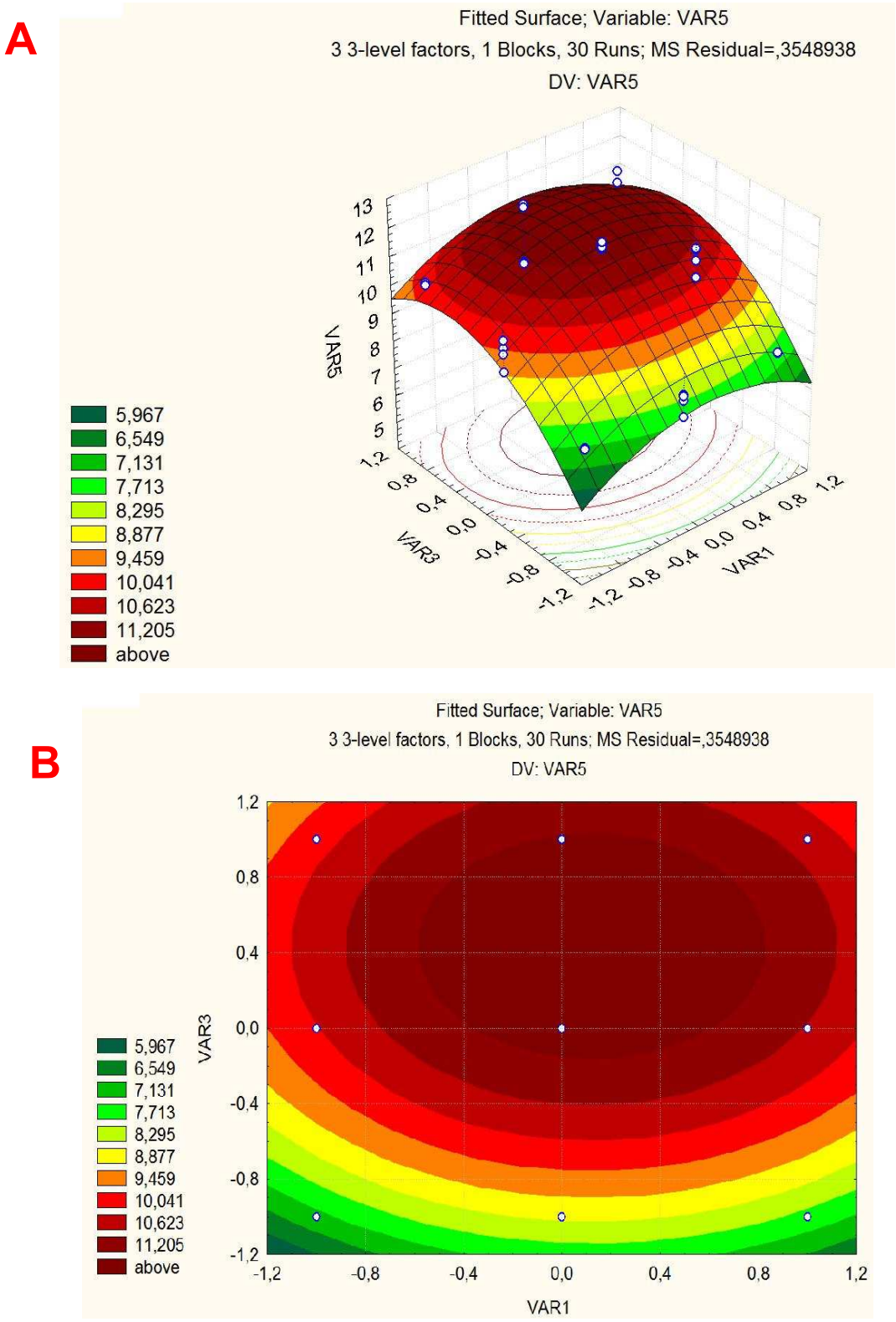


Figure 15 - Response surface (A) and contour surface (B) plots of the biomass yield: effects of glucose concentration (VAR1) vs. initial pH (VAR3) and their interactive effect with agitation speed (VAR2) set at centre level

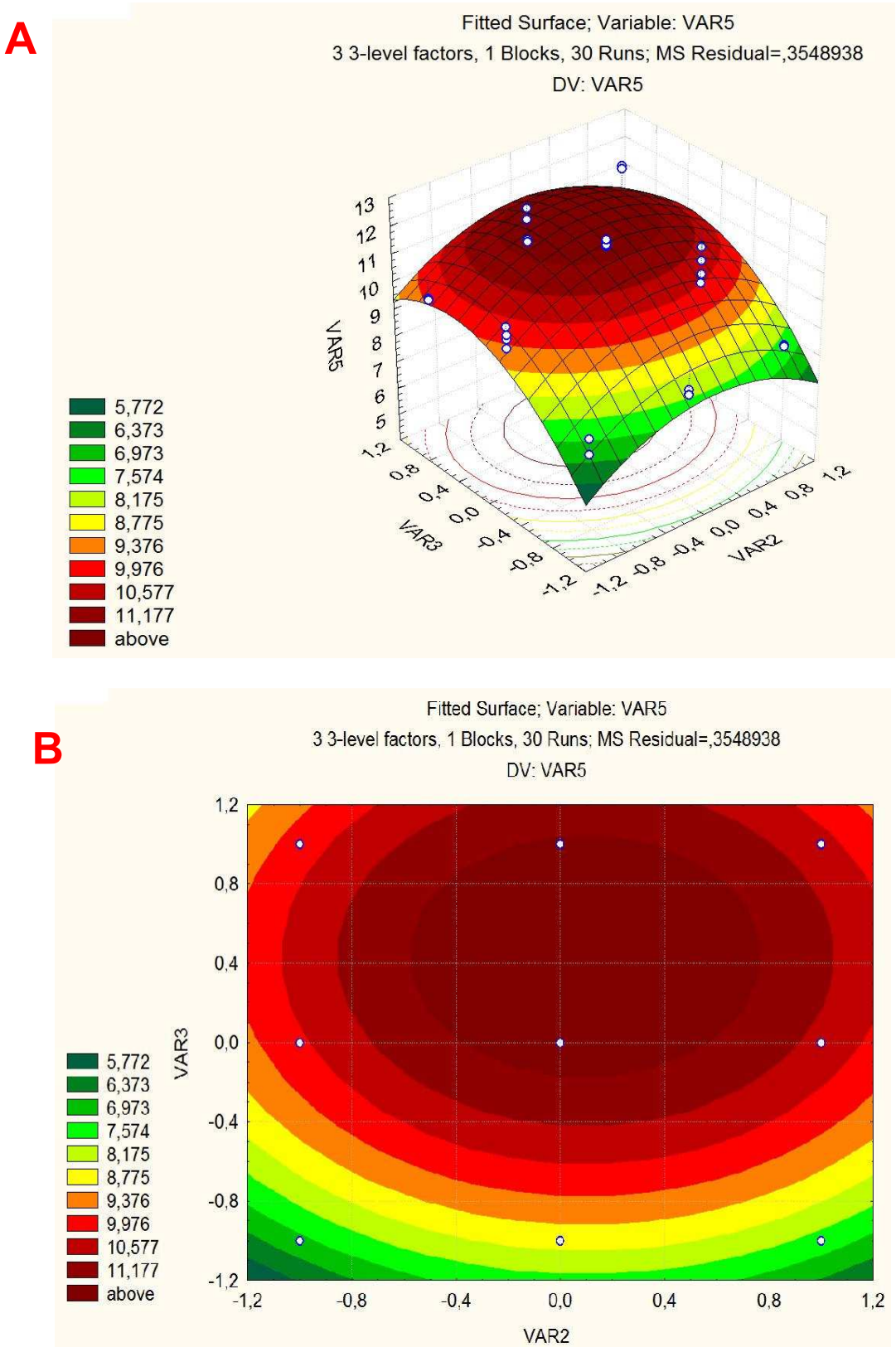


Figure 16 - Response surface (A) and contour surface (B) plots of the biomass yield: effects of agitation speed (VAR2) vs. initial pH (VAR3) and their interactive effect with glucose concentration (VAR1) set at centre level

3.5 Conclusion

The conditions for simultaneous production of EPS and biomass by macromycetes and microalgae were optimized utilizing experimental designs. The optimized fermentation conditions increased the exopolysaccharide and biomass yields by 33% and 61%, as compared with the unoptimized fermentation conditions. The present study indicates that sequential methodology based on the application of Plackett–Burman design and Box–Behnken design would be a viable and effective alternative for the optimization of similar fermentation process.

4 PRODUCTION AND PARTIAL CHARACTERIZATION OF EXOPOLISACCHARIDES EXTRACTED FROM SUBMERGED FERMENTATION BY CO-CULTURE OF BASIDIOMYCETES, *Agaricus blazei* AND *Trametes versicolor*, AND MICROALGAE, *Spirulina platensis* AND *Chlorella vulgaris*

4.1 Abstract

Some polysaccharides isolated from microorganisms show various important biological activities, such as antitumor, immunomodulatory, and anti-inflammatory effects, which are strongly affected by their chemical structures and chain conformations. Physicochemical properties, composition, and structure of the exopolysaccharides (EPS) synthesized by basidiomycetes, *A. blazei* and *T. versicolor*, and microalgae, *S. platensis* and *C. vulgaris* in submerged co-culture were studied. A combination of component analysis, ^{13}C NMR spectroscopy and CG-MS chromatography shows that the microorganisms produce different polysaccharides when they are kept in co-culture. The polysaccharides released are complex heteropolymers which are composed of six to ten different monosaccharides. This characteristic, quite unusual in microbial polysaccharides of industrial interest which are in most cases composed by a lower number of monomers, is of great significance. Mannose was a predominant monosaccharide in every sample, except for *Chlorella vulgaris* EPS, where glucose and fucose dominated. Among 10 tested samples of the microorganisms separately or co-cultured, all of them produced significant amounts of exocellular polysaccharide (EPS). The maximum production of EPS was 7.10 g/L after 8 days found in fermentation broth of *T. versicolor* with *C. vulgaris* co-culture. The kinetics of growth, substrate consumption, and EPS production in the batch, aerobic, submerged culture of this fungus were investigated in detail.

Keywords: exopolysaccharides characterization; NMR spectroscopy; gas chromatography; monosaccharide composition; co-culture.

4.2 Introduction

Fungi and algae are being researched as potential sources of many macromolecules, mainly polysaccharides. The possible application of these compounds on human health, as well as in other areas, has led to intensive studies related to its extraction and characterization (GERN et al., 2007). Algal and fungal EPS are widely used in the food industry as viscosifying, stabilizing, gelling, or emulsifying agents, due to their characteristic physical and rheological properties (DE VUYST; DEGEEST, 1999). In this context, a growing interest has developed in the use of EPS produced by microorganisms which carry the GRAS (Generally Recognized as Safe) status. To gain a better insight into the relationship between the structures of EPS and their physical properties, structural studies are currently performed on EPS produced by different kind of microorganisms, such as bacteria, algae, mushrooms, yeast.

Over the years, several polysaccharides from mushrooms and microalgae have been characterized, being mainly isolated from the biomass.

Here, we report on the structural determination of the EPS produced by basidiomycetes, *A. blazei* and *T. versicolor*, and microalgae, *S. platensis* and *C. vulgaris*. Physicochemical properties, composition, and structure of the EPS synthesized were studied.

4.3 Materials and methods

4.3.1 EPS extraction

Samples collected at various intervals from shake flask were vacuum filtered by filter-paper (Whatman 1). The resulting culture filtrate was concentrated to ¼ of the original volume by vacuum oven below 50 ºC. The filtrated was dialyzed in a membrane with cutoff of 12-14 KDa, mixed with four times its volume of absolute ethanol, stirred vigorously and left overnight at 4°C. The precipitated EPS was centrifuged at 10000 g for 10 min, discarding the supernatant. The precipitate of pure EPS was lyophilized and the weight of the polymer was estimated. Dry weight of mycelium was measured after repeated washing of the mycelial pellet with distilled water and drying overnight at 70°C to a constant

weight. The extracellular polysaccharide concentration was determined according to the classical method of DUBOIS et al. (1956) using glucose solution as a standard reference.

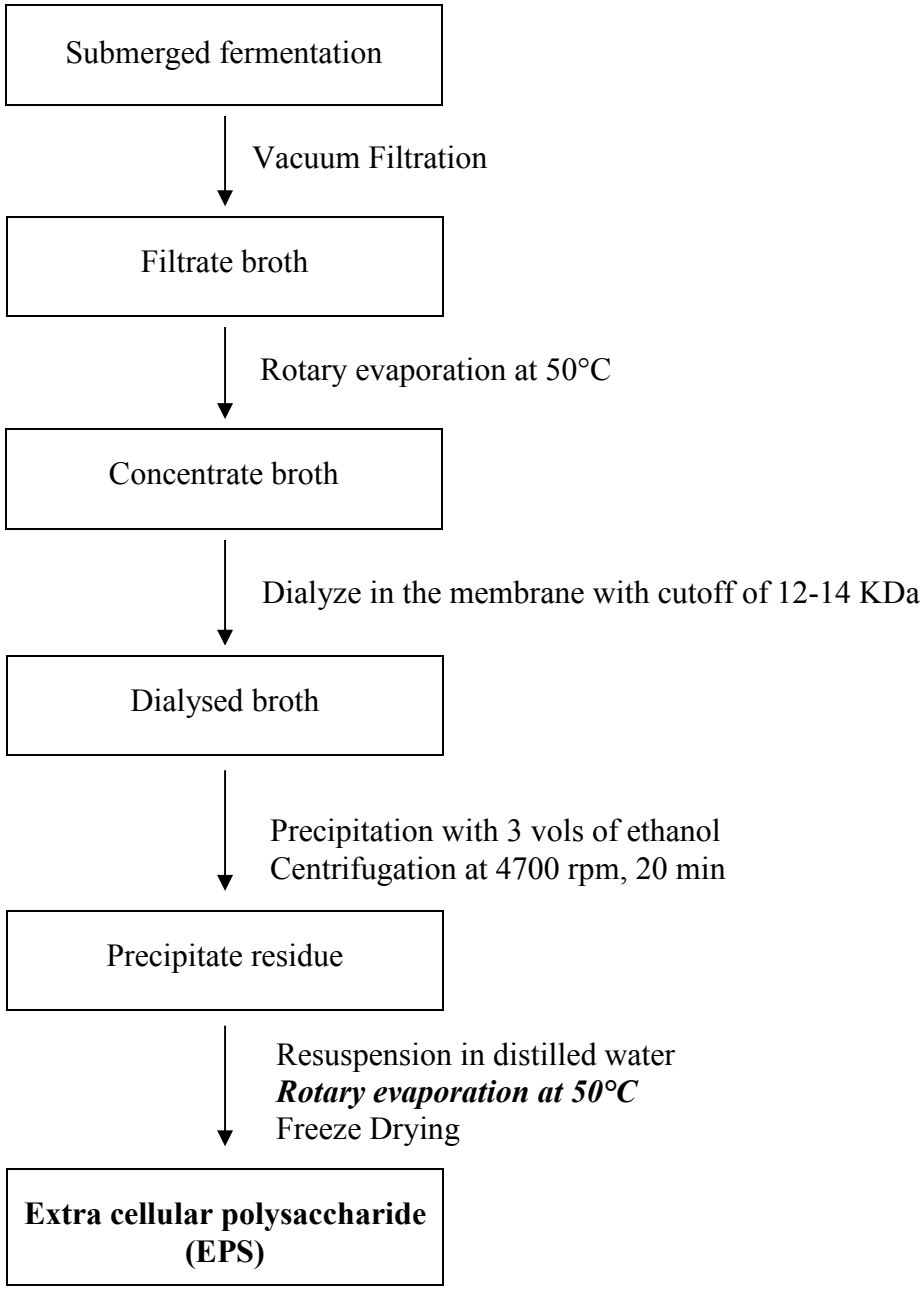


Figure 17 - Schematic extraction system of extra-cellular polysaccharides extracts from submerged fermentations

4.3.2 Sugar content

The carbohydrate quantification was done by phenol-sulfuric method (DUBOIS, 1956), and the quantification of residual sugar (glucose) was carried out according Somogyi-Nelson (1954). The reading of the absorbance for both methods was performed in spectrophotometer (Power Wave XS, BioTek).

4.3.3 Proteins content

The quantification of protein contents was performed according to LOWRY et al. (1951). The determination of absorbance was also carried out in spectrophotometer (Power Wave XS, BioTek).

4.3.4 Monosaccharide composition

Monosaccharide composition was performed second YANG et al. (1987). Approximately 1 mg of EPS was treated with 0.5 ml of trifluoracetic acid (TFA), 2 M, for 1 hour and 121°C (YANG, 2000). The acid was eliminated by evaporation until dissected, followed by NaBH4 reduction, acetylation and analyzed by Gas chromatography (CG) and CG coupled to mass spectrometry (CG-MS). GC-MS analysis was performed using VARIAN 3.300 gas chromatography equipped with DB-225 (30 m x 0.25 mm) column interfaced to Finnigan Mat ITD 800 mass spectrometry. A fused silica capillary column (30 m x 0.25 mm) coated with DB-225 was used to analyze the alditol acetates. The injector and FDI temperatures were 250 °C. Helium was used as carrier gas (1.0 ml.min-1).

4.3.5 ^{13}C NMR spectroscopy

The EPS comparison was assessed by ^{13}C-NMR spectroscopy. Resolution-enhanced 1D/2D 100.16 MHz NMR spectra were recorded in D2O on a Bruker spectrometer, 30.000 pulses with a pulse repetition time of 0.1 s, at a probe temperature of 50 to 70°C for polysaccharides. Prior to analysis, samples

were dissolved to a polysaccharide concentration of 0.6% (w/v) in D2O/H2O. Chemical shifts are expressed in ppm by reference to the α-anomeric signal of external [1-13C] glucose (dC-1 92.9) for ^{13}C. All NMR data were processed using TopSpin® software.

4.3.6 Microscopic Morpho-structural Observation

Scanning electronic microscopy was used to observe the microstructure of the isolated EPS using an EVE Jeol JSM-6360 LV scanning electron microscope.

4.4 Results

The EPS formation can start between 1 and 3 days after inoculation. The extent of production depends on the quantity of cells present, their specific activity, and the span of their product-forming capacity. Figure 18 shows the typical time courses of mycelial biomass and EPS production by the microorganisms cultivated separately and under a co-culture. In this case, the pH was not controlled, initial pH was 7.0 and changed according to the microorganisms in submerged fermentation.

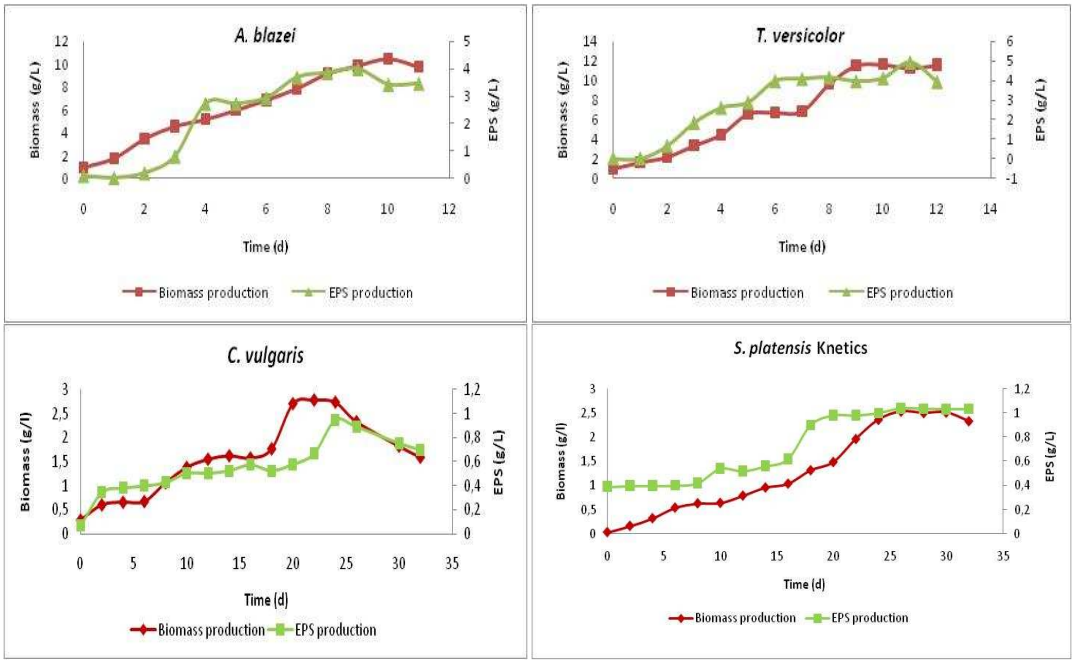


Figure 18 - Time profiles of EPS production and mycelial growth of microalgae and macromycetes mono-culture

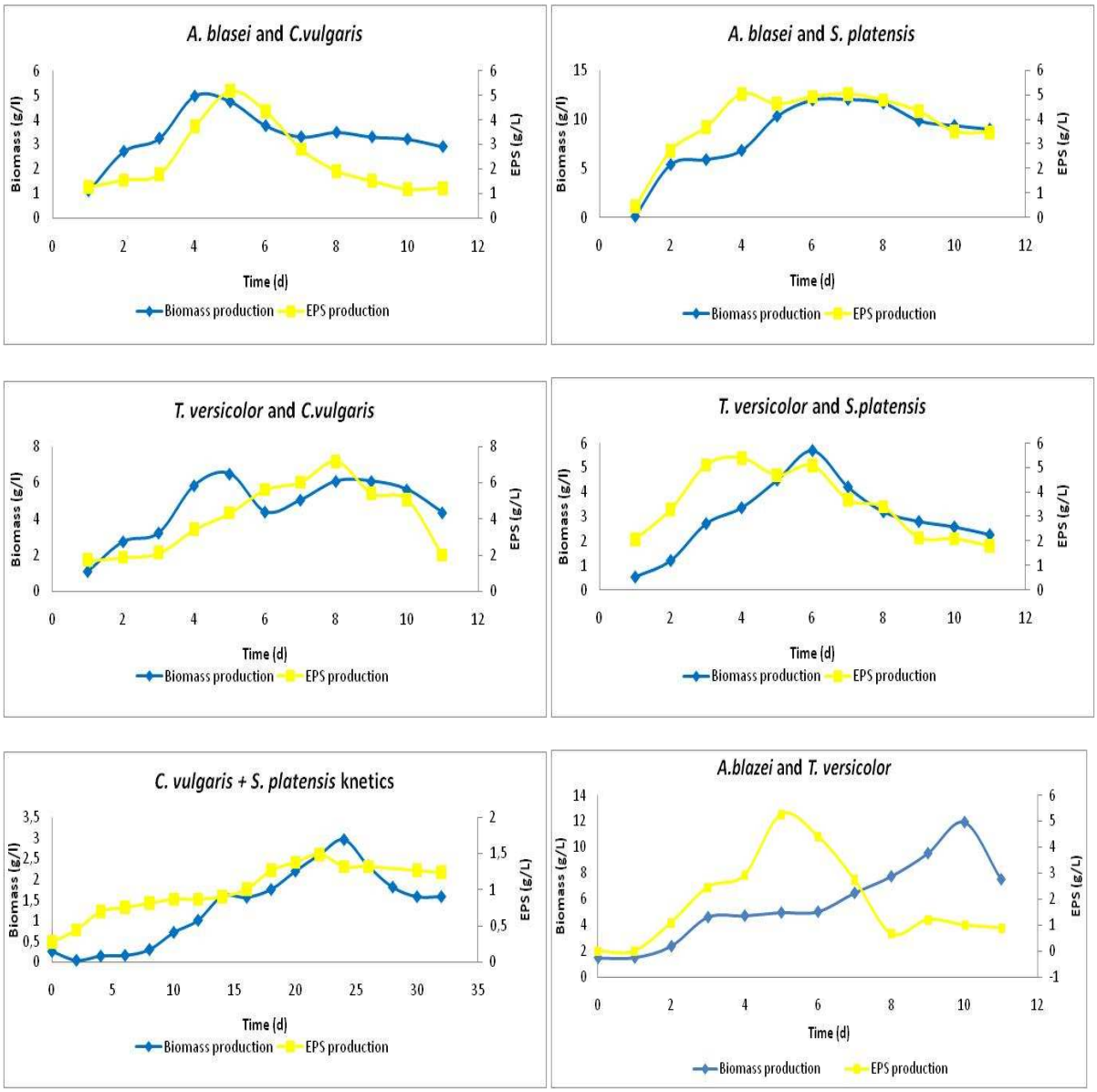


Figure 19 - Time profiles of EPS production and mycelial growth of microalgae and macromycetes co-culture

The highest EPS concentration were 4.95 g/L for *T. versicolor,* which was obtained after 11 days of culture; 4.00 g/L for *A. blazei* after 9 days of culture; 5.26 g/L for the co-culture of *A. blazei* with *T. versicolor* , after 5 days; 0.95 g/L after 24 days of *C. vulgaris* culture; 1.04 g/L after 26 days of *S. platensis* culture; 1.48 g/L after 22 days of *C. vulgaris* with *S. platensis* co-culture*;* 5.17 g/L after 5 days of *A. blazei* with *C. vulgaris* co-culture; 5.04 g/L after 4 days of *A. blazei* with *S. platensis* co-culture; 7.10 g/L after 8 days of *T. versicolor* with *C. vulgaris* co-culture and 5.42 g/L after 4 days of *T. versicolor* with *S. platensis* co-culture.

The results demonstrated that there is a EPS increase with the co-culture, showing that the contact with different microorganisms grown in a mixture is desired in this case and improving the EPS production and reducing the fermentation time.

Figure 20 - *Trametes versicolor* culture *and Trametes versicolor* with *Chlorella vulgaris* co-culture

The freeze-dried EPS (Figure 21) appeared as a yellow cotton polymer in the most part of cultures, but it is next to white for *Chlorella vulgaris* EPS and next to orange for *Trametes versicolor* EPS.

This crude EPS was then analyzed for its protein, neutral sugar, and total sugar contents by colorimetric analyses. The results demonstrated that EPS contain high levels of nitrogen and protein. The protein content in the samples was not determined, but protein moieties were found in almost all of the algal and fungal polysaccharides investigated. These moieties ranged from 8.6 to 23.1% of the EPS dry weight, and according some researchers depending on the species and culture conditions (HUNG et al., 2007).

Figure 21 - Photo of *Agaricus blazei* EPS after freeze drying process

Overall, in the literature, the fungal and microalgal carbohydrates contain 2 to 12 types of monosaccharides (HU et al., 2003). This is a striking difference from polymers that are synthesized by other microorganisms, which generally contain less than four monomers. In the present study, the microorganisms carbohydrate portion was formed 6 to 8 different types of monomer units (Table 10), including seven neutral sugars (xylose, galactose, glucose, fucose, arabinose, rhanmose, ribose and mannose).

The carbohydrates portions are complex due to the abundant presence of mannose, galactose, glucose, rhamnose and fucose. However, arabinose, ribose and xylose were present in small amounts. Pentoses, which are usually absent from other polysaccharides of microbial origin, are often found in macrofungies and microalgae (HUNG et.al 2007). In the present study, three pentoses were found, xylose, arabinose and ribose.

Mannose was a predominant monosaccharide in every sample, except for *Chlorella vulgaris* EPS, where glucose and fucose dominated.

Table 10 - Protein content, carbohydrate content, and monosaccharide composition of extracellular polysaccharides produced by the microorganisms separately and in co-culture
(—) Not detected.
bCompositional analyses determined after hydrolysis with M TFA for 4 h at 100ºC
cA 21-day-old culture was used for EPS biochemical analysis and to determine the monosaccharide composition.
dA 10-day-old culture was used for EPS biochemical analysis and to determine the monosaccharide composition.

EPS	Protein content (% EPS* dw)	Carbohydrate content (% EPS* dw)	Monosaccharide (mol %)b							
			Rha	Fuc	Rib	Ara	Xyl	Man	Glu	Gal
A_d	13.2	55.3	----	0.4	2.1	0.2	6.5	**87.3**	3.1	0.4
T_d	42.6	28.3	0.7	1.3	0.9	0.2	0.2	**24.0**	**70.3**	2.4
S_c	49.2	22.1	11.2	13.6	1.9	1.4	6.1	**25.3**	**24.1**	16.4
C_c	53.1	18.7	16.0	**20.5**	1.6	0.5	6.7	18.0	**21.2**	15.5
AT_d	51.3	25.3	2.0	4.3	2.2	0.4	0.6	**67.2**	5.8	17.5
AS_d	52.3	17.3	1.6	1.3	2.1	0.5	0.5	**66.2**	18.5	9.3
AC_d	15.3	37.2	3.1	3.3	2.8	2.7	4.0	**57.2**	6.1	**20.8**
TS_d	38.6	27.3	0.5	12.0	2.4	0.4	0.3	**46.8**	16.0	**21.6**
TC_d	30.0	45.0	1.2	7.9	2.1	----	0.6	**60.8**	15.8	11.6
SC_d	52.4	15.6	1.5	----	----	2.4	2.9	**55.0**	8.3	**29.9**

Figures 22 and 23 show the optical microscopy co-cultures images. We can see the fungi and microalgae structures. In most of the cultures the presence of crystals with different forms was observed, and thought these pictures we can see the crystals in the co-cultures of *A. blazei* with *S. platensis* and *T. versicolor* with *C. vulgaris*. It could indicate, in some cases, the presence of excreted metabolites.

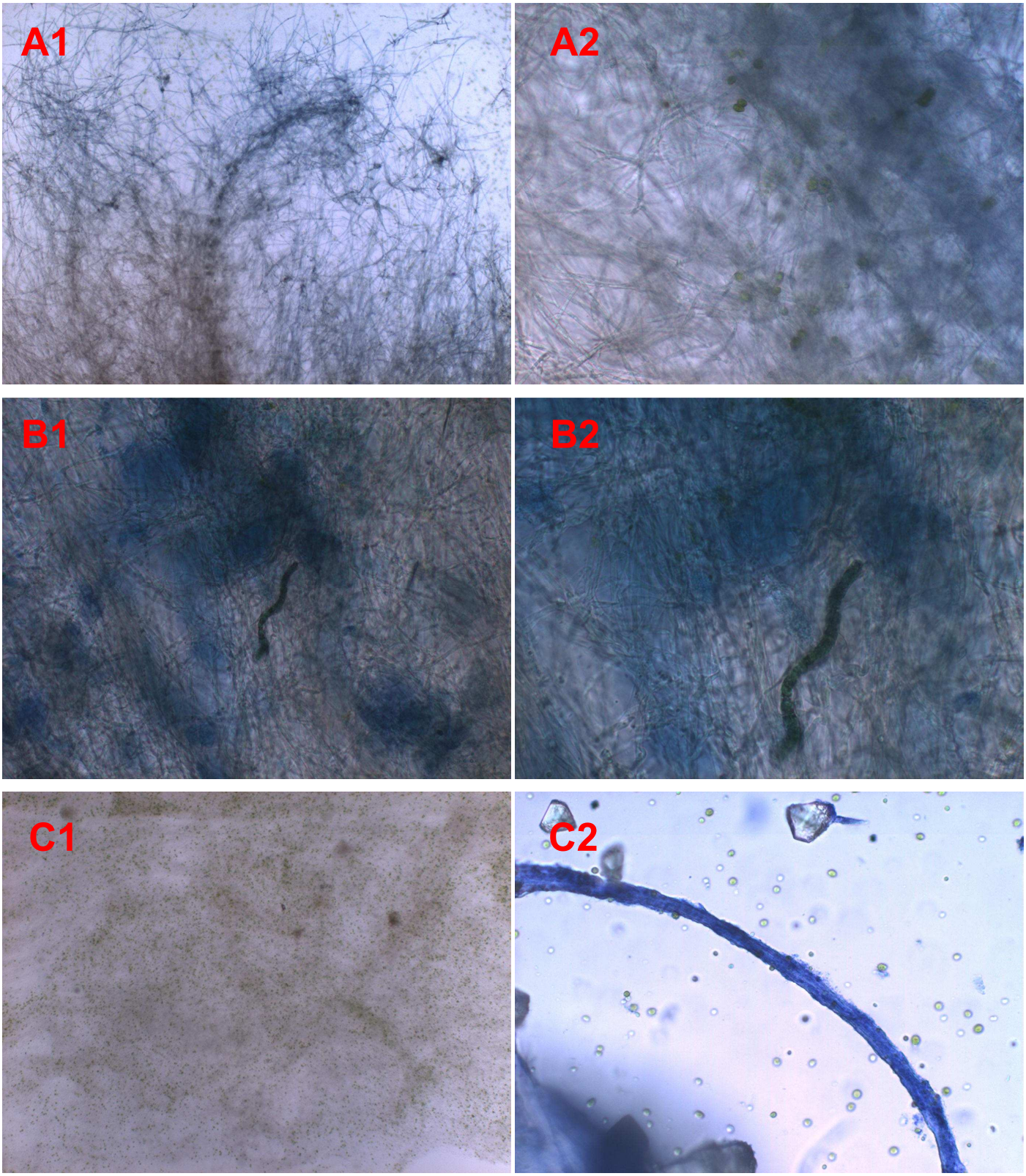


Figure 22 - Comparison between optical micrographs of the co-cultures of (A) *Agaricus blazei* and *Chlorella vulgaris* (amplification of (1) 40x and (2) 200x); (B) *Agaricus blazei* and *Spirulina platensis* (amplification of (1) 200x and (2) 400x) and (C) *Trametes versicolor* and *Chlorella vulgaris* (amplification of (1) 40x and (2) 200x)

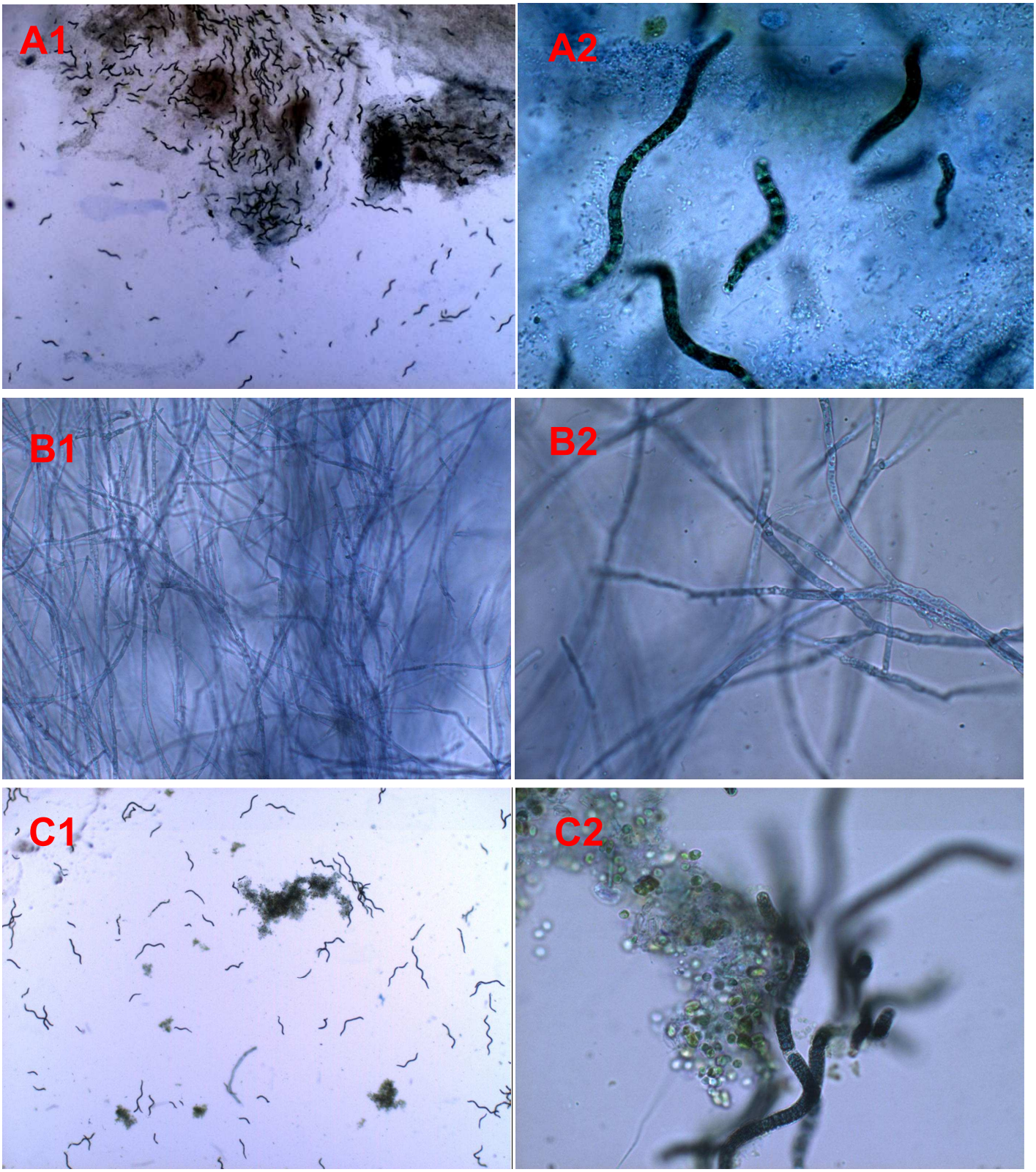


Figure 23 - Comparison between optical micrographs of the co-cultures of (A) *Trametes versicolor* and *Spirulina platensis* (amplification of (1) 40x and (2) 200x); (B) *Agaricus blazei and Trametes versicolor* (amplification of (1) 200x and (2) 400x) and (C) *Chlorella vulgaris* and *Spirulina platensis* (amplification of (1) 40x and (2) 400x)

Figures 24 to 26 show the morphological structure of the EPS produced by the microorganisms alone and by the co-culture. It appeared as constituted by the heterogeneous rod type components and globular structures.

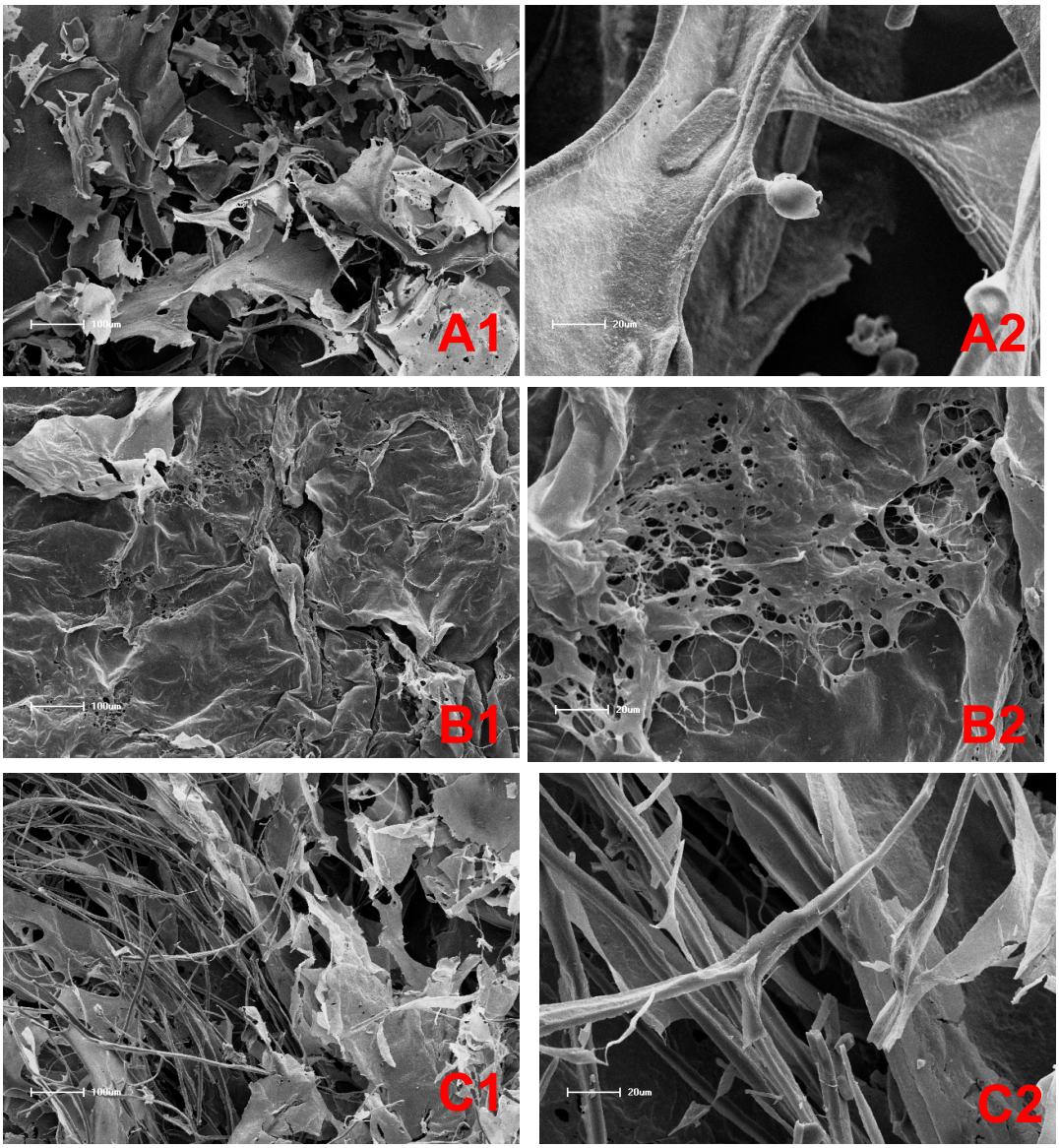


Figure 24 - Comparison between electron micrographs of extracellular polysaccharides produced by (A) *Agaricus blazei*; (B)*Trametes versicolor and* (C) co-culture of *Agaricus blazei and Trametes versicolor* (amplification of (1) 100x and (2) 500x)

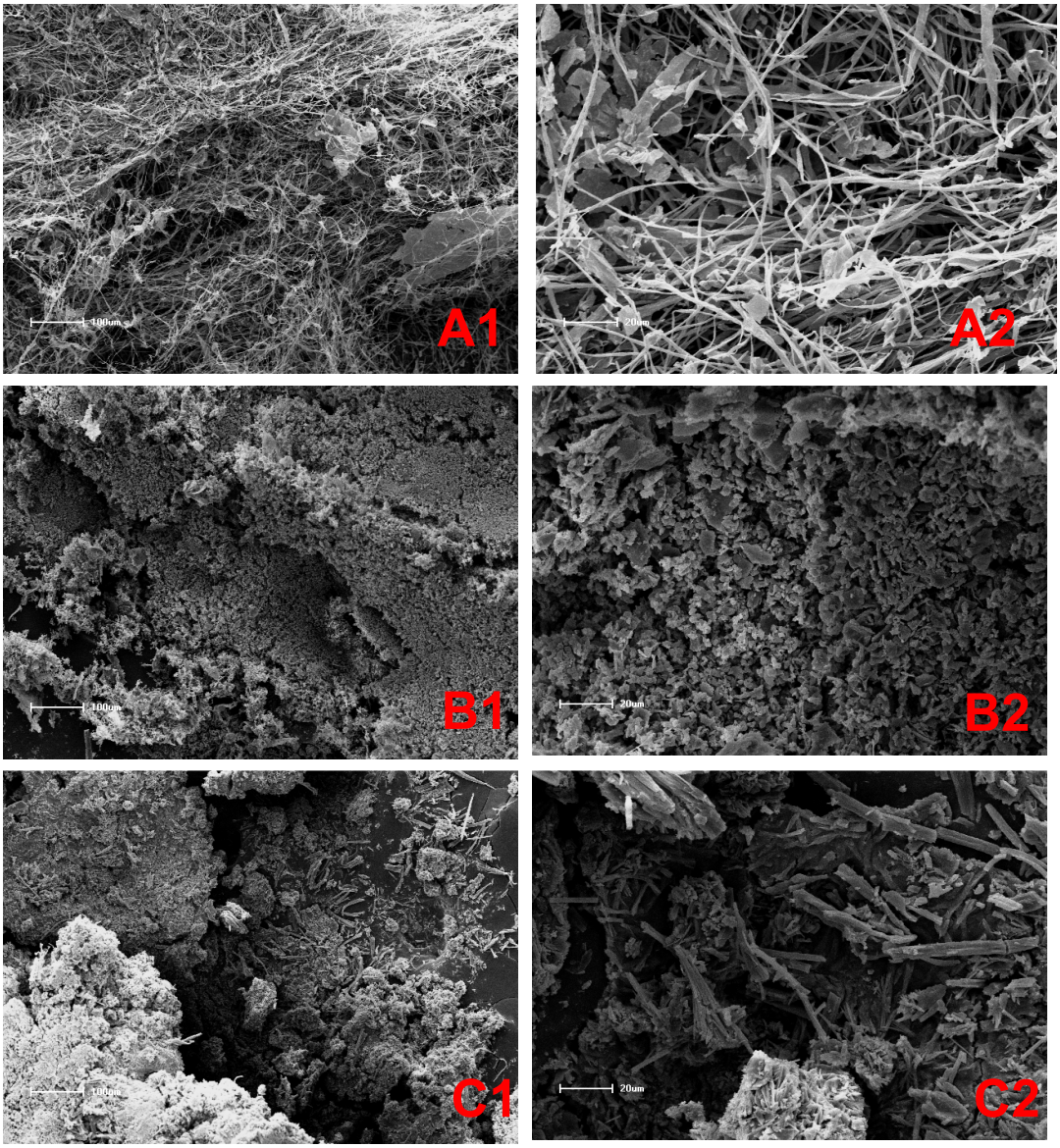


Figure 25 - Comparison between electron micrographs of extracellular polysaccharides produced by (A) *Chlorella vulgaris*; (B) *Spirulina platensis* and (C) co-culture of *Chlorella vulgaris* and *Spirulina platensis* (amplification of (1) 100x and (2) 500x)

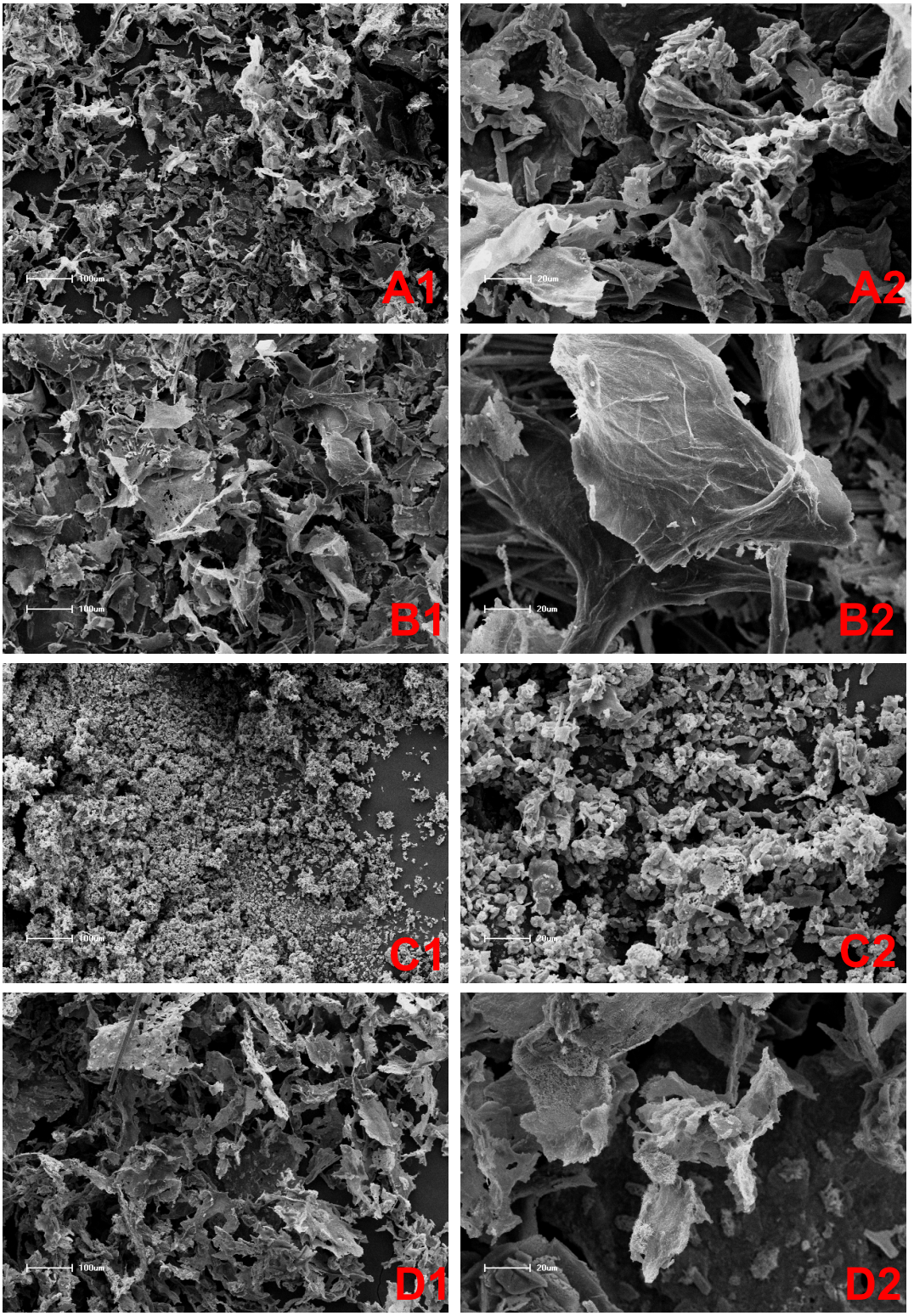


Figure 26 - Comparison between electron micrographs of extracellular polysaccharides produced by (A) co-culture of *Agaricus blazei* and *Chlorella vulgaris*; (B) co-culture of *Agaricus blazei* and *Spirulina platensis*; (C) co-culture of T*rametes versicolor* and *Chlorella vulgaris*; (D) co-culture of *Trametes versicolor* and *Spirulina platensis* (amplification of (1) 100x and (2) 500x)

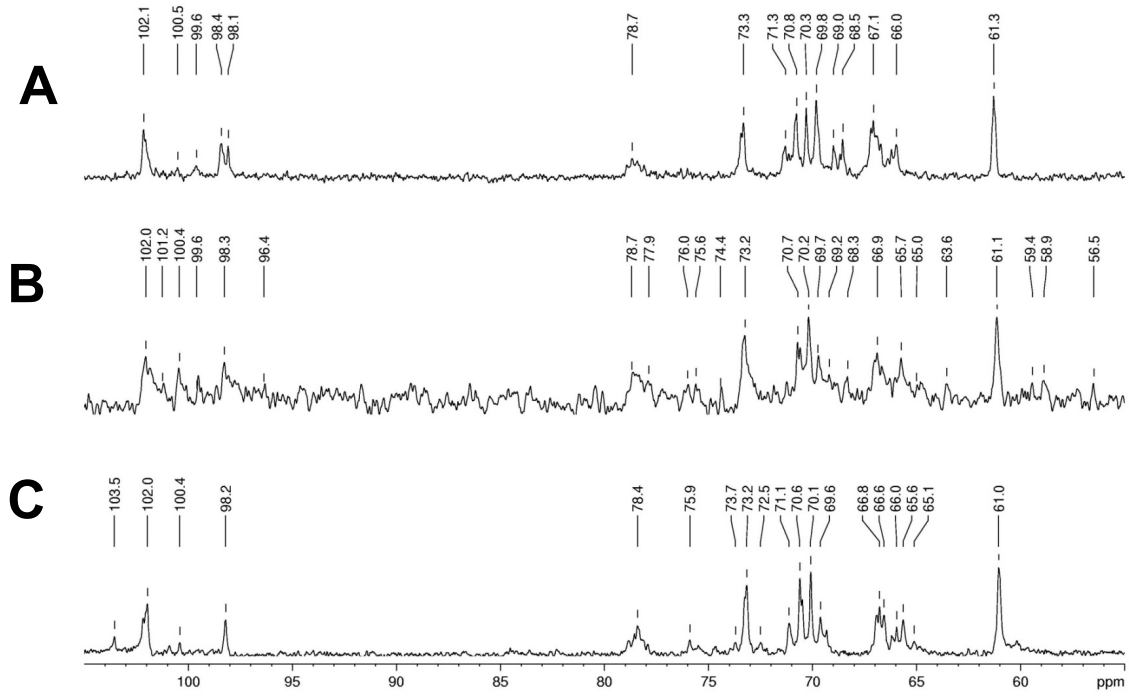


Figure 27 - ^{13}C NMR spectra of EPS produced by *Agaricus Blazei* with *Trametes versicolor* (A), *Trametes versicolor* (B) and *Agaricus Blazei* (C). Acetone was used as internal standard, chemical shifts - ppm (δ)

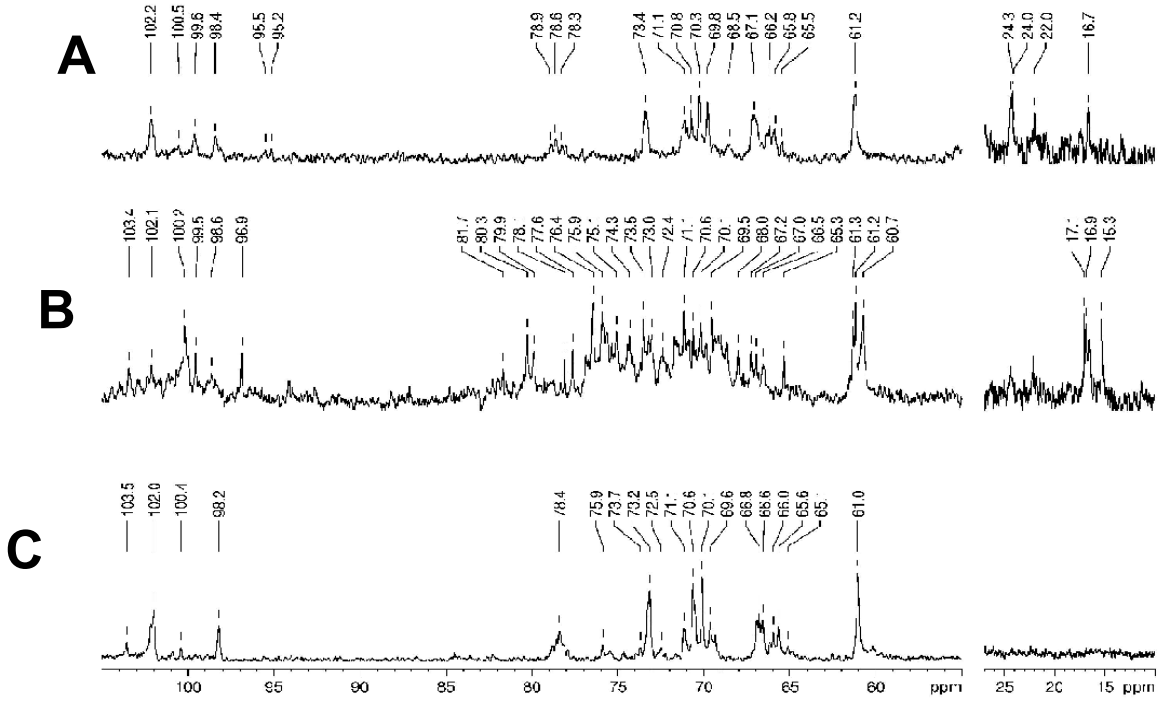


Figure 28 - ^{13}C NMR spectra of EPS produced by *Agaricus blazei* with *Spirulina platensis* (A), *Spirulina platensis* (B), and *Agaricus blazei* (C). Acetone was used as internal standard, chemical shifts - ppm (δ)

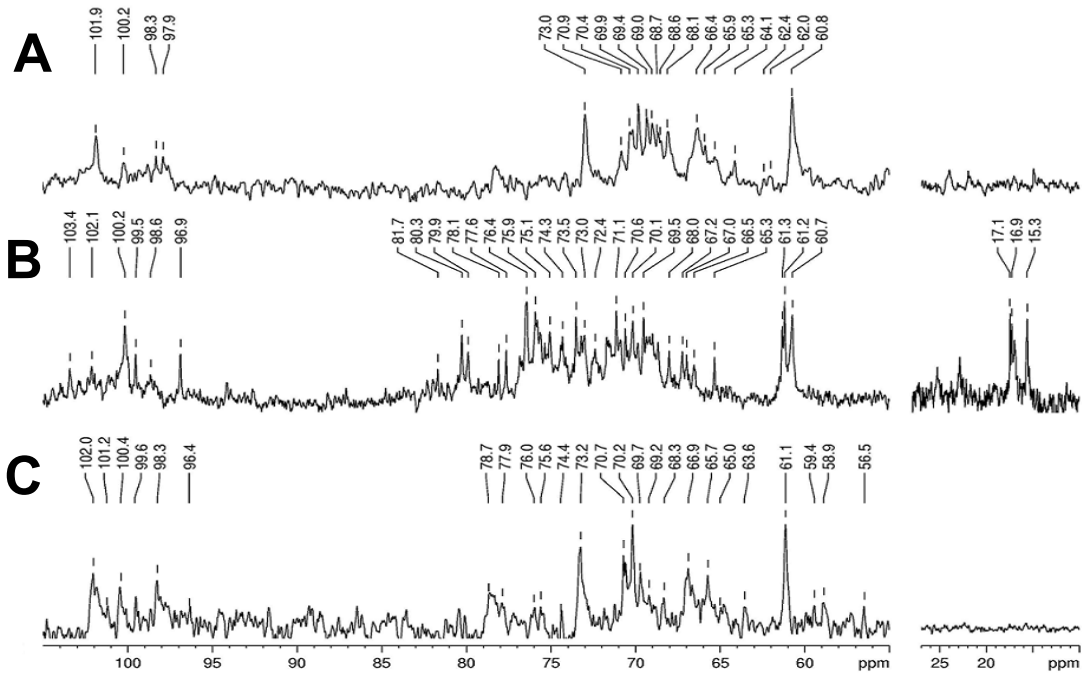


Figure 29 - ^{13}C NMR spectra of EPS produced by *Trametes versicolor* with *Spirulina platensis* (A), *Spirulina platensis* (B), and *Trametes versicolor* (C). Acetone was used as internal standard, chemical shifts - ppm (δ)

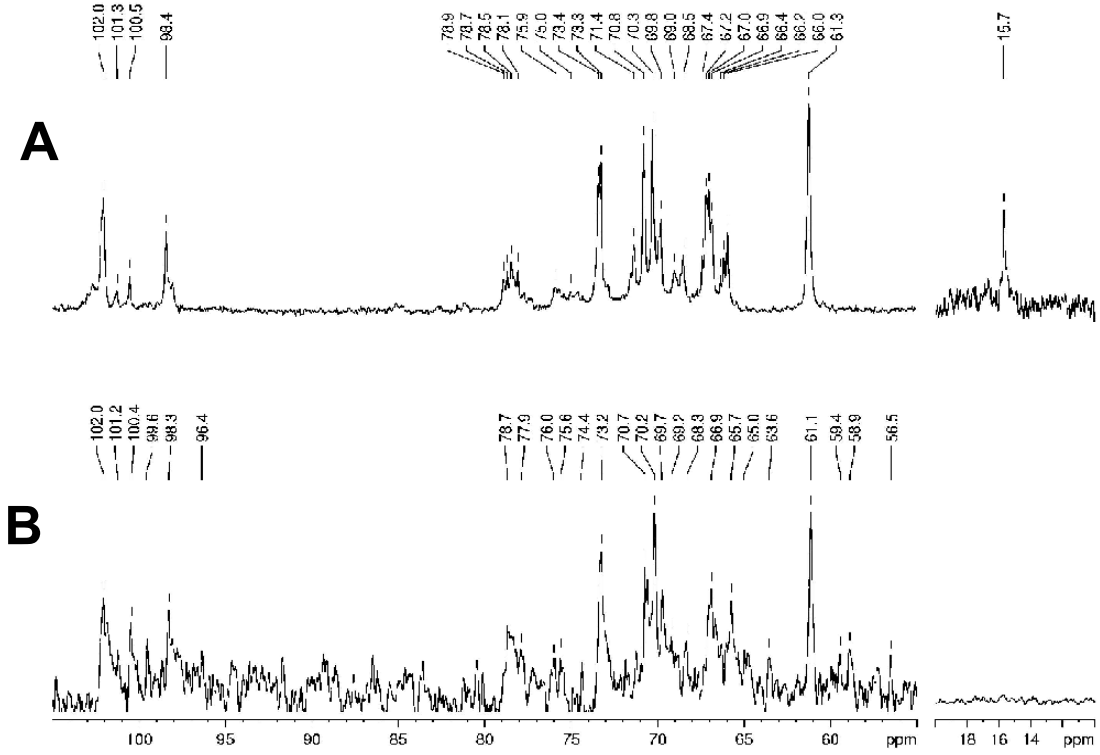


Figure 30 - ^{13}C NMR spectra of EPS produced by *Trametes versicolor* with *Chlorella vulgaris* (A), and *Trametes versicolor* (B). Acetone was used as internal standard, chemical shifts - ppm (δ)

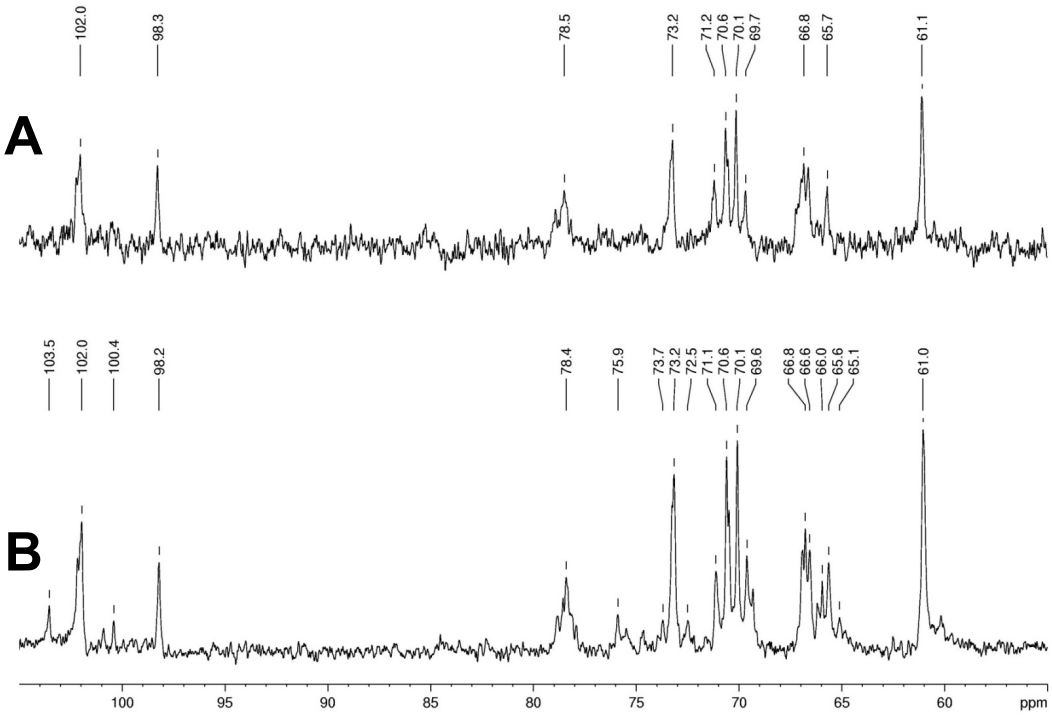


Figure 31 - ^{13}C NMR spectra of EPS produced by *Agaricus blazei* with *Chlorella vulgaris* (A), and *Agaricus blazei* (B). Acetone was used as internal standard, chemical shifts - ppm (δ)

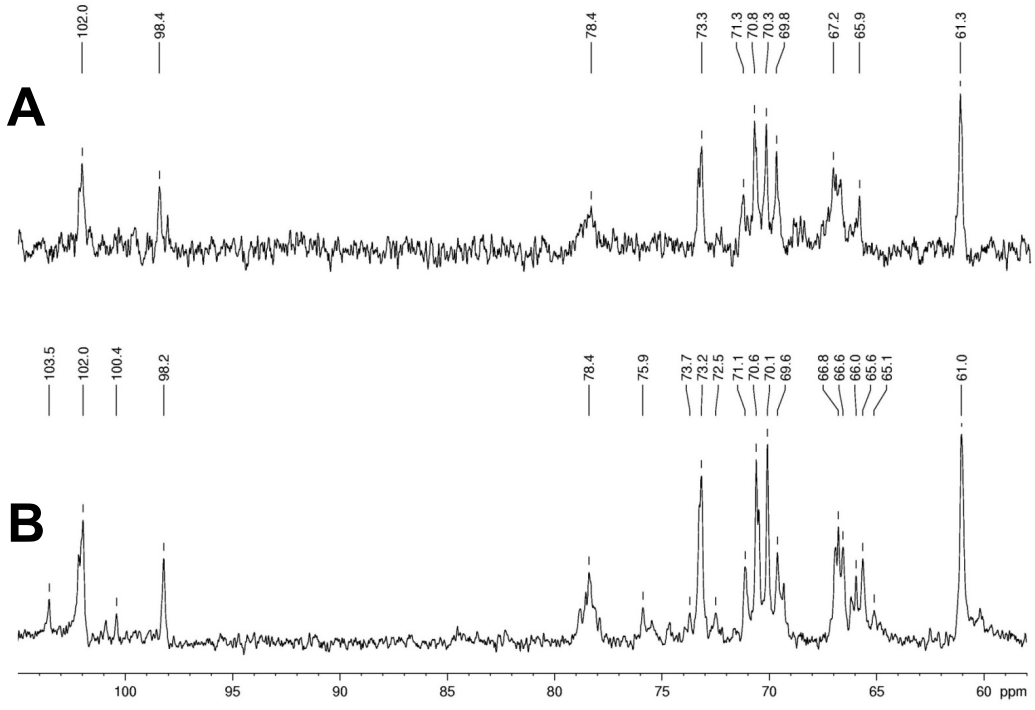


Figure 32 - ^{13}C NMR spectra for control. With different nitrogen sources: EPS produced by *Agaricus blazei using triptophan in the medium composition* (A), EPS produced by *Agaricus blazei* using yeast extract (B). Acetone was used as internal standard, chemical shifts - ppm (δ)

In the present study, we report on the partial characterization of water-soluble EPS produced by macromycetes and microalgae under submerged monoculture and co-cultures. Water-soluble EPS have been extensively studied and researchers have been shown that up to four different EPS can be produced by a single strain (HISAMATSU et al., 1982). We can't find about EPS produced by macromycetes and microalgae in a co-culture in the literature.

Knowing that we have EPS produced by yeasts and as we used yeast extracts in the culture medium, in order to eliminate any doubt about the interference of the yaest EPS on our results, we prepared a control (Figure 32), when we can observe in ^{13}C NMR spectra that *A.blazei* produced practically the same EPS using yeast extract or tryptophan as a nitrogen source, despite of the culture appearance was very different, as we can confirm in Figure 33.

Figure 33 - Photo of Agaricus blazei produced with yeast extract in the medium composition (A), Agaricus blazei produced with tryptophan in the medium composition (B)

The polysaccharides released are complex heteropolymers which are composed of six to ten different monosaccharides that we can confirm with the ^{13}C spectra. This characteristic, quite unusual in microbial polysaccharides of industrial interest which are in most cases composed by a lower number of monomers, is of great significance.

For ^{13}C NMR spectra, it was previously reported that the anomeric carbon (C-1) of a glycoside generally appears at 100~104 ppm, and that C-2, C-3, C-4, C-5, and C-6 of the glycosidic ring appear at 60~80 ppm (GONZAGA et. al, 2005). The ^{13}C NMR spectra of all the samples showed the characteristic peak for C-1 at 100~104 ppm, and for the spectra of *A. blazei* and *S. platensis*, we can see the typical peak for C-1 with β-(1→3) linkage at 103~103.8 ppm. (Fig. 9A and 7B).

The signals at 73 to 63 ppm were also assigned to C-2, C-3, C-4, C-5, and C-6.

The NMR spectra showed the mushroom EPS predominating in co-cultures, but we could detected some differences that could be related to a differences in the structure.

Figure 28 shows a highly complex spectrum (28B), with a variety of signals in the anomeric region, with five main signals at 100.2, 99.5, 96.9, 102.1 and 103.4 ppm. Signs in 17.1 and 15.3 ppm corresponding to CH3 and they confirm the presence of 6-desoxy sugars (fucose and rhamnose) in agreement with the monosaccharide composition results. For co-cultures we can see a simpler spectrum than *Spirulina* spectra, showing the fungal EPS predominance. We can identify some signals around 16 ppm and around 20~25 ppm showing probably a pyruvic acid acetal.

4.5 Conclusion

The present work showed a significant enhancement for EPS and biomass production by the co-culture. In addition, it suggests the presence of different structures in EPS obtained from the co-cultures and from the mono-cultures, what we can confirm with the monosaccharide composition. The NMR spectrum showed the predominance of fungal EPS in the co-cultures. Further studies are therefore required to better characterize the EPS.

5 ANTIOXIDANT ACTIVITIES OF EXTRACTS FROM SUBMERGED CO-CULTURE BY BASIDIOMYCETES, *Agaricus blazei* AND *Trametes versicolor*, AND MICROALGAE, *Spirulina platensis* AND *Chlorella vulgaris*

5.1 Abstract

Antioxidant activity of crude aqueous extracts and ethanolic extracts was investigated in this study, in the following submerged fermentations: *Agaricus blazei, Trametes versicolor, Spirulina platensis, Chlorella vulgaris*; and in the co-fermentations: *Agaricus blazei* with *Trametes versicolor*; *Agaricus blazei* with *Spirulina platensis, Agaricus blazei* with *Chlorella vulgaris*, *Trametes versicolor* with *Spirulina platensis, Trametes versicolor* with *Chlorella vulgaris*, *Spirulina platensis* with *Chlorella vulgaris*. Several biochemical assays were used to screen the antioxidant properties: DPPH free radical-scavenging, b-carotene/linoleic acid systems, total phenolic content**,** reducing power, radical scavenging capacities and inhibition of lipid peroxidation. Edible mushrooms and microalgae have potential as natural antioxidants. The extracts exhibited the most potent antioxidant activity at the maximum concentration tested of 1 mg/mL, but for every concentration, the extracts manifested significant antioxidant activity, which didn't exceede that of the positive controls (BHA and α-tocopherol). Increasing concentrations of the extract were found to cause progressively increasing activity. The data generated by this study strongly suggests that an crude and ethanolic extracts of the microorganisms in a co-culture or separately have potent antioxidant activity, suggesting that the extracts of the mushrooms and microalgae studied could serve as an easily accessible item of food rich in natural antioxidants, as a possible food supplement or even as a pharmaceutical agent.

Keywords: antioxidant activity; scavenging effect; reducing power; DPPH free radical-scavenging; β-carotene/linoleic acid systems; phenolic content; radical scavenging capacities; lipid peroxidation.

5.2 Introduction

Fungi and microalgae have played an important role as food, medicine, poison and for religious and other purposes in the life of man since remote times. For centuries, they have been used as food and for medicinal or functional purposes by various ethnic groups throughout the world, especially in Japan, China and Mexico (MOLITORI, 1994).

Studies have demonstrated that the regular consumption of mushrooms and algae or consumption of isolated bioactive constituents present in them is benefit to health. Some mushrooms and some microalgae may thus be considered as functional food (CHANG, 1989). The list of possible effects of mushrooms which promote good health is long and includes immunity improvement (INOUE, KODOMA & NAMBA, 2002), blood levels cholesterol and lipid reduction (CHENG; LU, 2002; FUKUSHIMA et al., 2001), blood pressure reduction (KABIR; KIMURA, 1987), blood glucose attenuation (KONNO et al., 2001), among several other actions (LEE et al. 1994; LEE et al. 1996; SONG et al. 1998; KIM et al. 1999; KIM et al. 2001; MAU et al. 2002; KIM et al., 2005). Recent literatures report that more than 270 medicinal fungi are used in traditional chinese medicine for their preventive and/or curative effects (CHENG; LEUNG, 2008).

Oxidation is essential to most living organisms for the production of energy to fuel biological processes. However oxidative stress occurs in a biological system after an increased exposure to oxidants, a decrease in the antioxidant capacity of the system, or both. It is often associated with or leads to the generation of reactive oxygen species (ROS), including free radicals, which are involved in the onset of many diseases such as cancer, rheumatoid arthritis, cirrhosis and arteriosclerosis as well as in degenerative processes associated with ageing. Reactive free radicals may come from endogenous sources through normal physiological and metabolic processes such as mitochondrial respiration. Alternatively, they could result from exogenous sources such as exposure to pollutants and ionizing irradiation, and particularly oxygen derived radicals are capable of oxidizing biomolecules, resulting in cell death and tissue damage (AMES, et al., 1993; CHEVION et al., 2000;

HALLIWELL; GUTTERIDGE, 2003). Free radicals are produced in normal and pathological cell metabolism.

Almost all organisms are well protected against free radical damage by oxidative enzymes such as superoxide dismutase and catalase or chemical compounds such as S-tocopherol, ascorbic acid, carotenoids, poliphenol compounds and glutathione (NIKI et al., 1994). However, these systems are many times insufficient to totally prevent the damage, resulting in diseases and accelerated ageing.

Oxidation is also one of the most important processes of food deterioration since it may affect food safety, colour, flavour and texture. Synthetic antioxidants have been used in stabilization of foods. The most commonly used synthetic antioxidants are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tert-butylated hydroxyquinone (TBHQ), that are applied in fat and oily foods to prevent oxidative deterioration (Löliger, 1991). On the other hand, BHA and BHT were found to be anticarcinogenic as well as carcinogenic in experimental animals. Originally, BHA appeared to have tumor-initiating as well as tumor-promoting action. Recently, it has been established that tumor formation appears to involve only tumor promotion caused by BHA and BHT (BOTTERWECK et al., 2000).

Natural products with antioxidant activity may be used to help the human body to reduce oxidative damage (HALLIWELL; GUTTERIDGE, 2003). Many fruits, vegetables, herbs, cereals, sprouts, seeds, microalgae and edible mushrooms have been investigated for antioxidant activity in the last years.

The antioxidant activity of plant materials was well correlated with the content of their phenolic compounds (VELIOGLU, et al., 1998). Phenolics are one of the major groups of nonessential dietary components that have been associated with the inhibition of atherosclerosis and cancer (WILLIAMS; IATROPOULOS, 1997). The bioactivity of phenolics may be related to their ability to chelate metals, inhibit lipoxygenase and scavenge free radicals (DECKER, 1997).

Many species of fruits, vegetables, herbs, cereals, sprouts and seeds have been investigated for antioxidant activity in the past (KUMAR et al., 2006). Components with antioxidant activities can be found in only a few species of algae. Although the occurrence of phenolic compounds in plants is well known and these

groups of compounds possess antioxidant activity in biological systems. Some studies reported that cancer was prevented by algae extracts (FEDKOVIC et al., 1993), because of their antioxidant properties. Mushrooms accumulate a variety of secondary metabolites, including phenolic compounds, polyketides, terpenes and steroids (CHEUNG et al., 2003). The antioxidants present in mushrooms are of great interest as protective agents to help the human body reduces oxidative damage without any interference. They are recognized as functional foods and as a source of physiologically beneficial components (WASSER; WEIS, 1999).

Therefore, our objective was to examine the antioxidant properties of the ethanolic and hot water extracts assayed included reducing capability, scavenging abilities of radicals and chelating abilities to metal ions. The contents of potential antioxidant components in these extracts were also determined.

5.3 Materials and methods

5.3.1 Antioxidant Activity

a) β-Carotene - linoleic acid assay

The antioxidant activity of extracts was determined, according to the b-carotene bleaching method described by DAPKEVICIUS and co-workers (1998). A stock solution of β-carotene–linoleic acid mixture was prepared as follows: 0.5 mg β-carotene was dissolved in 1 ml of chloroform (HPLC grade) and 25 µl of linoleic acid and 200 mg of Tween 40 were added. Chloroform was completely evaporated, using a vacuum oven. Then, 100 ml of distilled water, saturated with oxygen (30 min 100 ml/min), were added with vigorous shaking. 4000 µl of this reaction mixture were dispensed into test tubes and 200 µl portions of the extracts, prepared at 1 mg/ml, 500 µg/ml and 100 µg/ml concentrations, were added and absorbances were immediately measured at 490nm. The emulsion system was incubated for at 50 °C temperature, with periodic measurements at each 30 min. for 2h. The same procedure was repeated with synthetic antioxidants, BHT and α-tocopherol, as

positive control, and a blank. Antioxidative capacities of the extracts were compared with those of BHA, α-tocopherol and blank. The antioxidant capacity was expressed as percentage of inhibition of oxidation with the decrease in absorbance, measured in relation to control. Tests were carried out in triplicate.

Equation:

$$AA = 100 * [1 - \frac{A_0 - A_t}{A_0^0 - A_t^0}], \quad (1)$$

Where:

AA = antioxidant activity
A_0 = initial absorbance of the sample
A_t = final absorbance of the sample
A_0^0 = initial absorbance of the control
A_t^0 = final absorbance of the control

b) Reducing power activity

The reducing power of samples was determined by the method of OYAIZU (1986). Crude and ethanol extracts of various concentrations from the cultures were mixed with sodium phosphate buffer (2.5 mL, 200 mM, pH 6.6) and potassium ferricyanide (1%, 2.5 mL). The mixture was incubated at 50 8°C for 20 min, and then trichloroacetic acid (10%, 2.5 mL) (w/v) was added and centrifuged at 650 rpm for 10 min. The upper layer (5.0 mL) was mixed with deionized water (5.0 mL) and ferric chloride (0.1%, 1.0 mL), and the absorbance was measured at 700 nm in a spectrophotometer. Each value was expressed by the mean of triplicate measurements with standard deviations. The higher the absorbance value the higher the reducing power.

c) DPPH assay

According CUENDET and collaborators (1997), the hydrogen atom or electron donation abilities of the corresponding extracts and some pure compounds were measured from the bleaching of the purple-coloured methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH). This spectrophotometric assay uses the stable radical, DPPH* as a reagent. One thousand microlitre of various concentrations of the extracts in ethanol were added to 4 ml of 0.004% methanol solution of DPPH*. After a 30 min incubation period at room temperature, the absorbance was read against a blank at 517 nm. The capability to scavenge the DPPH radical was calculated using the capability to scavenge the DPPH radical was calculated using the following equation:

$$FRS(\%) = 100 * [\frac{A_0 - A_1}{A_0}], \quad (2)$$

Where:

$FRS\ (\%)$ = Free radical scavenging - DPPH* scavenging effect percentage

A_0 = absorbance of absorbance of the DPPH solution

A_1 = absorbance in the presence of the sample

The extract concentration providing 50% of radicals scavenging activity (EC50) was calculated from the graph of DSE percentage against extract concentration. BHA and a-tocopherol were used as standards (BARROS & BAPTISTA et al., 2007) Tests were carried out in triplicate.

d) Scavenging effect on superoxide anion

The ability of ethanolic extracts on scavenging the superoxide anion was determined by the method of NISHIKIMI et al. (1972). The reaction mixture, that contained the same volume of extracts, 60 mM phenazine methosulfate (PMS), 468 mM nicotinamide adenine dinucleotide (NADH), and 150 mM nitroblue tetrazolium (NBT) in a total volume of 3 mL of phosphate buffer (0.1 M, pH 7.4), was reacted at ambient temperature for 5 min and the absorbance measured, at 560 nm, against a blank in a spectrophotometer. Each value was expressed by the mean of triplicate measurements with standard deviation.

The inhibition percentage was calculated by using following formula:

$$inhibition\ rate\ (\%) = \frac{[A_0-(A_1-A_2)]}{A_0*100} \quad (3)$$

Where

A_0 = absorbance of control (using water instead of extract solution)

A_1 = absorbance in the presence of the sample

A_2 = absorbance in the presence of the sample under identical conditions as A_1 with 0.1 M phosphate

e) Determination of total phenolic compounds

Total soluble phenolics in the aqueous and ethanolic extracts were determined with Folin–Ciocalteu reagent, according to the method of Slinkard (SLINKARD; SINGLETON, 1977), using galic acid as a standard. Briefly, 1 ml of extract solution (contains 2000 µg) in a volumetric flask was diluted glass-distilled water (46 ml). Folin–Ciocalteu reagent (1 ml) was added and the contents of the flask were mixed thoroughly. After 3 min, 3 ml of $Na_2\,CO_3$ (2%) was added, then the mixture was allowed to stand for 2 h with intermittent shaking. The absorbance was measured at 760 nm. The concentration of total phenolic compounds in the mushroom ethanolic extract, determined as microgrammes of pyrocatechol

equivalents, by using an equation that was obtained from the standard galic acid graph, is given as:

$$Absorbance = 0{,}0003\ \mu g\ galic\ acid + 0{,}0428 \qquad R^2 = 0{,}9973 \qquad (3)$$

5.4 RESULTS

a) Antioxidant activity of extracts

The crude extracts: CEA (Crude extract of *A. blazei*), CEAC (Crude extract of *A. blazei* and *C. vulgaris*), CEAS (Crude extract of *A. blazei* and *S. platensis*), CEAT (Crude extract of *A. blazei* and *T. versicolor*), CET (Crude extract of *T. versicolor*), CETC (Crude extract of *T. versicolor* and *C. vulgaris*), CETS (Crude extract of *T. versicolor* and *S. platensis*), CEC (Crude extract of *C. vulgaris*), CES (Crude extract of *S. platensis*) and CECS (Crude extract of *C. vulgaris* and *S. platensis*); and ethanolic extracts: EEA (Ethanolic extract of *A. blazei*), EEAC (Ethanolic extract of *A. blazei* and *C. vulgaris*), EEAS (Ethanolic extract of *A. blazei* and *S. platensis*), EEAT (Ethanolic extract of *A. blazei* and *T. versicolor*), EET (Ethanolic extract of *T. versicolor*), EETC (Ethanolic extract of *T. versicolor* and *C. vulgaris*), EETS (Ethanolic extract of *T. versicolor* and *S. platensis*), EEC (Ethanolic extract of *C. vulgaris*), EES (Ethanolic extract of *S. platensis*) and EECS (Ethanolic extract of *C. vulgaris* and *S. platensis*), were subjected to screening for their possible antioxidant activity. Five complementary test systems, namely DPPH free radical-scavenging, β-carotene/linoleic acid systems, total phenolic compounds, reducing power activity and scavenging effect on superoxide anion, were used for the analysis.

b) β-Carotene–linoleic acid assay

The results of antioxidant activities show that some of the samples have antioxidant potential. To test the oxidation inhibition of β-carotene, the antioxidant potential of the sample is measured by its ability to taking the free radical generated during the peroxidation of linoleic acid, then, how easier the oxidation is, more it will compete with the β-carotene in reaction with the radicals, protecting it. The method is

based on spectrophotometric measures of discoloration (oxidation) of β-carotene, induced by oxidative degradation products of linoleic acid.

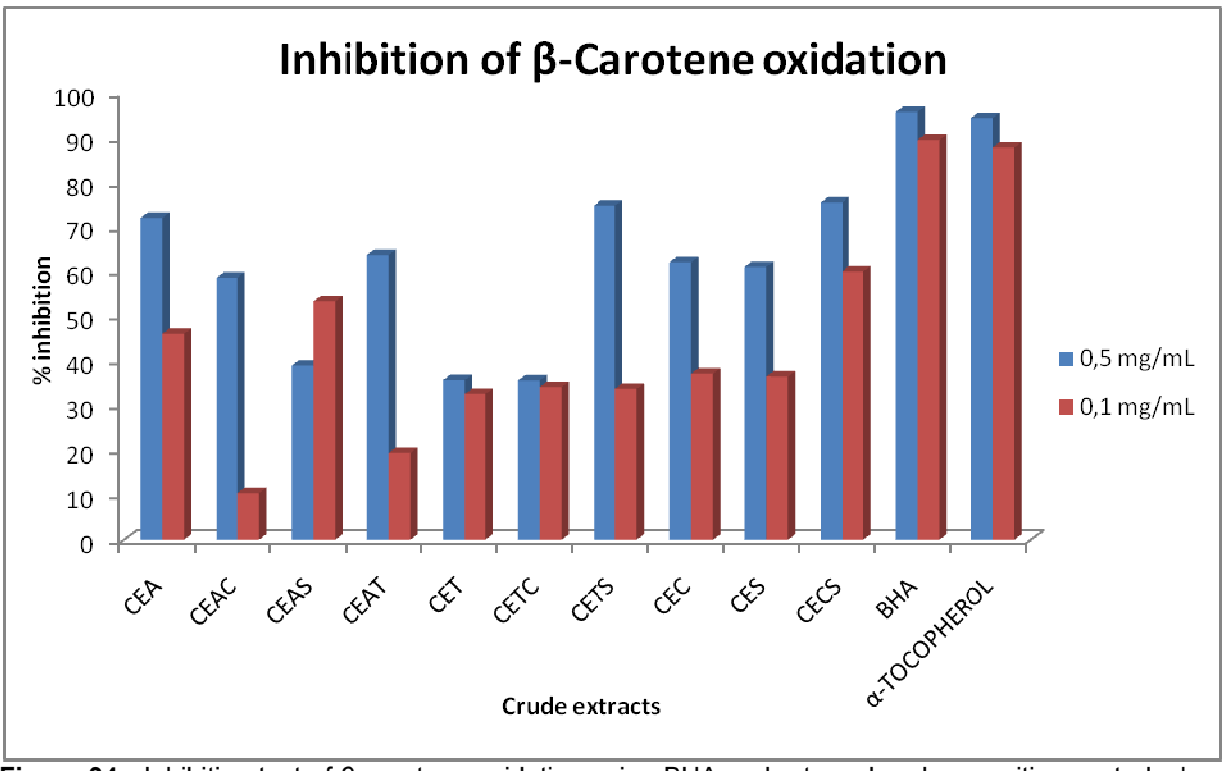


Figure 34 - Inhibition test of β-carotene oxidation using BHA and α-tocopherol as positive controls, by different doses of crude extracts (0.1 mg/mL and 0.l5 mg/mL) in the linoleic acid emulsion

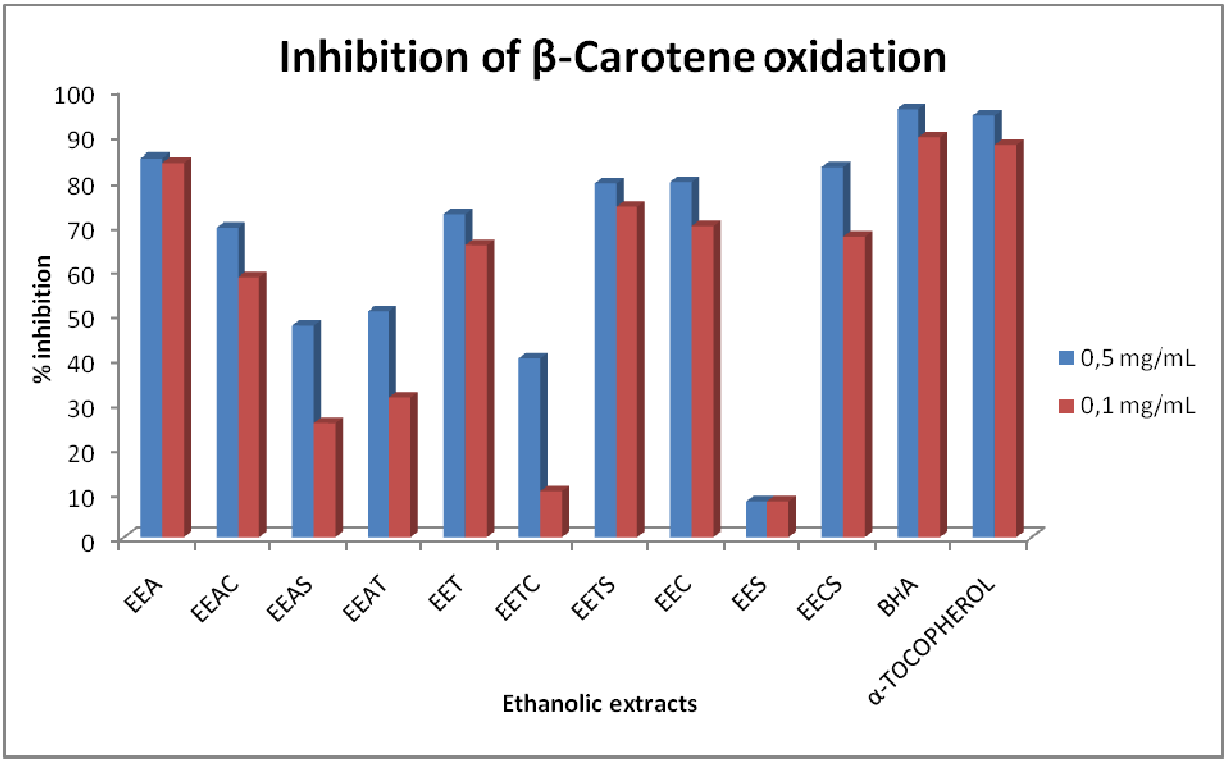


Figure 35 - Inhibition test of β-carotene oxidation using BHA and α-tocopherol as positive controls, by different doses of ethanolic extracts (0.1 mg/mL and 0.5 mg/mL) in the linoleic acid emulsion

The inhibition value increases with concentration. Linoleic acid oxidation was compared with those of ethanol extract, α-tocopherol and BHA. It was found that inhibition values of ethanol extracts, crude extracts and the standards increased with concentration. For example; at 0.5 mg/ml concentration, CETS extract, BHA and α-tocopherol showed 74.6%, 95.6%, 94.2% of inhibition whereas, at 0.1 mg/ml concentrations, these were 33.5%, 89.3%, 87.6%.

We can observe the decrease of the activity by some ethanol extracts comparing with the crude extracts. It can be attributed to heating that samples are submitted, it can degrade some important substances, sensitive to heating.

We can infer that the extracts could compete with BHA and α-tocopherol and increasing the concentrations probably they will have the same inhibition we can see in standards.

c) DPPH assay

We used the stable free radical diphenylpicrylhydrazyl (DPPH), with characteristic absorptions at 517 nm, to estimate the activity of antioxidants. The DPPH is characterised as a stable free radical by virtue of the delocalisation of the spare electron over the molecule as a whole, so that the molecules do not dimerise, as would be the case with most other free radicals. The delocalisation also gives rise to the deep violet colour, characterised by an absorption band in ethanol solution centred at about 517nm (SONGKLANAKARIN, 2004). As antioxidants donate protons to these radicals, the absorbance decreases. The decrease in absorbance is taken as a measure of the extent of radical-scavenging. Free radicial scavenging capacities of the extracts are shown in Figures 36 and 37. The scavenging effects extracts increased with their concentrations.

We could see a high scavenging ability, above 60%, at 1,0 mg/mL for the crude extracts from *A. blazei, A. blazei* and *C. vulgaris* co-culture and *T. versicolor* and *C. vulgaris* co-culture and for the ethanolic extracts from *T. versicolor*, *A. blazei* and *C. vulgaris*, *A. blazei* and *T. versicolor* and *C. vulgaris and S. platensis* co-cultures. HUANG et al. (1999) who found a high scavenging ability of 97.1 % at 2.5 mg/ml for the methanolic extract from *A. blazei*.

The standard BHA presented a scavenging effect of 91,29% at the concentration of 1,0 mg/ml. Free radical-scavenging is one of the known mechanisms by which antioxidants inhibit lipid oxidation. These tests are standard assays of antioxidant activity and they provide a technique for screening the radical scavenging activity of specific compounds or extracts (AMATOWICZ et al., 2004).

The decrease verified with some ethanolic extracts could be related to the process that the samples were submitted. The heating can provide a considerable loss of the active principles in the samples, due to the heat instability of the antioxidant molecules.

The EC50 value expresses the amount of extract necessary to decrease the absorbance of DPPH by 50 % (ANTOLOVICH et al. 2002). The value can be determined graphically by plotting the absorbance against the used extract concentration or calculated by using the slope of the linear regression (Table 11)

Based on the values of DPPH scavenging effect against extracts concentration, we established the linear correlations that in some cases had a high level of significance with a R^2 above 0,8. Some of them didn't show a good linear correlation and, in these cases, we can't consider the EC50 calculated. The linear correlations and EC50 values are presented in Table 11.

Table 11 - Correlation between the samples concentration and their reduction capability. The extract concentration providing 50% inhibition (EC50) was calculated from the equation obtained from the graph of DPPH scavenging effect against extract concentration

Samples	Equation	EC50 (mg/mL)
CEA	$\%\text{reduction} = 28.197 * \text{C} + 35.194\ \text{R}^2 = 0.987$	0.52
CEAC	$\%reduction = 28.430 * C + 43.235\ R^2 = 0.954$	0.24
CEAS	$\%reduction = 10.263 * C + 29.046\ R^2 = 0.860$	2.04
CEAT	$\%reduction = 14.479 * C + 35.834\ R^2 = 0.990$	0.98
CET	$\%reduction = 11.101 * C + 34.424\ R^2 = 0.423$	1.40
CETC	$\%reduction = 27.759 * C + 33.667\ R^2 = 0.9874$	0.59
CETS	$\%\text{reduction} = 10.949 * \text{C} + 29.14\ \text{R}^2 = 0.6079$	1.90
CEC	$\%reduction = 12.228 * C + 29.643\ R^2 = 0.7730$	1.66
CES	$\%reduction = 10.872 * C + 30.076\ R^2 = 0.9984$	1.83
CECS	$\%reduction = 3.6495 * C + 34.10\ R^2 = 0.9869$	4.30
EEA	$\%reduction = 6.2142 * C + 34.031\ R^2 = 0.7735$	2.57
EEAC	$\%reduction = 18.59 * C + 46.761\ R^2 = 0.9705$	0.17
EEAS	$\%reduction = 10.339 * C + 40.712\ R^2 = 0.7022$	0.90
EEAT	$\%reduction = 25.018 * C + 38.263\ R^2 = 0.9734$	0.47
EET	$\%reduction = 2.3077 * C + 56.935\ R^2 = 0.7793$	-3.00
EETC	$\%reduction = 3.7352 * C + 45.26\ R^2 = 0.7596$	1.27
EETS	$\%reduction = 11.634 * C + 39.055\ R^2 = 0.9219$	0.94
EEC	$\%reduction = 2.0032 * C + 36.160\ R^2 = 0.9992$	6.91
EES	$\%reduction = 8.9691 * C + 32.852\ R^2 = 0.8949$	1.91
EECS	$\%reduction = 15.302 * C + 48.882\ R^2 = 0.9787$	0.07

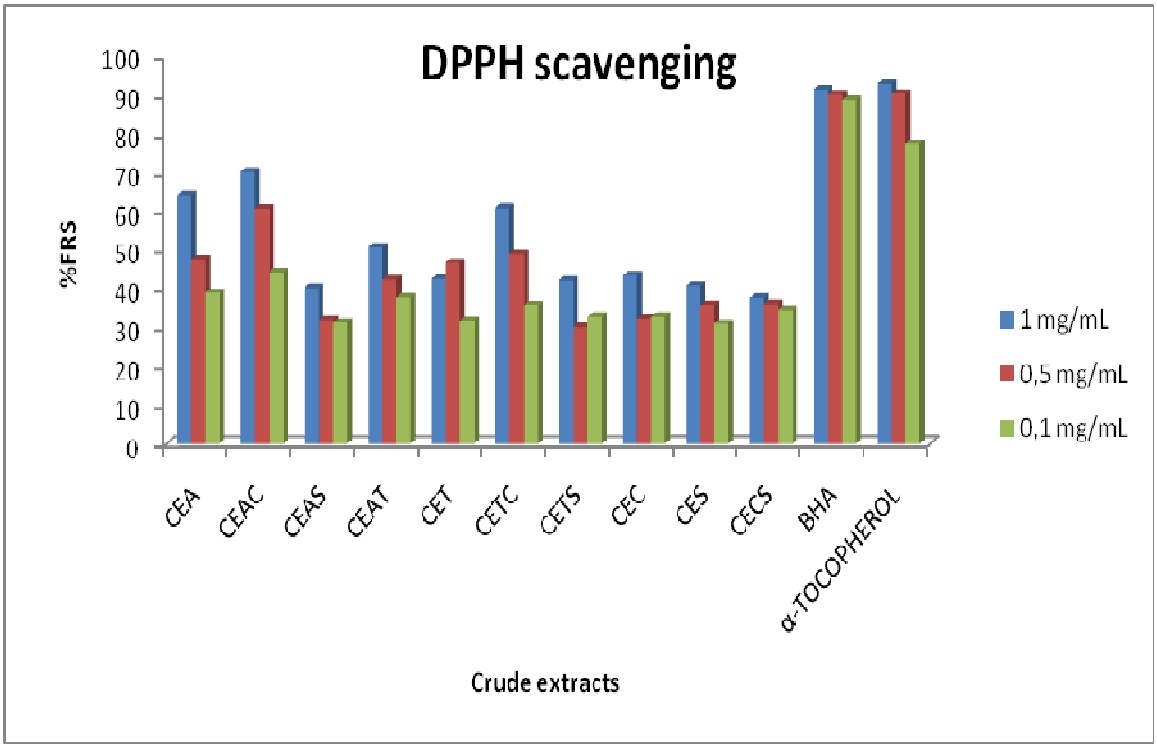


Figure 36 - Free radical-scavenging (FRS) capacities of crude extracts, measured by DPPH assay using BHA and α-tocopherol as positive controls, by different doses of crude extracts

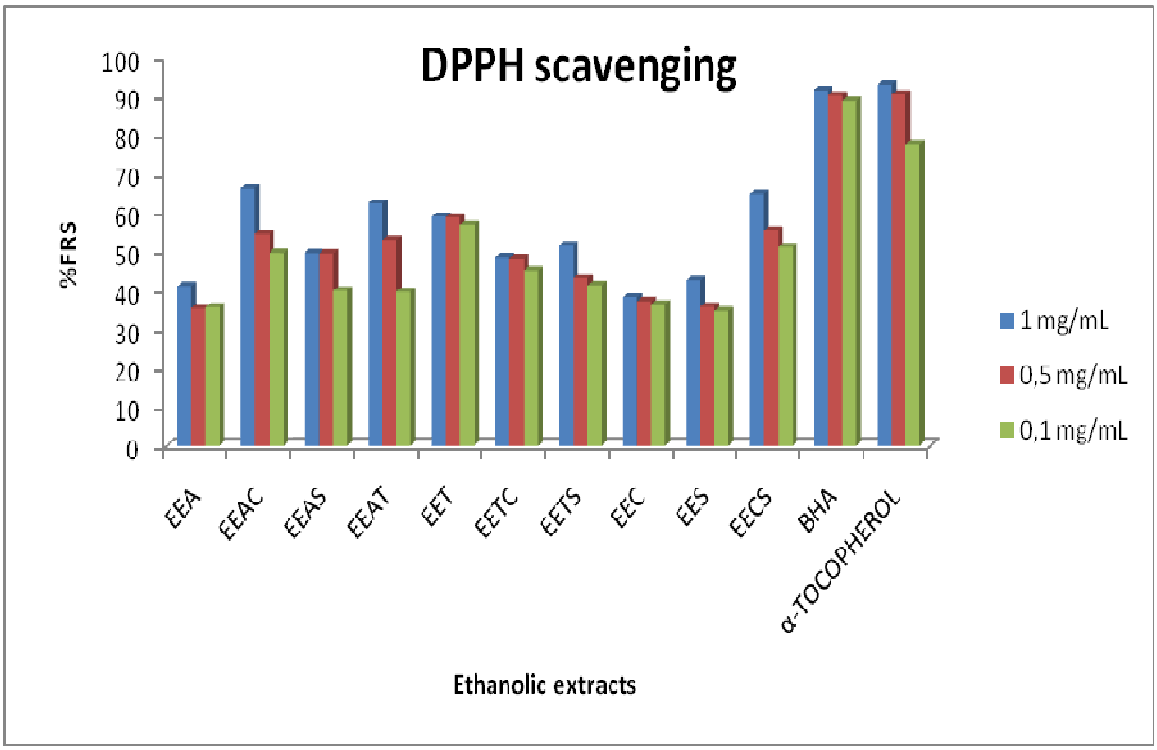


Figure 37 - Free radical-scavenging (FRS) capacities of ethanol extracts, measured by DPPH assay using BHA and α-tocopherol as positive controls, by different doses of ethanolic extracts

d) Reducing power activity

Figure 38 and 39 shows the reducing power of crude and ethanol extracts as a function of their concentration, respectively. In this assay, the yellow color of the test solution changes to various shades of green and blue, depending on the reducing power of each compound (BARROS et al., 2007). The presence of reducers causes the conversion of the Fe3+/ferricyanide complex used in this method to the ferrous form. By measuring the formation of Perl's Prussian blue at 700 nm, it is possible to determine the Fe2+ concentration. BHA and α-tocopherol (1.0 mg/mL) the positive control, used in this test, had a reducing power value of at 0,93 and 0.97 respectively. The reducing power of the extracts increased with their concentrations. At 0.1, 0.5 and 1.0 mg/mL, reducing powers from CETC, which one demonstrated more activity for crude extracts, were around 0.8, 0.69 and 0.52, respectively. For ethanolic extracts, the EEAT showed the highest activity: 0.91 at 1.0 mg/mL; 0.76 at 0.5 mg/mL and 0.70 at 0.1 mg/mL. HUANG et al. (1999) found that the methanolic extract from *A. blazei* showed a reducing power of 0.86 at 10 mg/ml, while a reducing power of around 0.7 was found to a ethanolic extract of the mushroom (TSAI, TSAI & MAU, 2007). With regard to reducing powers, the ethanolic extracts of were good as compared to edible and medicinal mushroom in general (CHOI et al., 2006). However, exceptionally high reducing power has been described by some medicinal mushrooms. MAU, LIN & SONG, (2002) showed that 4 mg/ml methanolic extracts of *Ganoderma tsugae* and *Ganoderma lucidum*, present reducing powers of 2.38 and 2.28, respectively.

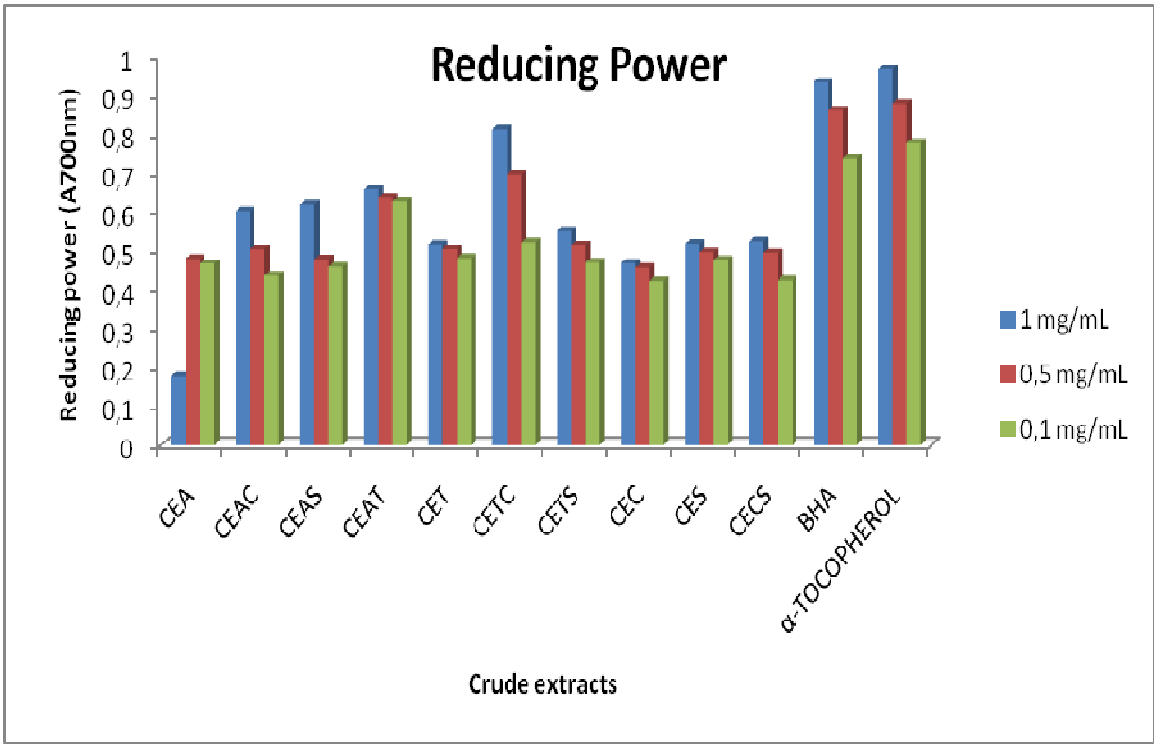


Figure 38 - Reducing power of ethanol extracts using BHA and α-tocopherol as positive controls, by different doses of extracts (0.1 mg/mL, 0.5 mg/mL and 1 mg/ mL)

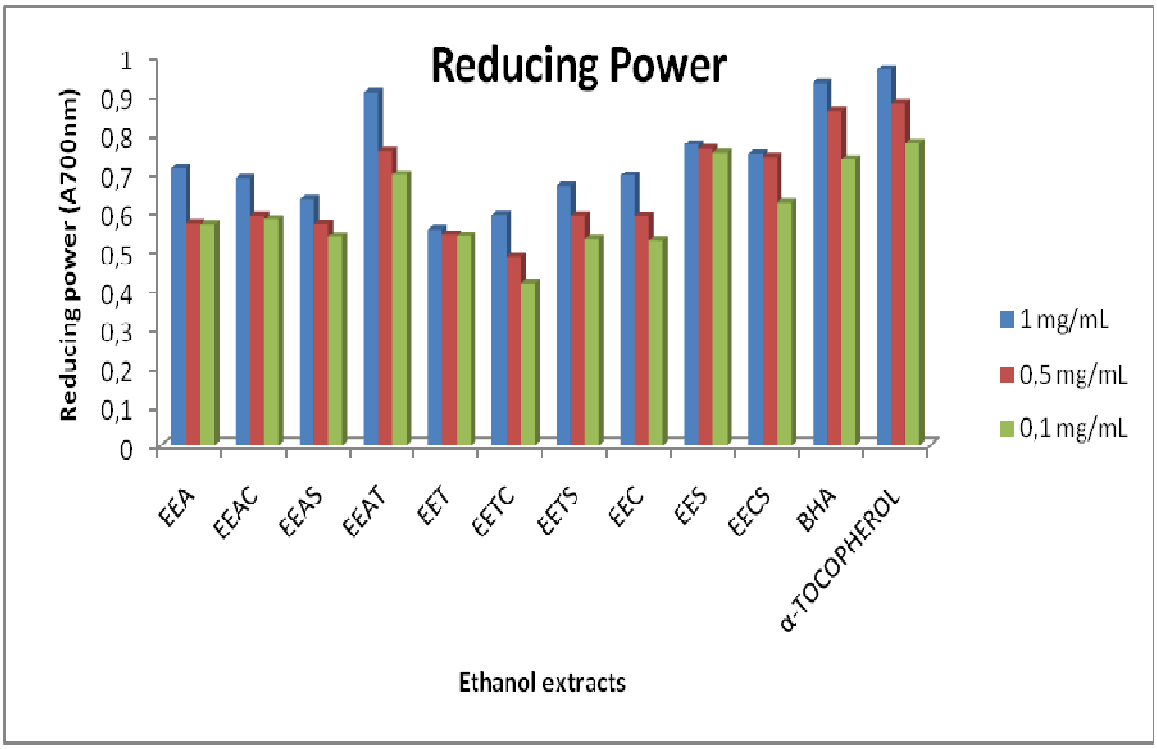


Figure 39 - Reducing power of ethanol extracts using BHA and α-tocopherol as positive controls, by different doses of extracts (0.1 mg/mL, 0.5 mg/mL and 1 mg/ mL)

e) Scavenging effect on superoxide anion

Superoxide anion and hydrogen peroxide are the main reactive oxygen species causing the oxidation of cells and tissues (STIEF, 2003).

Among different reactive oxygen species (ROS), $O_2\cdot$ is generated first. Although $O_2\cdot$ is a relatively weak oxidant, it may decompose to form stronger ROS, such as singlet oxygen and hydroxyl radical (OH·), which initiate peroxidation of lipids. $O_2\cdot$ is also known to initiate indirectly the lipid peroxidation as a result of the formation of H_2O_2, creating precursors of OH· (MEYER; ISAKSEN, 1995). Therefore, O2· scavenging is extremely important to antioxidant work.

Scavenging activities of crude and its ethanolic fractions against $O_2\cdot$ were presented in Figures 40 and 41. Notably, every sample showed stronger scavenging activity. However, the scavenging effects of CEA were significant weaker than that of its ethanolic extract. The scavenging effects of extracts didn't increase significantly with the increase of sample concentration. The results demonstrated that all the samples possessed $O_2\cdot$ scavenging activities, showing strong activities.

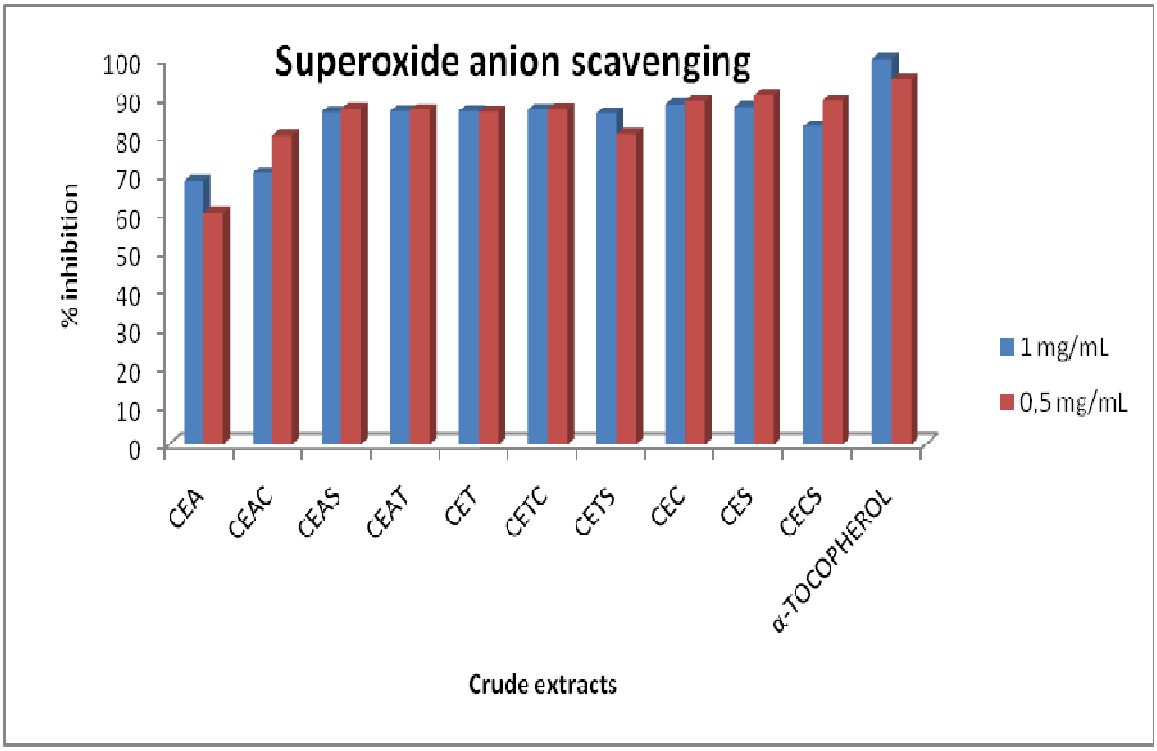


Figure 40 - Superoxide anion scavenging activity of crude extracts using α-tocopherol as positive controls, by different doses of extracts (0.5 mg/mL and 1 mg/ mL)

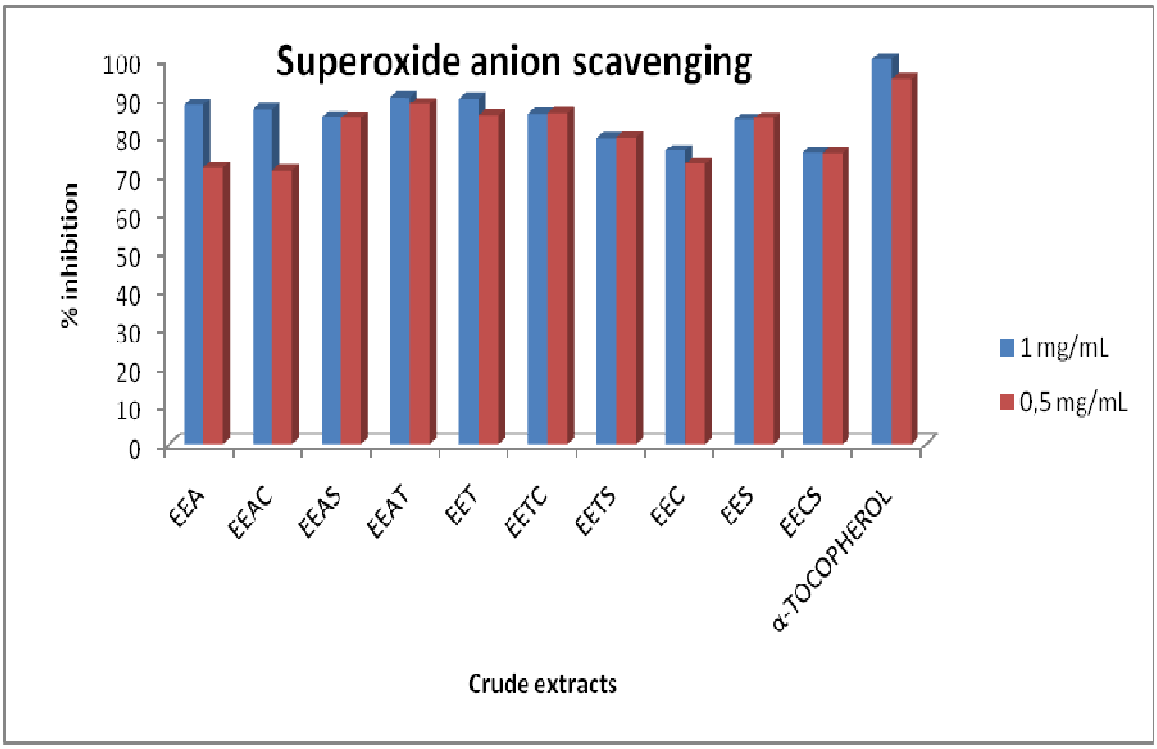


Figure 41 - Superoxide anion scavenging activity of ethanol extracts using α-tocopherol as positive controls, by different doses of extracts (0.5 mg/mL and 1 mg/ mL)

f) Determination of total phenolic compounds

Some authors have already reported a direct correlation between mushrooms antioxidant activity and total phenolic content, although the antioxidant action is raised by other substances such as tocopherols and b-carotene (CHEUNG; CHEUNG; OOI, 2003).

The bioactivity of phenolics may be related to their ability to chelate metals, inhibit lipoxygenase and scavenge free radicals (DECKER, 1997). Also, in food systems, flavonoids can act as free radical scavengers and terminate the radical chain reactions that occur during the oxidation of triglycerides. Therefore, they present antioxidative efficiency in oils, fats and emulsions (ROEDIG-PENMAN; GORDON, 1998).

The amount of phenolic compound was calculated as galic acid equivalents. The quantities of phenolic compounds present in each extract are presented in Tables 12 and 13.

Amounts of phenolic components of ethanol extracts were found to be from approximately 35 to 150 µg/µg galic acid equivalents and for crude extracts were around from 35 to 360 µg/µg galic acid equivalents. The higher values have been obtained in the crude extracts. This result suggests that the heat treatment could promote a loss of some phenolic compounds (CHOI et. al., 2006).

The phenolic compounds may contribute directly to antioxidative action (DUH; TU; YEN, 1999).

Table 12 - Amounts of total phenolic compounds in crude extracts. Data expressed as means ± s.e.m. of three samples analysed separately.(1) Galic acid equivalents (2) Standard deviation

Extracts	Phenolic Compounds $(\mu g/mg)^{(1)}$
CEA	357.33 ± 0.21 (2)
CEAC	170.77 ± 0.13 (2)
CEAS	57.33 ± 0.11 (2)
CEAT	99.00 ± 0.22 (2)
CET	74.00 ± 0.35 (2)
CETC	82.33 ± 0.08 (2)
CETS	72.33 ± 0.20 (2)
CEC	44.00 ± 0.13 (2)
CES	35.67 ± 0.41 (2)
CECS	36.67 ± 0.92 (2)

Table 13 - Amounts of total phenolic compounds in ethanolic extracts Data expressed as means ± s.e.m. of three samples analysed separately.(1) Galic acid equivalents (2) Standard deviation

Extracts	Phenolic Compounds $(\mu g/mL)^{(1)}$
EEA	85.67 ± 0.67 (2)
EEAC	129.00 ± 0.54 (2)
EEAS	79.00 ± 0.32 (2)
EEAT	162.33 ± 0.41 (2)
EET	87.33 ± 0.85 (2)
EETC	77.33 ± 0.12 (2)
EETS	67.33 ± 0.35 (2)
EEC	142.33 ± 0.12 (2)
EES	35.67 ± 0.42 (2)
EECS	35.67 ± 0.64 (2)

5.5 Conclusion

Mono-culture and co-culture crude and ethanol extracts showed significant antioxidant activity, namely DPPH free radical-scavenging, b-carotene/linoleic acid systems, reducing power, radical scavenging capacities and inhibition of lipid peroxidation, that can be related to the presence of phenolic compounds. Overall, all the species proved to have antioxidant properties. The crude extract of A. blazei showed to have a great amount of phenolic compounds. The work described in this study confirmed that microalgae and macromycetes can be considered as important sources of antioxidant compounds.

6 FINAL DISCUSSION AND CONCLUSION

6.1 Conclusions

Some algaes and fungis are considered as functional food and have many benefits to health, being rich sources of natural antioxidants. So called ‘immunomodulators’ (biological response modifier, immunopotentiators and immunostimulants) the EPS produced by some macromycetes and some algaes are very important for traditional medicine, especially in Japan, China, Korea and other East Asian countries today.

Microalgae and mushrooms may serve as a continuous and reliable source of natural products, including antioxidants, because they can be cultivated in bioreactors on a large scale. Furthermore, the qualities of these micoorganisms cells can be controlled, so that they contain no herbicides and pesticides, or any other toxic substances, by using clean nutrient media for growing. The value of microalgae and mushrooms as a source of medicinal EPS and natural antioxidants and is further enhanced by the relative ease of extraction and purification of target compounds.

Microalgae and mushrooms represent an almost untapped resource of natural antioxidants, due to their enormous biodiversity. However, not all groups of these microorganisms can be used for the purposes, due to their widely varied contents of target products, growth rate or yields, ease of cultivation, and/or other factors. Reports on the biological activity of these microorganisms are extensive.

Therefore, it was desirable to identify some rich sources of EPS and antioxidants. In these studies we proponed to identify new sources of safe and inexpensive EPS and antioxidants using the co-culture as a strategy to accelerate and increase the EPS production, knowing that in stress conditions, the microorganisms produce a great amount of EPS, being in that way a metabolic strategy of them for growth and development in unfavorable conditions.

Using statistical methods was possible to determine the optimal operating conditions to obtain higher biomass growth and EPS production. The validity of the model was proven by fitting the values of the variables in the second-order equation and by carrying out the experiment at those values of the variables. However, little is

known about the co-cultivation of microalgae and fungi. EPS characteristics and its activity were showed different from that produced in a monoculture and need to be deeply investigated. The current research has identified several key phenomena and opened avenues for future work, especially into underlying mechanisms that link the EPS production with co-culture metabolisms.

Summarizing, we have these scientific results:

a. The co-culture EPS and biomass production increase with increasing the glucose concentration till up to approximately 52 g/L, increasing the agitation speed till up to approximately 200rpm and in moderate initial pH (approximately 5.0).
b. Co-culture accelerates biomass and EPS production.
c. Mushroom EPS predominates in co-culture production.
d. The differences in monosaccharide composition are significant when we compare mono-cultures and co-cultures. These results could represent different EPS structures
e. Mono-culture and co-culture extracts showed significant antioxidant activity, that can be related to the presence of phenolic compounds.

6.2 Future Research Questions

1. Examine the antitumor activity in vivo and in vitro of EPS front tumor cells lines such as melanoma, tumor, Walker, leukemia, and others.
2. Test the anti microbial and anti viral activity of the extracts.
3. Test the sub acute and chronic toxicity of the EPS in animals.
4. Total Characterization of the EPS.
5. In vivo assays with diabetic animals to check the anti-hypoglycemic and hyperglycemia EPS effects.
6. Extraction, purification and characterization of others bioactive substances produced, testing in vitro and in vivo to verify its substance efficiency.

7. Check the effects of a supplemented diet with dry extract from the co-cultures on carbohydrates-lipid metabolism in animals with induced diabetes.
8. Define the optimal conditions for extracting and purifying the EPS from co-cultures.
9. Study the pigment production on tryptophan medium by *Agaricus blazei*

REFERENCES

ADACHI, Y. et al. TH1-Oriented Immunomodulating Activity of Gel-Forming (1→3)-β-Glucan. **International Journal of Medicinal Mushrooms**, v. 3, p. 95–109.

AKALEZI, C.O. et al. Combined effects of initial sucrose concentration and inoculation size on cell growth and ginseng saponin production by suspension cultures of Panax ginseng. **Process Biochem**, v.34, p. 639–642, 1999.

AMERICAN DIETETIC ASSOCIATION. Position of the American Dietetic Association: functional foods. **J. Amer. Diet, Assoc. U.S.A.,** v. 104, p. 814-826, 2004.

AMES, B. M.; SHIGENAGA, M. K.; HAGEN, T. M. Oxidants, antioxidants and the degenerative diseases of aging. **Proceedings of the National Academy Sciences**, Washington, v.90, p. 7915–7922, 1993.

ANTOLOVIC, M. et al. Methods for testing antioxidant activity. **Analyst**, London, v. *127*, p. 183–198, 2002.

BABITSKAYA, V. G.; SHCHERBA, V. V.; LKONNIKOVA, N. Melanin complex of the fungus *Inonotus obliquus.* **Applied Biochemistry and Microbiology**, Moscow, v. 36, 2002, p. 377-381.

BAE, J.T. et al. Optimization of submerged culture conditions for exo-biopolymerproduction by *Paecilomyces japonica*. **Journal of Microbiology and Biotechnology**, v. 10, p. 482-487, 2000.

BAO, X.-F. et al. Structural features of immunologically active polysaccharides from *Ganoderma lucidum.* **Phytochemistry,** Maryland Heights, v. 59, p. 175–181, 2002.

BARROS, L.; BAPTISTA, P.; FERREIRA, I.C.F.R. Effect of *Lactarius piperatus* fruiting body maturityl stage on antioxidant activity measured by several biochemical assays. **Food and chemical toxicology**, v. 9, p.1731-1737, 2007.

BARROS, L. et al. Antioxidant activity of *Agaricus sp.* mushrooms by chemical, biochemical and electrochemical assays. **Food Chemistry,** v.111, p. 61-66, 2008.

BELLINI, M.F. et al**. Anticlastogenic effect of aqueous extracts of *Agaricus blazei* on CHO-K1 cells, stying different developmental phases of the mushroom.** Toxicology in Vitro, v. 17, p. 465-469.

BOBEK, P.; GALBAVÝ, S. Hypocholesterolemic and antiatherogenic effects of oyster mushroom (*Pleurotus ostreatus*) in rabbits, **Nahrung**, v. 43, p. 339–342., 1999.

BOBEK, P.; HROMADOVÁ, M; OZDÍN, L. (1995). Oyster mushroom (Pleurotus ostreatus) reduces the activity of 3-hydroxy-3-methylglutaryl CoA reductase in rat liver microsomes**. Experientia,** v. 51, p. 589–591, 1995.

BOBEK, P.; OZDÍN, L.; GALBAVÝ, S. Dose- and time-dependent hypocholesterolemic effect of oyster mushroom (Pleurotus ostreatus) in rats. **Nutrition**, v. 14, p. 282–286, 1998.

BOBEK, P.; OZDÍN, L.; KUNIAK, L. Mechanism of hypocholesterolemic effect of oyster mushroom (*Pleurotus ostreatus*) in ratsreduction of cholesterol absorption and increase of plasma cholesterol removal. **Z. Ernährungswiss,** v. 33, p. 44–50, 1994.

BOTTERWECK, A. A. M. et al. Intake of butylated hydroxyanisole and butylated hydroxytoluene and stomach cancer risk: results from analyses in the Netherlands cohort study**. Food and Chemical Toxicology**, v.38, p. 599–605, 2000.

BOX, G.E.P.; HUNTER, J.S.; HUNTER, W.G. **Statistics for Experimenters.** 2. ed**.** New York: Wiley-Interscience, 2005.

BRAUER, D.; KIMMONS, T.; PHILLIPS. Effects of management on the yield and high-molecular-weight polysaccharide content of Shiitake (*Lentinula edodes*) mushrooms. **Jounal of Agricultural and Food Chemistry**, v. 50, p. 5333-5337. 2002.

BRUNS, R.E.; SCARMINIO, I.S.; B. NETO, B. **Statistical Design.** Amsterdam: Chemometrics, Elsevier, 2006.

CALPIS, CO. et al. Physiologically functional food having brain function-improving, learning ability-enhancing, and memory-enhancing functions. **A23C 009/12; A01N 063/00; A23L 001/31; C12N 001/00 U.S**. n. 6, p. 284,243, 2001.

CATLEY, B.J. The biochemistry of some fungal polysaccharides with industrial potencial. In: Arora, D.K.; Elander, R.P.; Mukerji, K.G. **Handbook of applied mycology**: fungal biotechnology. New York: Marcel Dekker, 1992. p.1114.

CHANG, S. T. **Edible mushroom and their cultivation**. Florida: CRC Press, 1989.

CHENG K.F.; LEUNG P.C. General review of polysaccharopeptides (PSP) from *C. versicolor*: pharmacological and clinical studies**. Cancer Therapy**, California, v. 6, p. 117-130, 2008.

CHENG, H.H.; HOU, W.C.; LU, M.L. Interactions of lipid metabolism and intestinal physiology with *Tremella fuciformis* berk edible mushrooms in rats fed a high-cholesterol diet with or without Nebacitin. **Jounal of Agricultural and Food Chemistry**, Washington, v. 50, p. 7438-1743, 2002.

CHEN, X.; LI, Y. DU. G.C.; CHEN, J. Application of response surface methodology in medium optimization for spore production of Coniothyrium minitans in solid-state fermentation. **World J. Microbiol. Biotechnol**, v. 21, p. 593–599, 2005.

CHEVION, S.; ROBERTS, M. A.; CHEVION, M. The use of cyclic voltammetry for the evaluation of antioxidant capacity. **Free Radical Biology and Medicine**, v. 28, p. 860–870, 2000.

CHIHARA, G. et al. Fractionation and purification of the polysaccharides with marked antitumor activity, especially, lentinan, from *Lentinus edodes*. **Cancer Res**, v. 30, p. 2776–2781, 1970.

CHU, K.K.W.; HO, S.S.S.; CHOW, A.H.L. Coriolus versicolor: a medicinal mushroom with promising immunotherapeutic values**. J. Clin. Pharmacol,** v. 42, p. 976–984, 2002.

CHUNG K.S., KIM S.S. et al. Effect of Kp, an antitumor protein polysaccharide from mycelia culture of Phellinus linteus on the humoral immune response of tumor bering ICR mice to sheep red blood cells**. Arch. Pharm. Res**, v. 16, p. 336-338, 1993.

CLEMENT, C. et al. The development of Spirulina algae cultivation**. Chem Eng Sci**, v. 35, p. 119-126, 1980.

COCKSHOTT, A.R; SULLIVAN, G.R. Improving the fermentation medium for Echinocandin B production. Part I: sequential statistical experimental design. **Process Biochem**, v. 36, p. 647–660, 2001.

CUENDET, M.; HOSTETTMANN, K.; POTTERAT, O. Iridoid glucosides with free radical-scavenging properties from Fagraea blumei. **Helvetica Chimica Acta**, v. 80, p. 1144–1152, 1997.

CUI, F. J. et al. Optimization of the medium composition for porduction of mycelial biomass and exopolymer by Grifola frondosa GF 9801 using response surface methodology. **Bioresource Technology**, v. 97, p. 1209-1216, 2006.

DAPKEVICIUS, A. et al. Antioxidant activity of extracts obtained by different isolation procedures from some aromatic herbs grown in Lithuania**. Journal of the Science of Food and Agriculture**, v. 77, 140–146, 1998.

DAVYDOV, M.; KRIKORIAN, A.D. Eleutherococcus senticosus (Rupr & Maxim.) Maxim. (Araliaceae) as an adaptogen: a closer look. J**. Ethnopharmacol**, v. 72, p. 345-393, 2000.

DECKER, E. A. Phenolics: prooxidants or antioxidants? **Nutrition Reviews**, v. 55, p. 396-407, 1997.

DE PHILIPPIS, R.; VINCENZINI, M. Exocellular polysaccharides from cyanobacteria and their possible applications**. FEMS Microbiol,** v. 22, p.151-175, 1998.

DIKEMAN, L.L. BAUER. et al. Effects of stage of maturity and cooking on the chemical composition of select mushroom varieties. **J. Agric. Food Chem**, v. 53, 1130-1138., 2005.

DONG Y., C. Y. et al. Antitumor effects of a refined polysaccharide fraction isolated from *Coriolus versicolor*: In vitro and in vivo studies. **Research Communications in Molecular Pathological Pharmacology**, v. 92, p. 140-148, 1996.

DUBOIS, M.; et al. Colorimetric method for determination of sugars and related substances. **Anal. Chem**, v.28, p. 350-356, 1956.

FAN, L. et al. Effect of nutritional and environmental conditions on the production ofexo-polysaccharide of Agaricus brasiliensis by submerged fermentationand its antitumor activity. **LWT - Food Science and Technology**, v. 40, p. 30-36, 2005.

FIRENZUOLI, F.; GORI, L.; LOMBARDO, G. The Medicinal Mushrooms Agaricus blazei Murrill: review of literature and pharmaco-toxicological problems. **Evid Based Complement Alternat Med**, v. 5, p. 3-15, 2008.

FUJIMIYA, Y. et al. Tumorspecific cytocidal and immunopotentiating effects of relatively low molecular weight products derived from the basidiomycete *Agaricus blazei* Murrill. **Anticancer Research**, v. 19, p. 113–118, 1999.

FUJITA, H. et al. Effect of PSK, a protein-bound polysaccharide from *Coriolus versicolor*, on drug-metabolizing enzymes in sarcoma-180 bearing and normal mice. **Int. J. Immunopharmacol**, v. 10, p. 445–450, 1988.

FUKUSHIMA, M. et al. Hepatic LDL receptor mRNA in rats is increased by dietary mushroom (Agaricus bisporus) fiber and sugar beet fiber, **J. Nutr.**, v. 130, p. 2151-2156, 2000.

FUKUSHIMA, M. et al. Cholesterol-lowering effects of maitake (Grifola frondosa) fiber, shiitake (Lentinus edodes) fiber, and enokitake (Flammulina velutipes) fiber in rats**, Exp. Biol. Med,** v. 226, p. 758–765, 2001.

GALVEZ, A.O. **Crescimento das diatomaceas Bacillariophyceae: *Chaetocerus sp., Skeletonema costatum* e *Thalassiosira fluviatilis* em diferentes meios de cultura e em condições controladas de temperatura e salinidade**. Master grade monograph in Water Culture, Department of Water Culture, Federal University of Santa Catarina, Florianopolis, Brazil, 1993.

GERN, J. C. **Production and characterization of extra-cellular polysaccharide from submerged fermentation by *Agaricus brasiliensis.*** Master grade monograph in Biotechnology, Department of Chemical Engineering, Federal University of Parana, Curitiba, Brazil, 2005.

GONZAGA, M. L., et al. Isolation and characterization of polysaccharides from Agaricus blazei Murill. **Carbohydr.Polym,** v. 60, p. 43-49, 2005.

GRAY, A.M.; FLATT, P.R. Insulin-releasing and insulin-like activity of Agaricus campestris (mushroom). **J. Endocrinol,** v. 157, p. 259-266, 1998.

GREGORY P.H.; HIRST, J.M. The summer airspora at Rothasmsted in 1952. **J Gen Microbiol**, v. 17, p. 135, 1957.

GRUBE, J. et al. White button mushroom phytochemicals inhibit aromatase activity and breast cancer cell proliferation, **J. Nutr**, v. 131, p. 3288-3293, 2001.

GUTERREZ, Z. R., et al. Variation of antimutagenicity effects of water extracts of Agaricus blazei Murill in vitro. **Toxicology in Vitro**, v. 18, p. 301-309, 2004.

GUTIÉRREZ, A. **Exopolisacaridos y metabolitos aromaticos de Pleurotus: naturaleza y función en la degradación de la lignina**. Sevilla, 1995. 121 p. (Ph.D. Thesis Facultad de Farmacia, Universidad de Sevilla),

HALLIWELL, B.; GUTTERIDGE, J. M. C. **Free radicals in biology and medicine.** Oxford, UK: Oxford University Press, 2003.

HAN, S.N. et al. Effect of mushroom (Coriolus versicolor) extract on the immune response of young and old mice. **FASEB J**, v. 10, p. 3200, 1996.

HATTORI, T.S. et al. Protein-boundpolysaccharide K induced apoptosis of the human Burkittlymphoma cell line. **Biomed Pharmacother** , v. 58,p. 226-230, 2004.

HETLAND G. et al. Effects of the medicinal mushroom Agaricus blazei Murill on immunity, infection and cancer. **Scand J Immunol,** v. 68, p. 363-370, 2008.

HIRASE, S. et al. **Studies on antitumor activity of polysaccharides**. [S.l]: Proc. Jpn. Canc. Assoc. 29th Annu. Meet, 1970. 288p.

HUANG, Q., Y. et al. Structure, molecular size and antitumor activities of polysaccharides from Poria cocos mycelia produced in fermenter. **Carbohydr. Polym**, v.70, p. 324-333, 2007.

HYDE H.A.; ADAMS K.F. Airborne allergens at Cardiff 1942-1959. **Acta Allergol**, v.15, p. 159, 1960.

INOUE, A.; KODAMA, N.; NAMBA, H. Effect of maitake (*Grifola frondosa*) D-fraction on the control of the T lymph node Th-1/Th-2 proportion. **Biol. Pharm. Bull**, v. 25, p. 536-540, 2002.

ITOH H, ITO H.; AMANO H, NODA H. Inhibitory action of a (1 fi 6)-beta-D-glucan-protein complex (F III-2-b) isolated from *Agaricus blazei* Murill (‘‘himematsutake’’) on Meth A fibrosarcoma-bearing mice and its antitumor mechanism. **Jpn J Pharmacol**, v. 66, p. 265–271, 1994.

JAYAKUMAR , T.; RAMESH, E.; GERALDINE, P. Antioxidant activity of the oyster mushroom, Pleurotus ostreatus, on CCl4-induced liver injury in rats. **Food Chem. Toxicol**, v. 44, p. 1989-1996, 2006.

JONG, S.C.; BIRMINGHAM J.M. Medicinal and therapeutic value of the Shiitake mushroom. **Adv Appl Microbial**, v. 39, p. 153-184, 1993.

JONG S.C.; YANG X.T. **PSP- a powerful biological response modifier from the mushroom *Coriolus versicolor*. Yang,Q.Y.** Hong Kong: Advanced Research in PSP - Association for Health Care, p.16-28, 1999.

JONG, S.C.; DONOVICK, R., Antitumor and antiviral substances from fungi. **Adv. Appl. Microbiol.**, v. 34, p. 183–262, 1989.

KABIR, Y.; YAMGUCHI, M.; KIMURA, S. Effect of shiitake (*Lentinus edodes*) and maitake (*Grifola frondosa*) mushrooms on blood pressure and plasma lipids of spontaneously hypertensive rats. **Journal Nutr. Sci. Vitaminol.,** v. 33, p. 341-346, 1987.

KAGEYAMA, H. et al. Simple isolation of phycocyanin from Spirulina platensis and phycocyanobilin-protein interaction. **J Mar Biotechnol,** v. 1, p. 185-188, 1994.

KIHO, T. et al. Polysaccharide in fungi. Anti-diabetic activity and structural features of galatomannan elaborated by Pestalotiopsis species. **Biol. Pharm. Bull**, v. 20, p. 118-121, 1997.

KIM, B.K.; PARK, E.K. Studies on the constituents of the higher fungi of Korea 12. Anti-neoplastic components of *Coriolus versicolor*, *Pleurotus ostreatus* and *Lentinus edodes*. **J. Nat. Prod.,** v. 42, p. 684, 1979.

KIM, D.H. et al. Production of a hypoglycemic, extracellular polysaccharide from the submerged culture of mushroom, Phellinus linteus*.* **Biotechnology Letters,** v. 23, p. 513-517, 2001.

KIM, D.H.; SHIM, S.B.; KIM, N.J.; JANG, I.S.(1999). **Beta-glucoronidase-inhibitory activity and hepatoprotective effect of *Ganoderma lucidum***. Biol. Pharm. Bull., v. 22, n. 2, p. 162-164.

KIM, Y. W.; KIM, K. H.; CHOI, H. J.; LEE, D. S. **Anti-diabetic activity of β-glucans and their enzymatically hydrolyzed oligosaccharides from *A. blazei.*** Biotechnol. Let., v. 27, p. 483-487, 2005.

KNIGHT, J.A. The biochemistry of aging. **Adv Clin Chem**., v. 35, p. 1-62, 2000.

KONNO, S. et al. A possible hypoglycemic effect of maitake mushrooms on Type 2 diabetic patients. **Diabetic Medicinal**, v. 18, p. 1007- 1010, 2001.

KULICKE, W. M.; LETTAU, A.I.; THIELKING, H. Correlation between immunological activity, molar mass, and molecular structure of different (1→3)-β-D-glucans. **Carbohydrate Research,** v. 297, p. 135-143, 1997.

LEE C.L., YANG X.T.; WAN J.M.F. The culture duration affects the immunomodulatory and anticancer effect of polysaccharopeptide derived from *Coriolus versicolor*. **Enzyme and Microbial Technology,** v. 38, p. 14-21, 2006.

LEE Y-LMING -TSUNG, Y.; MAU, J-L. Antioxidant properties of several medicinal mushrooms. **Journal of Agricultural and Food Chemistry**, v. 50, p. 6072-6077, 2007.

LEE, J.H. et al. Immunostimulating activity of polysaccharides from mycelia of *Phellinus linteus* grown under different culture conditions. **Journal of Microbiology and Biotechnology**, v. 6, p. 52-55, 1996.

LEE, K.H. et al. Correlation between anticomplementary and antitumor activity of crude polysaccharide from *Ganoderma lucidum* IY009. **Korean Journal of Applied Microbiology and Biotechnology,** v. 22, p. 45-51, 1994.

LEUNG, M.Y.K. et al. Polysaccharide biological response modifiers. **Immunology Letters,** v. 105, p. 101-114, 2006.

LI, J.F. Biological characteristics and pharmacological activities of Yun Zhi and its application. **J. Auhui Agricultural Sciences,** v. 31, p. 509-510, 2003.

LI, X.Y. et al. Immune enhancement of a polysaccharides peptides isolated from *Coriolus versicolor***. Acta Pharmacol. Sínica,** v. 11, p. 542-545, 1990.

LINDEQUIST, U.; NIEDERMEYER, T. H. J.; JÜLICH, W.-D. The pharmacological potential of mushrooms. **eCAM,** v. 2, p. 285-299, 2005.

LIU, F. et al. Immunomodulation and antitumor activity of polysaccharide–protein complex from the culture filtrates of a local edible mushroom, **Tricholoma lobayense. Gen. Pharmacol**., v. 27, p. 621-624, 1996.

LOWRY, O. H. et al. Protein measurement with the folin phenol reagent. **J. Biol. Chem.**, v. 193, p. 265, 1951.

MAO, X. W.; ARCHAMBEAU, J. O.; GRIDLEY D., S. Immunotherapy with low-dose interleukin-2 and a polysaccharopeptide derived from Coriolus versicolor. **Cancer Biotherapy and Radiopharmaceuticals***, v.* 11, p. 393-403, 1996.

MAO, X.W.; GRIDLEY, D.S. **Effects of extract of *Coriolus versicolor* and IL-2 against three tumor lines. FASEB J.,** v. 12, p. 5149, 1998.

MATSUNAGA, T. et al. An ultraviolet (UV-A) absorbing biopterin glucoside from the marine planktonic cyanobacterium *Oscillatoria sp.* **Appl Microbiol Biotechnol,** v. 39, p. 250–253, 1993.

MATTILA, P. et al. Basic composition and amino acid contents of mushrooms cultivated in Finlands**. Journal Agricultural and Food Chemistry**, v. 50, p. 6419-22, 2002.

MAU, J.L.; CHAO, G.R.; WU, K.T. Antioxidant properties of methanolic extracts from several ear mushrooms. **Journal of Agricultural and Food Chemistry**, v. 49, p. 5461-5467, 2001.

MAU, J.L.; LIN, H.C.; SONG, S. F. Antioxidant properties of several specialty mushrooms. **Food Research International**, v. 35, p. 519–526, 2002.

MAU, J.L.; LIN, H.C.; SONG, S.F. Antioxidant properties of several specialty mushrooms. **Food Research International,** v. 35, p. 519-526, 2002.

MAU, J. L.; LIN, H. C.; CHEN, C. C. Antioxidant properties of various extracts from *Hypsizigus marmoreus* . **Food Chemistry**, v. 104, p. 1-9, 2002.

MAYER, P.; DREWS, J. Effect of a protein-bound polysaccharide from Coriolus versicolor on immunological parameters and experimental infections in mice. **Infection,** v. 8, p. 13-21, 1980.

MENOLI, R.C.R.N. et al. Antimutagenic effects of the mushroom *Agaricus blazei* Murill extracts on V79 cells. **Mutation Research,** v. 496, p. 5-13, 2001.

MIZUNO, M. et al. Antitumor polysaccharide from the mycelium of liquid-cultured *Agaricus blazei* Murill**. Biochem Mol Biol Int**., v. 47, p. 707-714, 1999.

MIZUNO, M. et al. Polysaccharidesfrom Agaricus blazei stimulate lymphocyte T-cell subsets in mice. **Bioscience Biotechnology and Biochemistry**, v. 62, p. 434-437, 1998.

MIZUNO, T. Health foods and medicinal usages of mushrooms. **Food Rev. International**, v. 11, p. 69-81, 1995.

MOLITORIS, H.P. Mushrooms in Medicine. **Folia Microbiol**., v. 39, p. 91-98, 1994.

MOSMANN T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxic assay. **J. Immunol Methods,** v**.** 65, p. 55-63, 1983.

MOUHIM J.R. et al. Production, isolation and preliminary characterization of the exopolysaccharide of the cyanobacterium *Spirulina platensis.* **Biotechnol Lett**, v. 15, p. 567-572, 1993.

NARUSE, S.; TAKEDA, S. Studies on antitumor activity of Basidiomycete polysaccharide. II. Antitumor effects of polysaccharides prepared from cultures of Basidiomycetes**. Mie Med. J.,** v. 23, p. 207-231, 1974.

NELSON, N. A fotometric adaptation of Somogyi method for a determination of glucose. **J. Biol. Chem**., v. 153, p. 375-380, 1954.

NG, T.B. et al. Polysaccharopeptide (PSP) from mycelia of the mushroom *Coriolus versicolor*: an updated review. **Yang QY, Advanced Research in PSP - Hong Kong Association for Health Care**, v. 80-87, p. 1999.

NGAI, P.H.K.; NG, T.B. Lentin, a novel and potent antifungal protein from shitake mushroom with inhibitory effects on activity of human immunodeficiency virus-1 reverse transcriptase and proliferation of leukemia cells, **Life Sci**., v. 73, p. 3363-3374, 2003.

NIKI, E.; SHIMASKI, H.; MINO, M. **Antioxidantism – free radical and biological defence**. Tokyo: Gakkai Syuppan Center, 1994.

NISHIKIMI, M., N. A. RAO; K. YAGI. The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. **Biochem. Biophys. Res. Commun**., v. 46, p. 849, 1972.

OOI VE, LIU F. Immunomodulation and anti-cancer activity of polysaccharide-protein complexes. **Curr Med Chem**, v. 7, p. 715-729, 2000.

OYAIZU, M. Studies on products of browning reactions: antioxidative activities of products of browning reaction prepared from glucosamine. **Jpn. J. Nutr.,** v. 44, p. 307, 1986.

PARK, Y. K.; et al. Comparison of the flavonoid aglycone contents of Apis mellifera propolis from various regions of Brazil**. Arquivos de Biologiae Technologia**, v. 40, p. 97-106, 1997.

PAULSEN, B.S.; OLAFDOTTIR, E.S.; INGOLFSDOTTIR, K., J. Chromatography and electrophoresis in separation and characterization of polysaccharides from lichens. **Chromatogr**., v. 967, p. 163-171, 2002.

PUJARI, V.; CHANDRA, T.S. Statistical optimization of medium components for enhanced riboflavin production by a UV-mutant of Eremothecium ashbyii. **Process Biochem.**, v. 36, p. 31-37, 2000.

R.L, PLACKETT; J.P. BURMAN. The Design of Optimum Multifactorial Experiments. **Biometrika,** v. 33, p. 305-325, 1946.

RAU, U; et al. Production and structural analysis of the polysaccharide secreted by *Trametes (Coriolus) versicolor* ATCC200801**. Appl Microbiol Biotechnol**, v. 8, p. 1700-1712, 2008.

RIBEIRO, L. R.; SALVADORI, D. M. F. Dietary components may prevent mutation-related diseases in humans. **Mut. Res**., v. 544, p. 195-201, 2003.

RICHMOND, A. **Handbook of microalgal mass culture**. Boston: CRC, 1990.

SAKAGAMI, H. et al. Induction of immunhopotentiation activity by a protein-bound polysaccharide, PSK. **Anticancer Research**, v. 1, p. 993-1000, 1991.

SHIMADA, K. et al. Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion. **J. Agric. Food Chem**., v. 40, p. 945, 1992.

SLINKARD, K.; SINGLETON, V. L. Total phenol analyses: automation and comparison with manual methods. **American Journal of Enology and Viticulture**, v. 28, p. 49–55, 1977.

SOARES, A.A. et al. Antioxidant activity and total phenolic content of *Agaricus brasiliensis* (*Agaricus blazei* Murril) in two stages of maturity. **Food Chemistry**, v. 112, p. 775-781, 2009.

SOMOGYI, N. A new reagent for the determination of sugars**. J. Biol. Chem.**, v. 195, p. 19, 1952

SONG, C.H. et al. Anti-complementary activity of endo-polymers produced from submerged mycelial culture of higher fungi with particular reference to *Lentinus edodes*. **Biotechnol. Lett**., v.20, p. 741–744, 1998.

SOUZA-QUEIROZ, J. et al. Hematopoietic response of rats exposed to the impact of an acute psychophysiological stressor on responsiveness to an in vivo chalenge with Listeria monocytogenes: Modulation by Chlorella vulgaris prophylactic treatment. **Brain Behavior and Immunity**, v. 22, p. 1056-1065, 2008.

STIEF, T.W. The physiology and pharmacology of singlet oxygen. **Med. Hypotheses***, v.* 60, p. 567-572, 2003.

SUGANO, N. et al. Anti-carcinogenic action of an alcohol-insoluble fraction (LAP1) from culture medium of *Lentinus edodes* mycelia. **Cancer Lett**, v. 27, p. 1-6, 1985.

TANAKA, K. et al. Augmentation of host defense by a unicellular green alga, Chlorella vulgaris, to Escherichia coli infection. **Infect. Immun**., v. 53, p. 267–271, 1986.

TANAKA, K. et al. Oral administration of an unicellular green algae. Chlorella vulgaris, prevents stress-induced ulcer. **Planta Med**., v. 63, p. 465-466, 1997.

TANAKA, K. et al. A novel glycoprotein obtained from Chlorella vulgaris strain CK22 shows antimetastatic immunopotentiation. **Cancer Immunol. Immunother, v.** 45, p. 313-320, 1998.

TSANG, K.W. et al. *Coriolus versicolor* polysaccharide peptide slows progression of advanced non-small cell lung cancer. **Respiratory Medicine,** v. 97, p. 618-642, 2003.

TURKOGLU, A. et al. Antioxidant and antimicrobial activities of *Laetiporus sulphureus* (Bull.) Murrill. **Food Chemistry**, v. 101, p. 267-273, 2007.

UENO, S. et al. Method of producing nitrogen-containing polysaccharides**. U.S. Patent** , v. 4, n. 202, p. 969, 1980.

ULRIKE, L.; NIEDERMEYER, T.H.J.; JÜLICH , W.D. The Pharmacological Potential of Mushrooms. **eCAM**, v. 2, p. 285-299, 2005.

VELIOGLU, Y.S. et al. Antioxidant activity and total phenolics in selected fruits, vegetables and grain products. **Journal of Agricultural and Food Chemistry**, v. 46, p. 4113-4117, 1998.

WACHI, Y. et al. Effect of ultraviolet-A (UV-A) light on growth, photosynthetic activity and production of biopterin glucoside by the marine UV-A resistant cyanobacterium *Oscillatoria sp.* **Biochim Biophys Acta,** v. 1244, p. 165-168, 1995.

WANG, H.X. et al. Immunomodulatory and antitumor activities of a polysaccharide–peptide complex from a mycelial culture of Tricholoma sp., a local edible mushroom. **Life Sci**, v. 57, p. 269-281, 1995.

WANG, H.X. et al. Polysaccharide–peptide complexes from the cultured mycelia of the mushroom *Coriolus versicolor* and their culture medium activate mouse lymphocytes and macrophages. **Int. J. Biochem. Cell Biol**., v. 28, p. 601–607, 1996.

WANG, H.X. et al. A polysaccharide–peptide complex from cultured mycelia of the mushroom Tricholoma mongolicum with immunoenhancing and antitumor activities. **Biochem. Cell. Biol**., v. 74, p. 95-100, 1996.

WASSER, S. P. et al. Is a widely cultivated culinary-medicinal Royal Sun Agaricus (the Himematsutake mushroom) indeed *Agaricus blazei* Murrill? **International Journal of Medicinal Mushroom**, v. 4, p. 267-290, 2002.

WASSER, S.P.; WEIS, A.L. Therapeutic effects of substances occurring in higher Basidiomycetes mushrooms: a modern perspective. **Crit. Rev. Immunol**., v. 19, 65-96, 1999.

WATANABE, A. List of algal strains in collection at the Institute of applied microbiology University of Tokyo. **Journal of General and Applied Microbiology, London**, v. 6, p. 1-4, 1960.

WILLIAMS, G. M.; IATROPOULOS, M. J. **Antiocarcinogenic effects of synthetic phenolic antioxidants. In Oxidants, antioxidants, and free radicals.** USA: Taylor & Francis, 1997.

XIONG, Y.H. et al. Enhanced production of extracellular ribonucleic from Aspergillus niger by optimization of culture conditions using response surface methodology, **Biochem. Eng. J**., v. 21, p. 27–32, 2004.

YAN, J. et al. β-Glucan, a “specific” biological response modifier that uses antibody to target tumors for cytotoxic recognition by leukocyte complement receptor type 3 (CD11b/CD18). **The journal of immunology**, v. 163, p. 3045-3052, 1999.

YANG, Q. Y.; PAI, S. S. The anti-ageing effects of *Ganoderma* essence. **Proc Int Meet Ganoderma Sci**, v. 30, 2000.

YANG, M.M.P.; CHEN, Z.; KWOK, J.S.L. The antitumor effect of a small polypeptide from *Coriolus versicolor* (SPCV). **Am. J. Chin. Med**., v. 20, p. 221-232, 1992.

YANG, Q.Y. et al. Antitumor and immunomodulating activities of the polysaccharide–peptide (PSP) of *Coriolus versicolor*. **J. Immunol. Immunopharmacol**, v. 12, p. 29-34, 1992.

YANG, Q.Y.; YONG, S.C.; YANG, X.T. The physio-chemical characteristics of the polysaccharidepeptide (PSP) of *Coriolus versicolor* (Yun-zhi). **Report on the Polysaccharide - Peptide (PSP) of *Coriolus versicolor,*** p.1-6, 1987.

YASSER, R.A. et al. Improved production of Pseudomonas aeruginosa uricase by optimization of process parameters through statistical experimental designs, **Process Biochem**, v. 40, p. 1707-1714, 2005.

ZARROUK, C. **Contribution a l'etude d'une cyanophycee: Influence de divers facterurs physiques et chimiques sur la croissance et photosynthese de *Spirulina maxima***. France: PhD Thesis, University of Paris,1956.

ZHOU, X.W. et al. Cytotoxic activities of Coriolus versicolor (Yun zhi) extracts on human liver cancer and breast cancer cell line. **Afr. J. Biotechnol,** v. 6, p. 1740-1743, 2007.

ZJAWIONY, J. K. Biologically active compounds from Aphyllophorales (Polypore) fungi. **Journal of Natural Products**, v. 61, p. 1053-1071, 2003.