Université de Provence
(Aix-Marseille I)

Influence of drying methods over *in vitro* immuno-modulatory effect of polysaccharides from submerged fermentation by *Agaricus blazei*

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
Supervisor

By

Marcelo Boscaro Alberca Fernandes
Biotechnologist and Bioprocesses Engineer

*****

Biotechnology and Bioprocesses Engineering Division
Federal University of Paraná

August / 2008
CURITIBA – BRAZIL

AGRADECIMENTOS

A Deus, por me dar saúde e disposição para completar mais esta etapa em minha vida.

À minha família, pelo apoio e incentivo nos momentos difíceis.

À minha namorada Greta, por estar sempre ao meu lado compartilhando comigo as dificuldades e os momentos felizes.

Ao professor Soccol, pela orientação e disposição sempre em auxiliar e sugerir novas idéias, e pela confiança que me foi depositada para fazer parte do grupo do Departamento de Biotecnologia da UFPR.

Aos professores do Master BIODEV, pela oportunidade que me foi dada em participar deste programa de Mestrado e pela presteza em responder às dúvidas durante as aulas.

À banca examinadora, professores Tholozan, Lorquin, Adriane e Herta, pelas correções e sugestões que enriqueceram e aperfeiçoaram minha dissertação.

Aos professores do Laboratório de Processos Biotecnológicos, pela orientação e auxílio na prática laboratorial.

Aos amigos e colegas do Laboratório, pela agradável companhia e ajuda nos procedimentos experimentais.

Por fim, os meus sinceros agradecimentos a todos aqueles que tiveram, de alguma forma, alguma colaboração direta ou indiretamente em meu trabalho.

RESUMO

Agaricus blazei é um cogumelo que pertence à biodiversidade brasileira e é considerado um importante produtor de compostos bioativos benéficos à saúde humana. Vários estudos têm demonstrado que tais compostos apresentam propriedades imuno-moduladoras, antioxidantes e antitumorais. Com a finalidade de comparar o método mais utilizado para a secagem de polissacarídeos fúngicos (liofilização), com outros métodos de escala industrial, o objetivo deste trabalho foi submeter extratos de polissacarídeos de *A. blazei* às secagens por Estufa a Vácuo, "*Spray dryer*" e Liofilização, e avaliar a manutenção de seus efeitos imuno-moduladores *in vitro*. Extratos de polissacarídeos extra-celulares (*EPS*) e intra-celulares (*IPS*) produzidos pela fermentação submersa de *A. blazei* foram extraídos com etanol e submetidos aos processos de secagem. Os três métodos de secagem apresentaram valores satisfatórios de rendimento e eficiência. O teor de umidade e a atividade de água dos produtos secos resultantes mostraram-se suficientes para a prevenção contra o crescimento de microrganismos e reações de degradação química. A porcentagem de açúcares totais e de proteínas sofreu um decréscimo após as secagens, porém, os extratos secos de polissacarídeos extra-celulares apresentaram atividade imuno-moduladora *in vitro* sobre macrófagos de camundongos fêmeas da raça Wistar. *EPS* secos por "*spray drying*" com temperatura de entrada de 180ºC e concentração de 20mg/ml obtiveram 63.24% de estimulação sobre os macrófagos.

Palavras-chave: *Agaricus blazei*, fermentação submersa, produção de polissacarídeos, Liofilização, Spray Drying, Estufa a Vácuo, atividade imuno-moduladora.

ABSTRACT

Agaricus blazei is a mushroom that belongs to the Brazilian biodiversity and is considered as an important producer of bioactive compounds beneficial to human health. Studies have demonstrated that these compounds present immuno-modulatory, antioxidant and antitumor properties. In order to compare the most used method for fungal polysaccharide drying (lyophilization) with other industrial scale methods, the aim of this work was to submit *A. blazei* polysaccharides extracts to Vaccum, Spray and Freeze Drying, and evaluate the maintenance of its immuno-modulatory effect *in vitro*. Extra-cellular (*EPS*) and intra-cellular (*IPS*) polysaccharides produced by *A. blazei* submerged fermentation were extracted with ethanol and submitted to drying processes. All three drying methods presented satisfactory values of yield and efficiency. Dried resultant products showed humidity contents and water activity that prevent the growth of microorganisms and reactions of chemical degradation. Contents of total sugars and proteins decrease after drying, nevertheless, dried extracts of extra-cellular polysaccharides showed *in vitro* immuno-modulatory activity over macrophages of Wistar female mice. *EPS* dried by spray drying with inlet temperature of 180ºC and concentration of 20 mg/ml reached 63.24% of macrophage stimulation.

Keywords: *Agaricus blazei*, submerged fermentation, polysaccharides production, Lyophilization, Spray Drying, Vaccum Drying, immuno-modulatory activity.

INDEX

TABLE LIST

FIGURE LIST

1 INTRODUCTION

Cancer is a disease whose causes are not well defined yet, and it is responsible for a high mortality rate in the world. In recent decades, the prevention of cancer is becoming a major concern among people and especially among the health authorities. In the field of science, researchers come searching, increasingly, to find substances with effects that can prevent, control or even provide a cure for some types of cancer. Treatments used in the combat against cancer, such as radiotherapy and chemotherapy, have undesirable side effects and, often, poor therapeutic responses.

Researches have shown that some diseases can be cured or controlled by the consumption of certain mushrooms as a functional food or through the use of bioactive compounds extracted from them. Many investigations with basidiomycetes currently are strategically focused on the therapeutic properties, prophylactic, nutrition and other benefits of this group of microorganisms.

Numerous bioactive polysaccharides, glycoproteins, glycopeptides, and proteoglycans from macrofungi are considered as immuno-modulators. They affect on proliferation and differentiation of immune cells and cytokines, interleukins and receptors production. These compounds are recognized by the certain receptors located on the leukocytes and other immune cells that lead to enhance the innate and cell-mediate immune responses. In virtue of these activities, the induction of different types of antitumor effectors cells, such as cytotoxic T cells, NK cells and macrophages occurs. In addition to these activities, some of these compounds possess antiviral, antibacterial, antifungal and antiprotosoal activities (WASSER & WEIS, 1999).

Fungal polysaccharides 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.

Agaricus blazei is a medicinal mushroom originating from Brazilian subtropical regions and is nowadays produced on industrial scale in some countries such as China, Japan and Brazil. In recent years, the submerged fermentation of *A. blazei* has received great attention in Asian regions as a promising alternative for efficient production of its valuable metabolites. It is obviously necessary and important to develop a process for efficient production of these valuable metabolites in bioreactors, as also develop economically viable methods of extraction, purification and drying.

The principal anti-tumor compound produced by *Agaricus blazei* is a protein-bound polysaccharide, the β-D-glucan (DONG et al., 2002). The presence of a protein in the compound of interest signals to the idea that the substance could not be submitted to high temperatures because it could ocasionate the protein denaturation or structural modifications on the complex, and thus resulting in the loss of anti-tumor bioactivity. Often, lyophilization (freeze drying) is used for drying of the extra and intra-cellular polysaccharides extracted from *A. blazei* and others mushrooms, and very few works utilize another method of drying.

Based on this, the present work had the following objectives: cultivation of *Agaricus blazei* on submerged fermentation; production and extraction of its extra-cellular and intra-cellular polysaccharides (called EPS and IPS, respectively); drying of polysaccharides extracts using three different methods: Lyophilization (Freeze Drying), Spray Drying and Vacuum Drying; and analysis of immuno-modulatory effects *in vitro* of dried extracts (through stimulation of mice macrophages).

In order to compare the drying methods, it was made a comparative study of efficiency and yield of each process, as also physical-chemical characteristics of the resultant powders, for examples the humidity percentage and the water activity.

Analysis of total sugar, residual sugar and total protein can indicate if there was any degradation or change in the compound. *In vitro* assays were made to verify the effect of these dried polysaccharides over mice macrophages. The spectrophotometry analysis provides qualitative results about immuno-modulatory effects.

2 BIBLIOGRAPHIC REVIEW

2.1 Medicinal Mushrooms and Bioactive Compounds

Several major substances with immune modulatory and/or anti-tumor activity have been isolated from mushrooms. Since 1966 extensive studies on the anti-tumor effects of Basidiomycetes have been performed in Japan (WASSER, 1999). These substances include mainly polysaccharides (in particular β-D-glucans), polysaccharopeptides, polysaccharide proteins, and proteins (LULL et al., 2005). In 1968, researchers described the anti-tumor activity in aqueous extract of some mushrooms. Two years later, works were published about purification of polysaccharides of shiitake with high anti-tumor activity. Then, one of these polysaccharides, lentinan, began to be marketed by a japanese company for the treatment of stomach cancer. Besides lentinan, two other drugs with medicinal properties were isolated in Japan: krestin (*Coriolus versicolor*) is used for the treatment of cancer of gastrointestinal system, lung and breast, and sonifilan extracted from *Schizophyllum commune* is administered in the treatment of cervical cancer (DIAS et al., 2004).

Furthermore, other bioactive substances, including triterpenes, lipids, and phenols, have been identified and characterized in mushrooms with proven medicinal properties. The major immune modulating effects of these active substances derived from mushrooms include mitogenicity and activation of immune cells, such as hematopoietic stem cells, lymphocytes, macrophages, dendritic cells and natural killer cells, resulting in the production of cytokines. The therapeutic effects of mushrooms, such as anticancer activity, suppression of autoimmune diseases, and allergy have been associated in many cases with their immune modulating effects (LULL et al., 2005).

β-glucan is a potent stimulator of the immune system and boosts the body's natural ability to fight infections, heal wounds, and destroy tumour cells. There is now growing recognition of the huge potential for beta-glucan enhancement of the natural immune response, both to improve general health and wellbeing, and to improve the efficacy of conventional drug therapies. Microbial beta-glucans (principally from yeasts and fungi) have been known for a long time to stimulate the immune response and are widely used in traditional Asian medicine. Macrophages are one of the most important immune cells in the body and are understood to play a central role in beta-glucan immune enhancement; firstly by showing increased ability to destroy invading microbes or infected cells and secondly, by stimulating many other cells of the immune response, via a cascade of cellular interactions (BROWN and GORDON, 2001).

The family of glucan containing the main chain of β-D-(1-3) units of glucopyranosyl, and a short side chain of β-D-glucopyranosyl in O-6 has received considerable attention for its anti-tumor activity and immune-modulator action. Esquizofilan, escleroglucan, epiglucan and lentinan are the most known of the group of polysaccharides (DU et al., 2004).

The β-glucan extracted from mushrooms operates on the biological response, improving the immune system. The lentinan extracted from *Lentinus edodes* and a protein linked to the polysaccharide K (PSK) from *Coriolus Versicolor*, are typical β-glucans that have a chain β-1-3 and a side chain β-1-6. A β-glucan (D-fraction) previously extracted from *Grifola frondosa* with chain β-1-6 and β-1-3 activated T immune-competent cells, macrophages to a large extent of lentinan or PSK. The experiments conducted have shown that the D-fraction can reduce the amount of Mitomycin-C (MMC) used on cancer treatment and improves the response of Th1 (KODAMA et al., 2005).

An extract isolated from the fruit body of *Grifola frondosa*, whose active component is an isolated β-glucan, caused direct enhancement of the colony-forming units of granulocytes and macrophages and enhanced the recovery of the granulocytes and macrophages response after induced hematopoietic suppression. These studies suggest that this β-glucan has the potential to reduce hematopoietic suppression induced by chemotherapy (LIN et al, 2004).

METHACANON et al. (2005) showed that the extra-cellular polysaccharides are considered biocompatible and inducers of IL-8, a cytokine responsible for improving the process of healing.

HUANG et al. (2007) extracted six polysaccharide fractions from *Poria cocos* mycelia (intra-cellular polysaccharide) with a solvent extract system. The water-soluble fractions were heteropolysaccharides composed of glucose, galactose, and mannose, whereas the water-insoluble fractions were (1-3)-α-D-glucans. All water-soluble polysaccharides exhibited strong antitumor activities against Sarcoma 180 solid tumor implanted in BALB/c mice *in vivo* and against HL-60 tumor cell *in vitro*.

2.2 Submerged Cultivation of Mushrooms

Traditionally, fruiting bodies of mushrooms are grown on solid substrates using wastes or ligno-cellulosic compounds and the cultivation is a long-term process requiring from one to several months for the first fruiting bodies to appear. The mycelium growth of mushroom conducted by submerged fermentation is a faster alternative method for obtaining quality biomass (CUI et al., 2006).

The growth of mushroom cell cultures in submerged conditions in a liquid culture medium accelerates the process, resulting in biomass yield within a few days and allows obtain standardized nutraceutical substances. The polysaccharides produced in submerged culture can be extracted from the mycelial biomass and the biomass-free culture broth. Polysaccharides isolated from different sources of mushrooms, mycelium, and biomass-free broth differ somewhat in structure, composition, and physiological activity (LULL et al., 2005).

According to XIAO et al. (2006) the carbohydrates are the formers components of the cytoskeleton and important nutritional requirements for the good growth of fungus. The use of carbon sources is variable among species, but all species need a specific carbon source.

2.3 *Agaricus blazei* mushroom

Agaricus blazei was discovered in 1960 in the city of Piedade (Brazil) by Furumoto, a researcher who sent the sample to Japan in 1965 for research. The mushroom was identified as *Agaricus blazei* Murril by the belgian scientist Dr. Heinemann in 1967. In Japan, it is popularly known as "Himematsutake" and in Brazil “Cogumelo Piedade”, but after the discovery of its medicinal properties, it passed to call “Cogumelo do Sol” and in other countries "Royal Sun Agaricus" (DIAS et al., 2004).

According to WASSER (2002) medicinal properties of *Agaricus blazei* were discovered after epidemiologists studied a population with very low incidence of a variety of illnesses, including cancers and bacteria induced diseases. That population was native from a very small area of the mountains of Brazil, near the town of São Paulo, and the absence of diseases in that population was associated to the constant consumption of *Agaricus blazei* mushroom in their normal diet.

In addition to the nutritional properties, *A. blazei* is used by about 500,000 people for prevention of cancer or as adjunct in the treatment with drugs for chemotherapy, after the removal of a malignant tumor (TAKAKU et al., 2001).

Several compounds of medicinal interest, including β-glucan, have already been characterized. Anticancer compounds include polysaccharides, complex protein-polysaccharides and steroids, present both in fruiting body as mycelium (DI PIERO, 2003).

The crude polysaccharide extracted from *A. blazei* contains 57.5% of carbohydrates and 21.6% of proteins associated. The analysis showed that the carbohydrates are composed predominantly of glucose and small amounts of raffinose, xylose, mannose and galactose (DONG et al., 2002).

According to DI PIERO (2003), in 1990 researchers noted the presence of high content of alanine and tyrosine in extracts of *A. blazei*, showing anti-tumor activity. The effect characterized by the inhibition of growth of Sarcoma 180 implanted in mice, probably occurred because of the properties immune-regulatory of the complex.

FAN et al. (2005) showed that extra-cellular polysaccharides extracted from *A. blazei* reached 72.19% inhibition of Sarcoma 180 in rats, whereas 50% of the animals tested showed complete regression of tumors.

According to CHANG et al. (2001), *A. blazei* contains 31.4 mg/g (dry weight) of arabitol, 45.3 mg/g of glucose and 23.9 mg/g of trehalose. Mannitol and trehalose are sugars commonly present in the fruiting bodies of fungi, however, mannitol is not found in most of mycelia.

MIZUNO et al. (1990a) isolated polysaccharides from the aqueous extracts of *A. blazei* mycelium, which show activity anti-tumor, and these ones were different from polysaccharides anticancer isolated from the mushroom fruiting body. The complex 1.6 and 1.4 α-glucan also stimulated the synthesis of lymphocytes in mice when administered in normal mice, suggesting that polysaccharides of *A. blazei* may be important to the preventive treatment against cancer.

Another indication is the stimulation of the immune system of the host showed by NAKAJIMA et al. (2002), in which the expression of messenger RNA by interleukins was increased by *A. blazei* extract, as in macrophages as in cells of the spleen. This suggests that the extract can stimulate macrophages and T cells releases interleukins, resulting in increase of antibodies production.

The insoluble polysaccharide from the hot-water extract of the fruiting body of *A. blazei* Murril was shown to have significant anti-tumor activity in mice, supressing Sarcoma 180, Erlich ascites carcinoma, Meth A fibrosarcoma, Shionogi carcinoma 42 and Lewis lung carcinoma. The mode of action involves activation of natural killers cells/activated macrophages or helper/inducer lymphocyte T-cells (MIZUNO et al., 1990a,b; MIZUNO et al., 1998; MIZUNO et al., 1999; ITO, 2000; ITO et al., 2002).

CHANG et al. (2001) characterized the free amino acids of the mycelium and the highest content found was alanine. The aspartic acid and glutamic acid components are similar to the sodium glutamate, what characterizes the typical flavor of the mushrooms, the taste “umami”. However, the data obtained shown that *A. blazei* contains relatively low concentrations of these components. According to CHANG et al. (2001), it is common to find amino acids as alanine, glycine, threonine and glutamic acid in mushrooms.

The intravenous administration of β-glucan-(1-6) extracted from *A. blazei* demonstrated an antitumoral activity more effective than oral application. However, after hydrolysis of the same molecule, forming fragments with molecular weight of 10 kDa, the antitumoral effect becomes significant, when administrated orally (SMITH; SULLIVAN; ROWAN, 2003).

2.4 *Agaricus blazei* submerged cultivation

On last years, submerged fermentation of *Agaricus* has received the attention of the Asian researchers like a promising alternative for the production of bioactive metabolites in bioreactors (ZOU, 2005).

According to results obtained by FAN et al. (2005), the cultivation media for the production of extra-cellular polysaccharide (EPS) from *A. blazei* was optimized with the pH adjusted to 6.1 and incubated at 30°C for 7 days under agitation (150 rpm), and the cultivation media was formed by sucrose and glucose 10.0 g/l, yeast extract 6.0 g/l, K_2HPO_4 1.4 g/l and $MgSO_4$ 0.3 g/l. At theses conditions, the EPS production was 3 mg/50 ml, using sucrose. The authors assume that the disaccharides are better than the monosaccharides, because those ones can encourage the polymerization. However, the glucose has been mentioned as the most likely carbon source for mycelium growth of most of mushrooms (FAN et al., 2005).

ZOU (2005) tested various carbon sources and sucrose shown to be efficient in the production of EPS. GERN (2005) achieved an extra-cellular polysaccharide production of 1.235 g/l with an optimized culture media, whose composition was: glucose 20.0 g/l, yeast extract 6.0 g/l, K_2HPO_4 0.6 g/l, MgSO4 0.3 g/L, $MnSO_4$ 0.3 g/l, $CaCl_2$ 0.05 g/l, $FeCl_3$ 0.05 g/l and olive oil 13.0 ml/l; aeration rate of 2.00 vvm and agitation of 120 rpm.

2.5 Drying

The most important operation in pharmaceutical industry is drying, since dried products are more stable than moist ones, and the growth of mold and bacteria decreases with lower moisture content. In addition, drying represents around eighty percent of the total industrial energy consumption (ALDEN et al., 1988). The removal of water from food provides microbiological stability and decrease of the cost of storage and transportation (SINGH, 1993).

Drying is defined as a process of moisture removal due to simultaneous heat and mass transfers. Heat, necessary for evaporation, is supplied to the particles of the material and moisture vapors are removed from the material into the drying medium. Heat is transported by convection from the surroundings to the particle surface, and from there, by conduction, further into the particle. The moisture can be either transported to the surface of the product and then evaporated, or transported in the opposite direction within the particle (as a liquid or vapor) to the surface and there it evaporates and passes by convection, to the surrounding. The heat transfer depends on the air temperature, relative humidity, airflow, exposed area and pressure (SERRANO, 2004).

Thermal drying has been recognized as an important unit operation as it is energy intensive and has a decisive effect on the quality of most products that are dried commercially (MUJUMDAR, 2007).

2.5.1 Spray drying

The drying by atomization is a very appropriate method of conservation for dehydrated thermo-labile substances as food, because it is a very fast drying. The rapid evaporation of water maintains the low temperature of the particles so that the high temperature of the drying air does not affect the product. The function of spray dryers is to dry a spray of droplets formed by atomization of a liquid feed formulation through contact with heated air or inert gas (MASTERS, 1991).

In a spray dryer, the flow of hot air is usually introduced in the chamber by dispersor air at the top of the same. The liquid sprayed can have the same flow of hot air, occurring in this case, a contact of the product with the more humid air warmer, system used for products more labile to heat. In counter-current flow, the liquid is sprayed in a position opposite to the entry of hot air, occurring contact of the particle more drought with the air warmer. This system uses the heat efficiently, and is suitable for products less thermo-labile. When the hot air comes in contact with humid particle, the dehydration of the droplet happens almost instantaneous and the water evaporation in the drying chamber, in the form of a mist. The air drying go out through the bottom of the unit, passing in cyclones to recover the fine particles by the force of gravity and allowing the output of clean air to the atmosphere through chimneys. The separation of the dry product from the drying air has great influence on the characteristics of the final powder due to the mechanical handling used in separation (GAVA, 1978).

HONG et al. (2007) studied the thermal stability of spray-dried powders of extra-cellular polysaccharide from *Agaricus blazei* extracts. According to their results using thermogravimetric analysis, the fractions of three different molecular weights of *A. blazei* extra-cellular polysaccharide showed a range of 200–400°C of decomposition temperature.

2.5.2 Freeze drying

Freeze-drying, or lyophilization, is sometimes preferred for unstable or heat labile foods or when product quality and structural integrity are critical. It is the best method for the water removal with final products of the highest quality compared with other methods of drying. The quality of freeze-dried products is considered excellent because of the protection of the product solids by evaporative cooling (SINGH et al., 1993).

Despite many advantages, freeze drying has always been recognized as the most expensive process for manufacturing a dehydrated product. Also the porosity and surface area of freeze-dried products are greater than those of dehydrated products dried by other methods (KING et al., 2001; RATTI, 2001). Furthermore, it requires a lengthy process time and has capital and energy costs three times those of other drying methods (SNOWMAN, 1997).

2.5.3 Vaccum drying

Vacuum drying is well known as a suitable process for heat-sensitive materials. Since the drying operates under vacuum, moisture can be removed from the materials at a low temperature. The basic vacuum dryer consists of a chamber containing heated shelves. Trays containing the wet materials are placed on the shelves, and water is removed by a vacuum pump and condensed at a condenser (HAYASHI, 1983).

LAI et al. (2007) did not notice any modifications caused by drying methods including hot air, vaccum and freeze drying, over the rheological properties of polysaccharide extracted from *Ganoderma lucidum* fruiting body and mycelium. Moreover, the convective drying air with a temperature of 50°C has less cost than vacuum or freeze drying, maintaining the rheological characteristics.

2. 6 Water activity and humidity content

The presence of water in chemicals or biological products occurs as water activity and water linked, resulting in total water content (humidity). The water contained in the product can be present in 2 ways: a) water linked: the water is closely linked to the molecules constituents of the product, and it can not be removed or utilized for any type of reaction; b) water activity: the water is available for physical reactions (evaporation), chemical and enzymatic reactions (darkening) and microbiological reactions, becoming the primary responsible for the product deterioration. This water can be measured and through its value, it can be determine the susceptibility of the product degradation (BEUCHAT, 1981).

The degree of availability of water in a product can be expressed as the water activity (Aw) and it is defined by the relationship between the steam pressure of water in the product (P) and the steam pressure of pure water in the same temperature (Po). The main factor in the stability of a product is not the humidity content, but the available of water for growth of microorganisms and chemical reactions (COULTATE, 1996).

Fungi are the most resistant microorganisms to decrease of water activity, and hence, they are the main responsible for deterioration of food in the range of Aw 0.61 – 0.70. This occurs due the fact that there is not bacteria competition at this band (BEUCHAT, 1983).

CHRISTENSEN & KAUFMANN (1974) studied various products of plant origin and verified the minimum water activity for survival of the major fungi under conditions of optimal temperatures (26°C – 30°C). The values obtained for the studied microorganisms are on Table 1.

Table 1. Minimum water activity (Aw) for fungi survivor.

Fungi	Water activity (Aw)
Aspergillus restrictus	0.70
Aspergillus halophilicus	0.68
Aspergillus glaucus	0.73
Aspergillus candidus	0.80
Aspergillus ochraceus	0.80
Aspergillus flavus	0.85
Penicillum (various species)	0.85

Source: CHRISTENSEN & KAUFMANN (1974).

The microbial contamination of medicines and cosmetics can cause changes to their sensory characteristics, making them unfit for use, and promote the degradation of components of the formulation; thus, the contamination may cause damage to health, depending on the type of microorganism, route of administration and the health status of the user of the product (BAIRD & BLOOMFIELD, 1996; PINTO et al., 2000).

3 MATERIALS AND METHODS

3.1 Strain of *A. blazei* LPB 03

The strain of *A. blazei* LPB 03 was isolated by Fan Leifa in 2000. The strain was maintained on potato-dextrose agar (PDA) medium in Petri dishes at room temperature. Sub-cultures were made at each three months to maintain the strain active. The pre-inoculum was made with 5 agar blocks (5 mm in diameter each) in 250 ml Erlenmeyer flasks containing 50 ml of medium (pH 6,0) and incubated in shaker at 30°C, 120 rpm, for 10 days. The medium was prepared with glucose 20 g/l, yeast extract 4 g/l, K_2HPO_4 0.6 g/l and $MgSO_4$ 0.3 g/l (FAN, 2005). This media was called basal media.

3.2 Inoculum

For the inoculum (Figure 1), 20 ml of the pre-inoculum was added to 180 ml of basal media in 500 ml Erlenmeyer flasks and incubated in shaker at 30°C, 120 rpm, for 7 days. After this period, the fermented broth was filtered using a screen with 2.0 mm pores size, thus the biomass was triturated with a spatula and washed with 150 ml of sterile distilled water. The mycelium suspension was used for the fermentation studies.

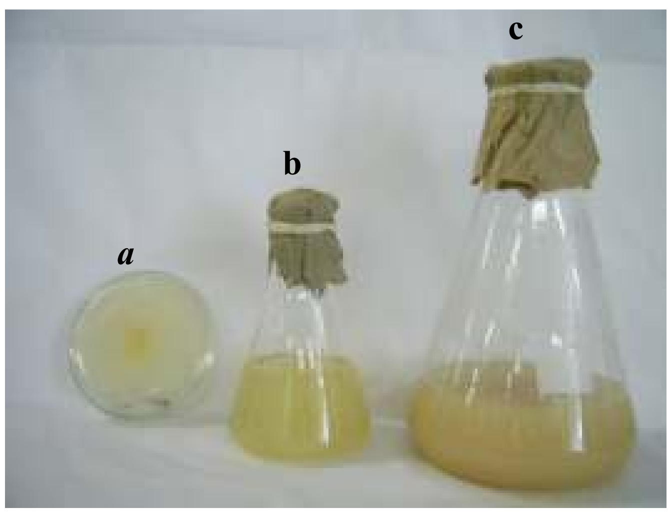

Figure 1. a) *Agaricus blazei* strain; b) pre-inoculum; c) inoculum

3.3 Submerged Cultivation

Submerged cultivation was carried out according to optimized conditions of extra-cellular polysaccharide production by GERN (2005). The medium was prepared with glucose 20 g/l, yeast extract 4g/l, K_2HPO_4 0.6 g/l, $MgSO_4$ 0.3 g/l, $MnSO_4$ 0.3 g/l, $CaCl_2$ 0.05 g/l, $FeCl_3$ 0.05 g/l and olive oil 13.0 ml/l. Inoculation rate was 4% (40 ml per liter of mycelium suspension). The temperature was controlled at 30°C; the air flow rate used was 2 vvm (air volume/broth volume/min) and the agitation was maintained at 120 rpm. The stired-tank fermentor used was an 8 liters bioreactor MARUBISHI, CO (Figure 2) with 6 liters of useful volume.

The pH of the medium was controlled at 6.0 during the first two days and then, the set point was modified to pH 7.0 in order to enhance the EPS production (data not published of experiments made in our laboratory); the pH was then maintained at 7.0 until the end of the fermentation (7 days). To control pH during the fermentation period, it was used H_3PO_4 2 N and NaOH 2 N. To avoid the excessive oscilation of the peristaltic pumps, a dead band of 0.05 was used.

Figure 2. Stirred-tank fermentor, MARUBISHI 8L, used for *Agaricus blazei* submerged fermentation.

3.4 Extra-cellular polysaccharide extraction

According to RUBEL (2004), after the submerged fermentation period, the culture was filtered under vacuum using Whatman 1 filter paper and washed with 20 ml of water distilled to remove the metabolites adhered to the mycelium. The filtrate was concentrated in a rotary evaporator at 55°C under low pressure and the extra-cellular polysaccharides (EPS) were precipitated with four volumes of 95% ethanol keeping, previously, over-night in the freezer. This mixture was left overnight at -10°C. The precipitate EPS was centrifuged at 4000 rpm, washed with 95% alcohol and centrifuged more two times (Figure 3).

Finally, distilled water was added until a final concentration of 1.72% of soluble solids, in order to recover the precipitate adhered to the bottom of the centrifugation bottle. From EPS collected, only the soluble fraction was used in drying studies.

3.5 Intra-cellular polysaccharide extraction

The filtered biomass obtained from the fermentation was hydrolyzed four times, of 3 hours each, in distilled water at 96°C (Figure 3). After hydrolysis, the aqueous extract was filtered under vacuum using Whatman 1 filter paper, and the extraction followed the same procedures described on item 3.4 (LUO et al. 2008).

Intra-cellular polysaccharide (IPS) collected after centrifugation, at a concentration of 2.82% of soluble solids after resuspension in distilled water, was used in drying studies (the soluble fraction).

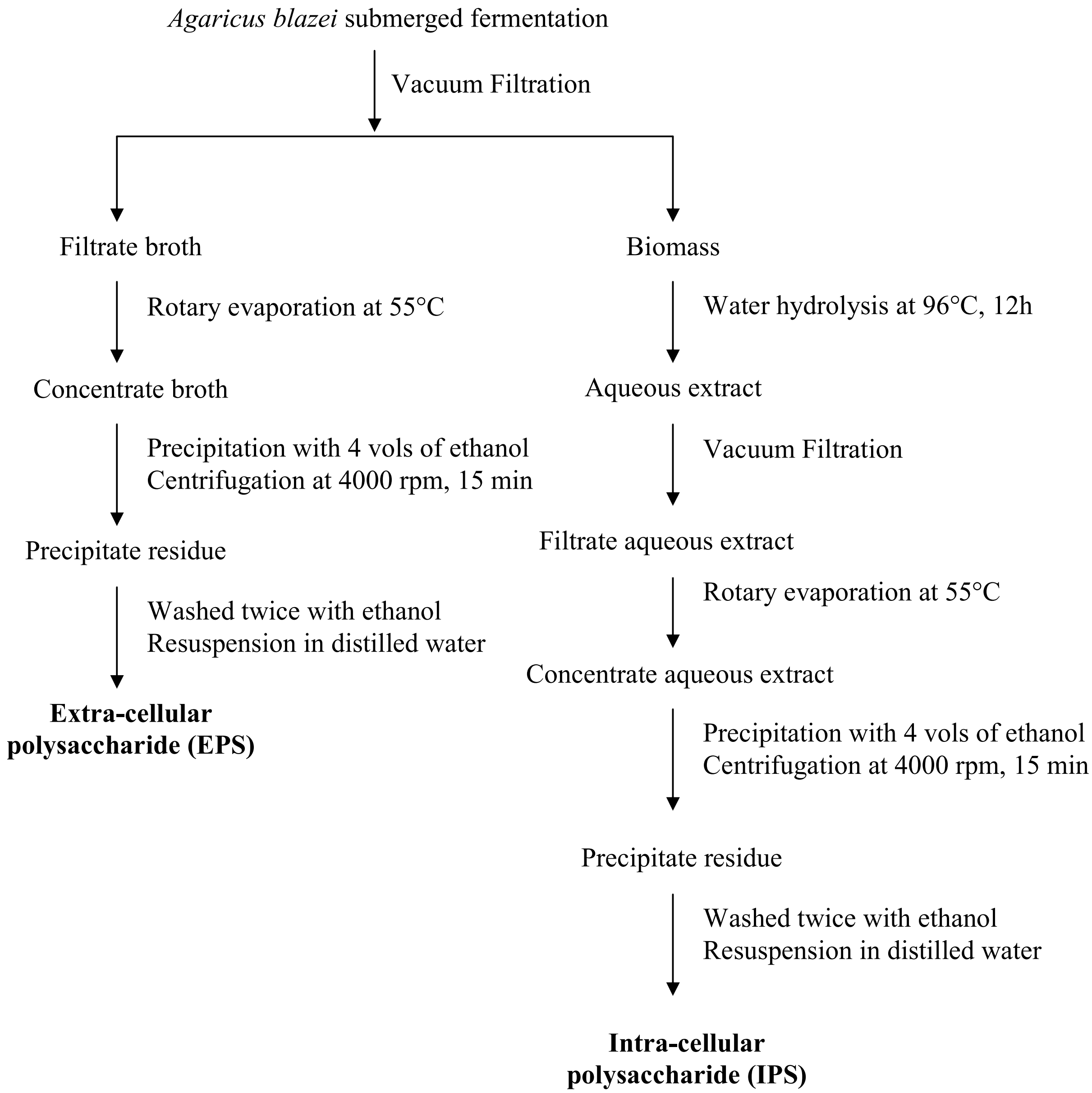

Figure 3. Schematic extraction system of extra-cellular and intra-cellular polysaccharides extracts from *Agaricus blazei* submerged fermentation

3.6 Drying Studies

Equal amounts of *A. blazei* extra-cellular polysaccharide (EPS) and intra-cellular polysaccharide (IPS) collected after centrifugation were submitted to three different methods of drying: Spray Drying, Vaccum Drying and Freeze Drying. 150 ml of each sample was used for drying processes.

Extra-cellular and intra-cellular polysaccharides not submitted to drying methods were called by *EPS initial* and *IPS initial*, and they were used as comparative groups.

3.6.1 Freeze Drying

EPS and IPS were freeze-dried in a Modulyod Freeze Dryer 230 (Thermo Electron Corporation), submitted to a temperature of –45°C and a negative pressure of 50 mBar.

Extra-cellular and intra-cellular polysaccharides dried by this method were called *EPS freeze* and *IPS freeze*, respectively.

Figure 4. Modulyod Freeze Dryer 230 (Thermo Electron Corporation), used for freeze drying of polysaccharides

3.6.2 Vacuum Drying

EPS and IPS were vacuum dried in a Vacuum cabinet line standard Vacucell 22, 55, 111 (MMM Group), submitted to a temperature of 50°C and reduced pressure of 0.1 bar.

Extra-cellular and intra-cellular polysaccharides dried by this method were called *EPS vacuum* and *IPS vacuum*, respectively.

Figure 5.Vacuum cabinet line standard Vacucell 22, 55, 111 (MMM Group), used for vaccum drying of polysaccharides

3.6.3 Spray Drying

EPS and IPS solutions were spray dried in a Lab Plant Spray Dryer SD 05 (Labplant, West Yorkshire, UK) with 0.5 mm jet, compressed air pressure of 0.7 bar, compressed air rate of 45 m^3/h, feed rate of 450 ml/h and three different inlet temperatures: 120°C, 180ºC and 240ºC. The amount of IPS obtained during submerged fermentation was very lower than amount of EPS, and consequently, a low quantity of IPS was available for drying studies; for this reason, it was tested only one inlet temperature during spray drying process, 180ºC.

Extra-cellular and intra-cellular polysaccharides dried by this method were called according to the inlet temperature used during drying: *EPS spray 120°C*, *EPS spray 180°C*, *EPS spray 240°C* and *IPS spray 180ºC*, respectively.

Figure 6. Lab Plant Spray Dryer SD 05 (Labplant, West Yorkshire, UK), used for spray drying of polysaccharides

3.7 Analytical methods

In order to evaluate if there was any variation on the amount of reducing sugars, total sugars and proteins in the samples, caused by the action of temperature during drying process, it was made the following analysis:

3.7.1 Analysis of reducing sugar

The reducing sugar was measured by SOMOGY-NELSON (SOUTHGATE, 1976), using glucose in the standard curve. The tests were made in triplicate.

3.7.2 Analysis of total sugar

The concentration of polysaccharide was determined by phenol-sulfuric method (DUBOIS et al, 1956), utilizing glucose as standard. The tests were made in triplicate.

3.7.3 Total protein dosage

The dosage of total protein was determined by LOWRY method (LOWRY et al., 1951), using bovine albumin as standard. The tests were made in triplicate.

3.7.4 Humidity content

After each drying processes, the humidity content of the resulting powder was measured following the methodology described of AOAC (1990). The samples were submitted to desiccation in oven-drying at 105ºC until constant weight.

3.7.5 Water activity

Water activity determination was made by direct measurement, following the procedure of equipment Aqualab CX-2 Water Activity-System, making up the calibration with saturated solution of NaCl, at 24ºC. It was made sequential readings per sample, until a maximum variation of 0.003 on the value of water activity.

3.8 *In vitro* immuno-modulatory effect

3.8.1 Preparation of mice macrophages

Macrophages were obtained from female Wistar mice with 20 days age, kindly disposed by professor Dr. Luis Claudio Fernandes from Laboratory of Cell Metabolism, Department of Physiology (Federal University of Parana, Brazil) and the cell culture followed the manufacturer's recommendations, using a culture medium containing 90% Roswell Park Memorial Institute (RPMI) 1640 (Himedia, Laboratories Pvt. Ltda. Mumbai – India), sterile, supplemented with 10% fetal bovine serum (Cultilab, Materials for Cell Culture Ltda., Campinas, SP, Brazil), 10 μg/ml streptomycin and 10 IU/ml penicillin (Gibco, Invitrogen Corporation, Grand Island, NY, USA). The monolayers of cell cultivation were obtained in appropriate bottles at 37°C, under humidified atmosphere of 5% carbon dioxide gas.

3.8.2 Macrophages culture conditions

These assays aimed to verify the stimulant effect of *A. blazei* polysaccharides over macrophages proliferation.

An aliquot was extracted from cell culture and total viable cells were quantified in Newbauer camera using "Trypan" blue dye. Assays were performed on cell culture plates of 96-well. Each well were seeded with 0.1 ml of cells suspension of macrophages (initial density of 3.2 x 10^5 cells/ml); 0.1 ml of culture medium (90% RPMI, 10% fetal bovine serum, 10 μg/ml streptomycin and 10 IU/ml penicillin); and 0.1 ml of polysaccharide samples at 20, 10 and 5 mg/ml. The samples used were EPS and IPS powders previously dried (*EPS freeze*, *EPS vacuum*, *EPS spray 120°C*, *EPS spray 180°C*, *EPS spray 240°C*, *IPS freeze*, *IPS vacuum* and *IPS spray 180ºC*), resuspended in ultra pure water and sterilized in filter 0.22 µm. All tests were carried out in triplicate. The results were compared to a negative control made with PBS (phosphate buffer solution, 0.2 M, pH 7.4).

The plates were incubated during 48 hours, at 37°C, under humidified atmosphere of 5% carbon dioxide gas (LIMA et al., 2006).

3.8.3 MTT reduction assay

It was used the colorimetric method MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, Aldrich Chemical Co. Inc., Milwaukee, WI, USA), described by MOSMANN (1983), which measures indirectly the cell viability through mitochondrial enzyme activity of living cells. The test consists of a colorimetric analysis based on conversion of salt MTT to formazan, through the activity of the enzyme succinil dehydrogenase present in the mitochondria of viable cell, allowing quantify the percentage of living cells.

At the end of incubation (48 h) of macrophages treated with the polysaccharides, it was added 10 µl of MTT (5 mg/ml). The plate was then incubated for more 3 hours, at 37°C, under humidified atmosphere of 5% carbon dioxide gas. After this period, it was added 50 µl of SDS 10% (Sodium Dodecyl Sulfate, an anionic detergent), and the plate was rolled with aluminium paper and incubated at 37ºC for more 15 hours. The SDS detergent solubilizes the crystals formed in the reaction, allowing the measure of the absorbance in spectrophotometer at a wavelength of 550 nm (LIMA et al., 2006).

4 RESULTS AND DISCUSSION

On Figure 7, there are extra-cellular polysaccharides extracts after drying, where its physical characteristics can be compared: *EPS freeze*, *EPS vaccum* and *EPS spray$_{180°C}$*.

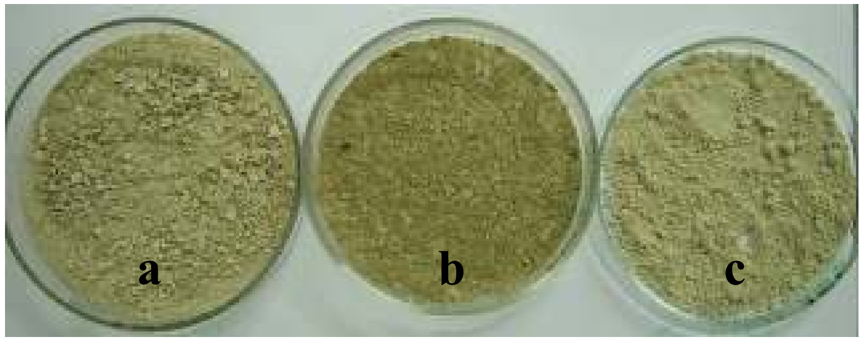

Figure 7. a) *EPS freeze*; b) *EPS vacuum*; c) *EPS spray$_{180°C}$*

On Figure 8, the dried intra-cellular polysaccharides extracts are showed: *IPS freeze*, *IPS vaccum* and *IPS spray$_{180°C}$*.

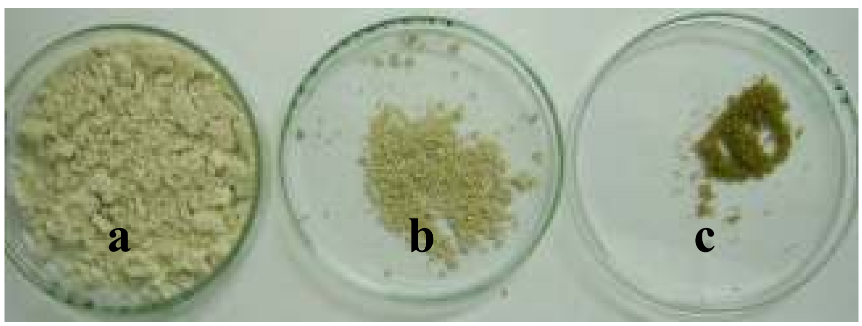

Figure 8. a) *IPS freeze*; b) *IPS vacuum*; c) *IPS spray$_{180°C}$*

4.1 Humidity content and water activity

On Table 2, there are the values of humidity content and water activity of polysaccharides extracts submitted to drying methods. EPS initial and IPS initial represent the samples that did not submitted to any drying process.

Table 2. Values of humidity content (%) and water activity (dimensionless) of *Agaricus blazei* extra-cellular polysaccharides (EPS) and intra-cellular polysaccharides (IPS) extracts submitted to freeze, vacuum and spray drying

Samples	Humidity content (%)	Water activity
EPS initial	98.28	0.964
EPS freeze	3.15	0.311
EPS vacuum	3.78	0.291
EPS spray 120°	8.58	0.427
EPS spray 180°C	3.92	0.371
EPS spray 240°C	3.16	0.336
IPS initial	97.18	0.961
IPS freeze	6.14	0.365
IPS vacuum	16.13	0.384
IPS spray 180°C	3.70	0.349

Values of water activity for extra-cellular and intra-cellular polysaccharides extracts, before being submitted to drying process (EPS initial and IPS initial), were very close to value corresponding to pure water (approximately 1.000), because there is a great content of humidity into these samples (almost 100%). The high contents of humidity can cause chemical degradation and loss of biological properties. Also, when the values of water activity are compared with those presented on Table 3 and Table 4, it is clear that, at these conditions, both polysaccharides extracts would be very susceptible to action of pathogenic microorganisms and non-pathogenic microorganisms. In pharmaceutical industry, the water activity in the formulations of products must be controlled to avoid reactions of darkening, oxidation hydrolytic or microbial proliferation, ensuring the quality and increasing the length of validity of these products.

However, it can be noticed that, after drying process, all drying methods tested managed to reduce the humidity content and the value of water activity to lower than the minimum value required for growth of microorganisms, about 0.80 (Table 4). It indicates that the samples are more stable than moist ones, because the growth of mold and bacteria decreases with lower moisture content.

For extra-cellular polysaccharides drying, the methods freeze drying, vacuum drying and spray drying, except for inlet temperature of 120ºC for spray drying, presented the best results concerning to humidity content and water activity, around 3% of humidity and less than 0.380 of water activity, respectively. For intra-cellular polysaccharides drying, only spray drying with 180ºC of inlet temperature presented similar results, being therefore, between the methods tested, the best drying process for these parameters.

Table 3.Water activity values of pathogenic microorganisms likely to multiply.

Water Activity Range	Pathogenic Microorganisms Capable of Growth
0.98-0.99	*Salmonella, Campylobacter, Yersinia, Escherichia coli, Shigella, Bacillus cereus, C. perfringens, C. botulinum, S. aureus*
0.93-0.97	The above cited organisms, all except *S. aureus* grow slower and cease multiplying within this range; *S. aureus* grows well within this range *V. parahaemolyticus*
0.85-0.92	*S. aureus*, but no enterotoxin production. Molds, including those that produce mycotoxins
0.60-0.84	No pathogens grow, no reports of mycotoxin production at values of 0.80 or below
<0.60	No microbial growth, but microorganisms remain viable for a long time

Source: IAMFES (1991).

Table 4. Approximate Minimum (Aw) Values for Growth.

Organisms groups	Water activity
Most spoilage bactéria	0.90
Most spoilage yeasts	0.88
Most spoilage moulds	0.80

Source: BEUCHAT (1983), adapted.

4.2 Yield and efficiency of drying methods

Yield and efficiency of each drying method were calculated by the following relations, respectively:

$$Yield(\%) = \left(\frac{\text{dry weight of solids after drying (g)}}{\text{dry weight of solids before drying (g)}} \right).100$$

$$Efficiency(\%) = \left(\frac{\text{humidity content before drying (\%) - humidity content after drying (\%)}}{\text{humidity content before drying (\%)}} \right).100$$

Yield and efficiency obtained from purified polysaccharides extracts submitted to drying methods are showed on Table 5.

Table 5. Values of yield and efficiency of freeze, vacuum and spray drying of *A. blazei* extra-cellular polysaccharides (EPS) and intra-cellular polysaccharides (IPS)

Samples	Yield (%)	Efficiency (%)
EPS freeze	97.36	96.15
EPS vacuum	93.23	96.79
EPS spray 120°	24.11	91.27
EPS spray 180°C	33.18	96.02
EPS spray 240°C	42.31	96.79
IPS freeze	85.76	93.68
IPS vacuum	72.67	83.40
IPS spray 180°C	30.09	96.19

Through the value of yield, it is possible to quantify the amount of material that was lost during the drying process. The more the value approximates to 100%, larger quantities of the final product can be obtained, and therefore, the method can be considered more economically viable. Based on this, for both extra-cellular and intra-cellular polysaccharides drying, the best results were obtained by freeze and vacuum drying.

On the other hand, spray drying of EPS and IPS presented low yield for all the three inlet temperatures tested, while the best result was obtained with 240°C of inlet temperature. It can be noticed that by increasing the temperature, yield rises. The probable reason for this effect is a faster atomization of the particulates caused by the increase of inlet temperature, thus, a larger amount of solid material is deposited in the chamber collector, and less material is removed by the exhaust pipe.

In a comparative analysis, VALDUGA et al. (2003) spray dried mate extract at 180ºC of inlet temperature, using an equal equipment Lab Plant SD-05, and obtained a yield of 28.95%; when these authors added an adjuvant, gum arabic at the concentration of 0.8% related to the total content of solid, the value of yield increase 25%. RATHANANAN et al. (2007) also used a spray dryer Lab Plant SD-05, with 140°C of inlet temperatures, to dry mucoadhesive microspheres and obtained a yield of 20.02%; the addition of adjuvants raised the yield value to 45.86% and 63.44%.

These studies show that the values of yield obtained on this work are acceptable for this specific equipment, and the addition of adjuvants could raise them. The adjuvants have the capacity to increase the amount of solid adhered to the wall of the equipment cyclone, through an adhesion effect. As a consequence, less material is removed by the exhaust pipe.

The value of efficiency represents the humidity content that was retired during the drying process. In this case, again, the best result is that one closer to 100%, because the final product (dried polysaccharide) presents minor humidity content. For this parameter, all drying methods tested had great results, except vaccum drying of intra-polysaccharides that showed efficiency around 10% lower than the other processes.

4.3 Reducing sugars contents

In all drying methods tested there was an increase in the amount of reducing sugars contents in extra-cellular polysaccharides extracts. The values of reducing sugars percentage present in extra-cellular polysaccharides (EPS) and intra-cellular (IPS) polysaccharides extracts, before and after drying methods, are showed on Figure 9 and Figure 10, respectively. *EPS initial* and *IPS initial* represent extra-cellular and intra-cellular polysaccharides extracts not submitted to drying.

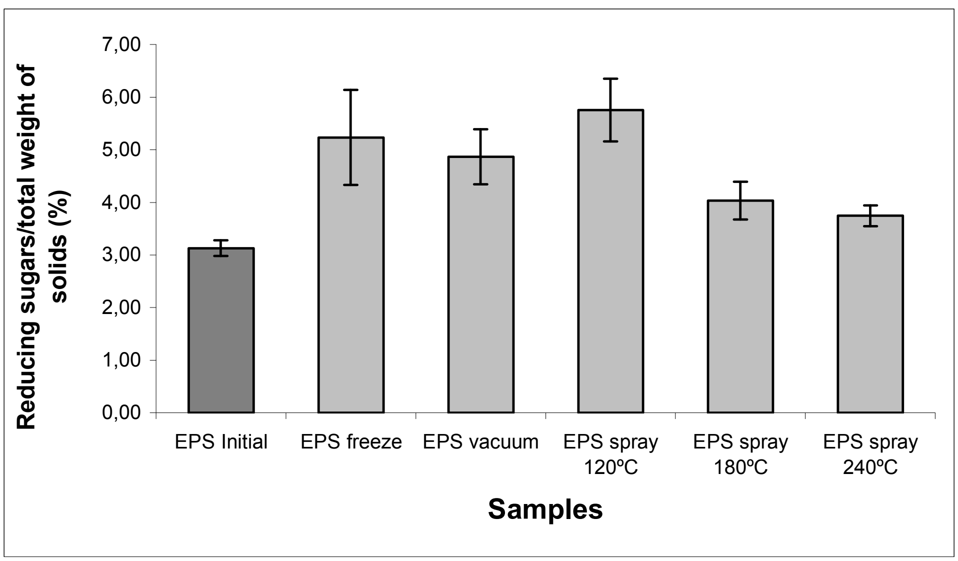

Figure 9. Percentage of reducing sugars on *A. blazei* extra-cellular polysaccharides extracts submitted to drying process. Results are mean ± standard deviation of three determinations.

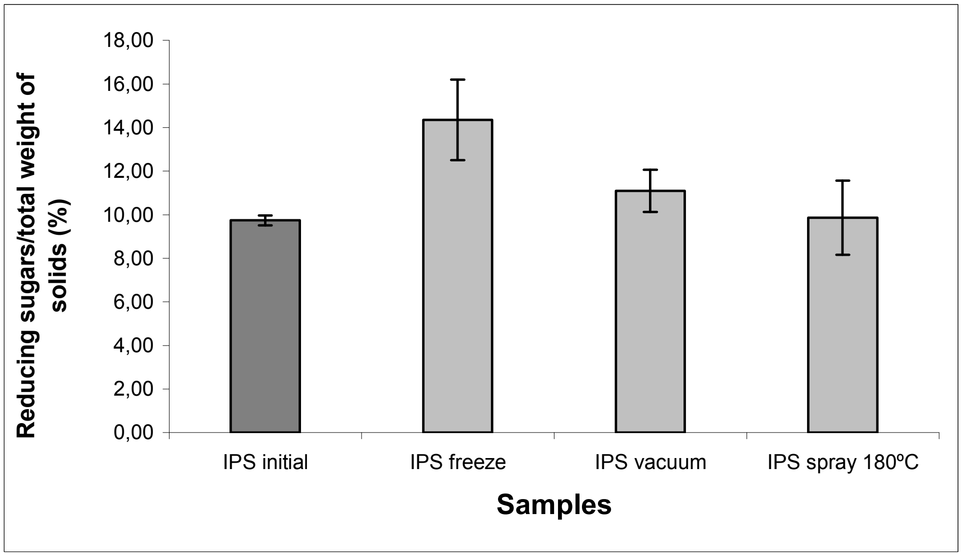

Figure 10. Percentage of reducing sugars on *A. blazei* intra-cellular polysaccharides extracts submitted to drying process. Results are mean ± standard deviation of three determinations.

The probable cause to the increase of reducing sugars contents is the broken of glycosidic linkages of the polysaccharide polymer during the drying process, possibly cause by the action of temperature, and the consequent release of monosaccharides (reducing sugars). This break in the chain of polysaccharide polymer can reduce its biological action. According to YOSHIYUKI et al. (1990) and MIZUNO et al. (1995), molecular weight of the polysaccharide is an important factor for antitumor activity, because the β-glucans such as lentinan (from *Lentinus edodes*), schizophyllan (from *Schyzophyllan commune*) and grifolan (from *Grifola frondosa*) could induce antitumor activity when they had high molecular weights. In this specific case, the method of drying most suitable for extra-cellular polysaccharide drying is spray-drying, using an inlet temperature of 240ºC (19.68% of relative increase), and the least suitable method is spray-drying using 120ºC (84.00% of relative increase). For intra-cellular polysaccharides drying, the spray-drying with 180ºC (1.26% of relative increase) is the most suitable method, and freeze drying (47.42% of relative increase) is the least one suitable (Table 6).

Table 6. Relative increase of reducing sugars contents on *A. blazei* polysaccharides extracts caused by drying methods, in relation to reducing sugars contents of polysaccharides not submitted to drying process (*EPS initial*)

Samples	Relative increase of reducing sugars contents (%)
EPS freeze	67,33
EPS vacuum	55,63
EPS spray 120°	84,00
EPS spray 180°C	28,96
EPS spray 240°C	19,68
IPS freeze	47,42
IPS vacuum	13,91
IPS spray 180°C	1,26

4.4 Total sugars contents

There was a decrease of total sugars contents in all polysaccharides extracts submitted by the drying processes. The values of total sugars percentage present in extra-cellular polysaccharides (EPS) and intra-cellular (IPS) polysaccharides extracts, before and after drying methods, are showed on Figure 11 and Figure 12, respectively. *EPS initial* and *IPS initial* represent extra-cellular and intra-cellular polysaccharides extracts not submitted to drying.

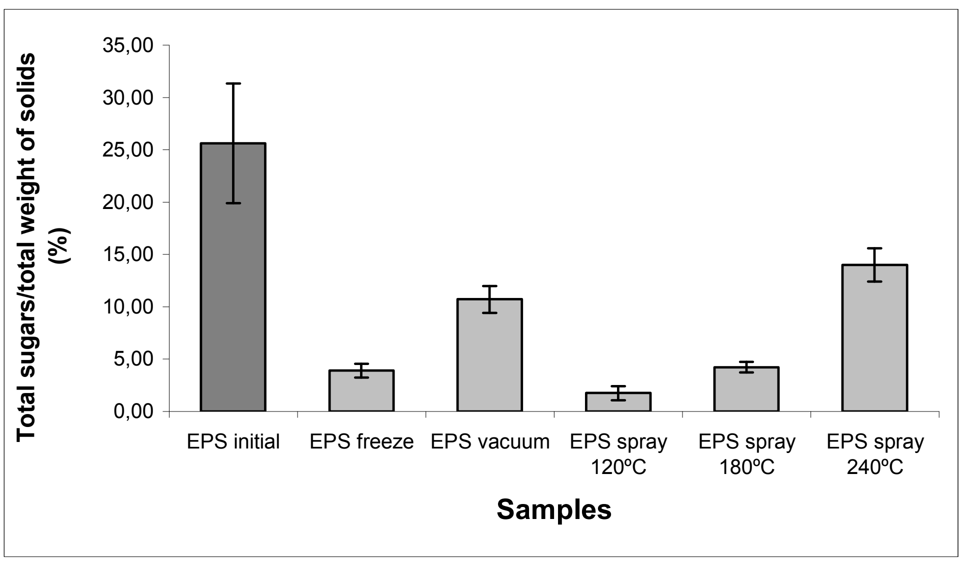

Figure 11. Percentage of total sugars on *A. blazei* extra-cellular polysaccharides extracts submitted to drying process. Results are mean ± standard deviation of three determinations.

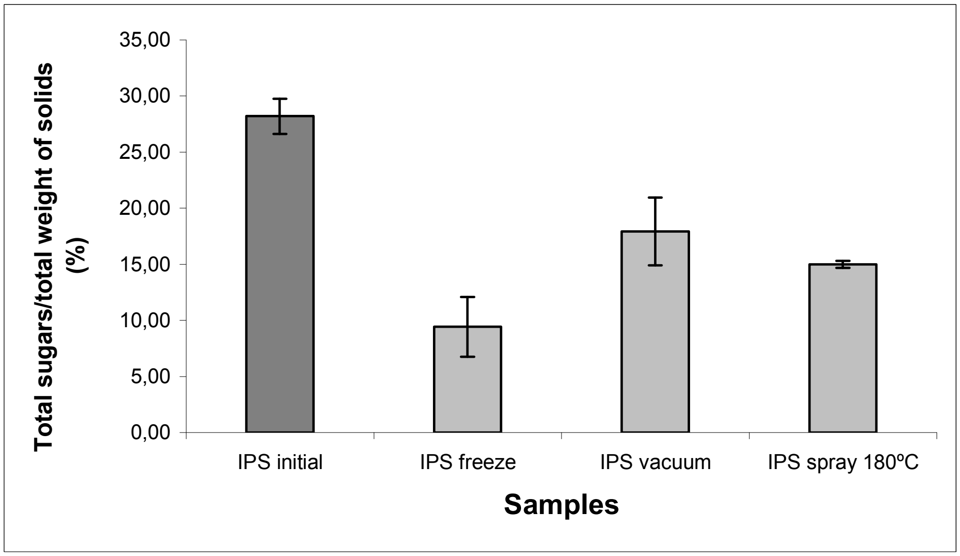

Figure 12. Percentage of total sugars on *A. blazei* intra-cellular polysaccharides extracts submitted to drying process. Results are mean ± standard deviation of three determinations.

One of the possible reasons for this decrease may have been caused by the degradation of polysaccharides during drying. However, this hypothesis must be considered carefully, since that, according to studies made by HONG et al (2005), the decomposition temperature for powders of extra-cellular polysaccharide from *Agaricus blazei* extracts was in the range of 200-400ºC. Therefore, as the temperature used in drying processes tested not reach this range (except in the process of spray drying), other factors must be associated with this decrease in the level of total sugars. Anyway, the decrease of total sugar caused by drying is not a good result, because this means that a smaller quantity of the product will have biological effective action. Based on this conclusion, and considering only this parameter, spray-drying using 240ºC as inlet temperature (45.36% of relative decrease) and vacuum drying (36.43% of relative decrease) are the most indicated methods for extra-cellular and intra-cellular polysaccharides drying, respectively. On the other hand, the least suitable drying methods for extra-cellular and intra-cellular polysaccharides are, respectively, spray drying with 120ºC of inlet temperature (93.19% of relative decrease) and freeze drying (66.57% of relative decrease) (Table 7).

Table 7. Relative decrease of total sugars contents on *A. blazei* polysaccharides extracts caused by drying methods, in relation to reducing sugars contents of polysaccharides not submitted to drying process (*EPS initial*)

Samples	Relative reduction of total sugars contents (%)
EPS freeze	84.81
EPS vacuum	58.24
EPS spray 120°	93.19
EPS spray 180°C	83.51
EPS spray 240°C	45.36
IPS freeze	66.57
IPS vacuum	36.43
IPS spray 180°C	46.86

4.5 Proteins contents

A decrease of proteins contents occurred in all polysaccharides extracts after submitted to the drying processes. The values of proteins percentage present in extra-cellular polysaccharides (EPS) and intra-cellular (IPS) polysaccharides extracts, before and after drying methods, are showed on Figure 13 and Figure 14, respectively. *EPS initial* and *IPS initial* represent extra-cellular and intra-cellular polysaccharides extracts not submitted to drying.

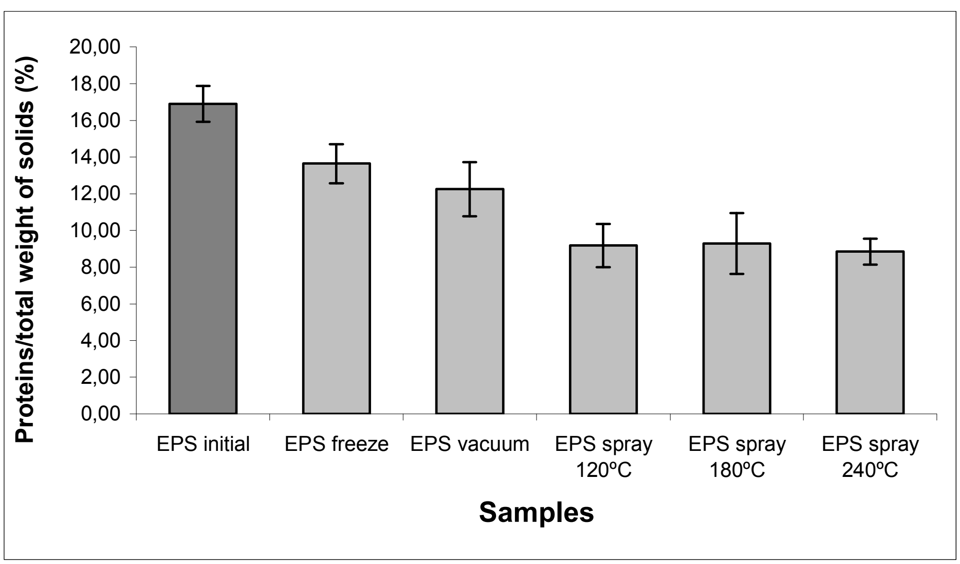

Figure 13. Percentage of proteins on *A. blazei* extra-cellular polysaccharides extracts submitted to drying process. Results are mean ± standard deviation of three determinations.

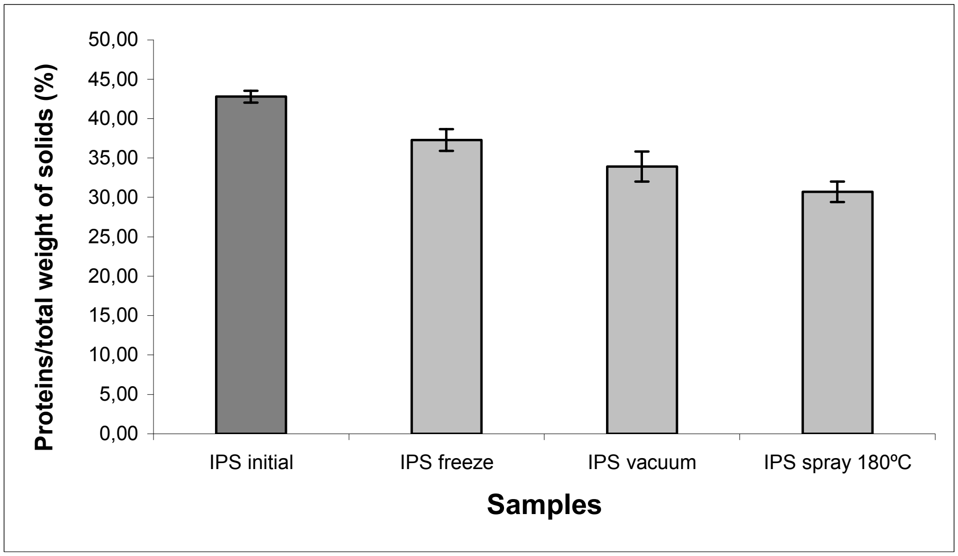

Figure 14. Percentage of proteins on *A. blazei* intra-cellular polysaccharides extracts submitted to drying process. Results are mean ± standard deviation of three determinations.

The decrease of proteins contents in polysaccharides extracts after drying was an expected result, because is very likely to happen denaturation of some proteins that are part of extract. In addition of temperature effects, changes on the solubility and pH of the extract can modify the secondary and tertiary structures of the protein or disruption of links peptide. Considering the fact that the principal anti-tumor compound produced by *Agaricus blazei* is a protein-bound polysaccharide, the β-D-glucan (TAKATU et al., 2001; YOSHIYUKI et al., 1990; DI PIERO, 2003; MIZUNO et al., 1990a), a loss of proteins could lead to a lesser antitumor and immuno-modulator activities. For this reason, and analyzing this parameter alone, freeze dryer presented the least value of proteins contents decrease for extra-cellular and intra-cellular polysaccharides (19.31% and 12.84% of relative decreases, respectively), being, therefore, the most suitable method of drying. The further decrease of proteins was caused by spray drying (180ºC of inlet temperature), with 28.23% of relative decrease for intra-cellular polysaccharides drying, and giving values very similar to the three temperatures tested for extra-cellular polysaccharides drying (around 46% of relative decrease) (Table 8). It is interesting to note that the variation in inlet temperature, during the spray drying, had no major effect on lowering the content of protein; it can be assumed with that outlet temperature has a greater participation on protein denaturation, because the time that the dry material is acceded to the walls of the collector pipe is much greater than the time the temperature of air entry is in contact with the particles of the sample.

Table 8. Relative reduction of proteins contents on *A. blazei* polysaccharides extracts caused by drying methods, in relation to proteins contents of polysaccharides not submitted to drying process (*EPS initial*)

Samples	Relative reduction of proteins contents (%)
EPS freeze	19.31
EPS vacuum	27.52
EPS spray 120°	45.72
EPS spray 180°C	45.01
EPS spray 240°C	47.66
IPS freeze	12.84
IPS vacuum	20.74
IPS spray 180°C	28.23

4.6 Immuno-modulatory effect

In order to facilitate the viewing, the results of *in vitro* MTT assay were divided by method of drying, and they are presented on Figure 15, Figure 16 and Figure 17. The control group is the same for all the three graphic representations. The treatments were added in three concentrations: 20 mg/ml, 10 mg/ml and 5 mg/ml.

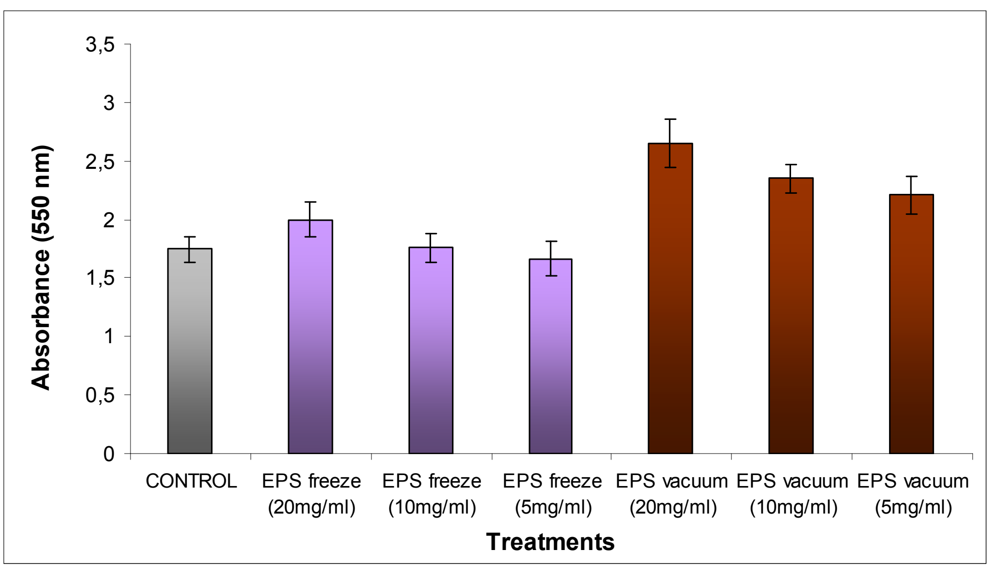

Figure 15. Immuno-modulatory effect *in vitro* of *Agaricus blazei* extra-cellular polysaccharides extracts submitted to freeze and vaccum drying processes. The control group was treated with PBS.

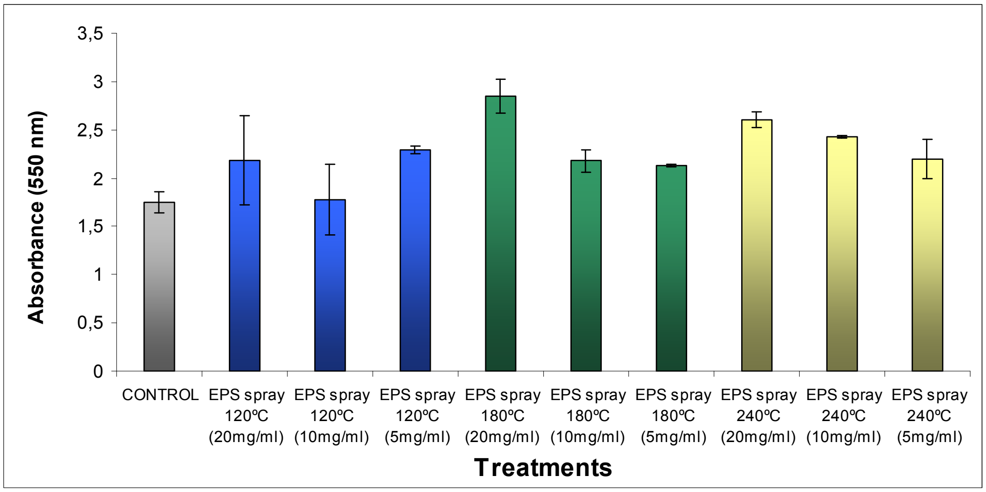

Figure 16. Immuno-modulatory effect *in vitro* of *Agaricus blazei* extra cellular polysaccharides extracts submitted to spray drying processes. The control group was treated with PBS.

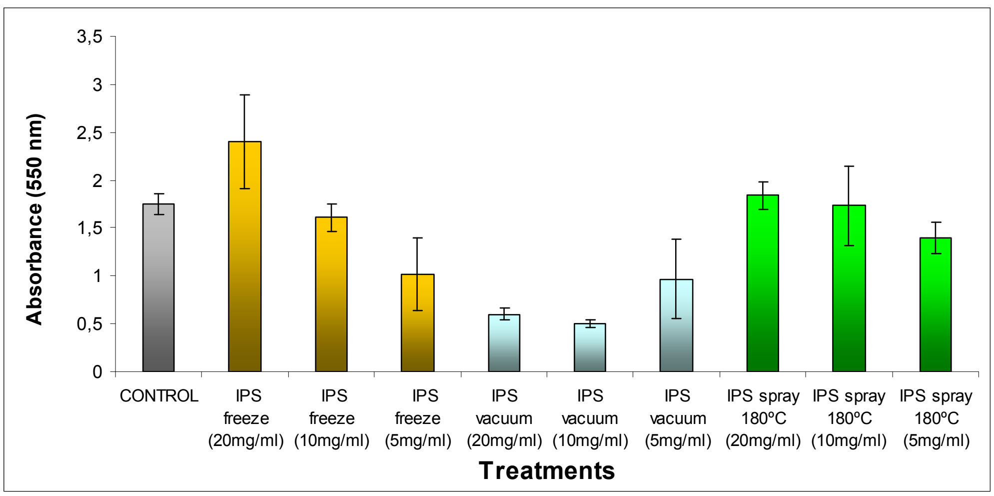

Figure 17. Immuno-modulatory effect *in vitro* of *Agaricus blazei* intra-cellular polysaccharides extracts submitted to freeze, vaccum and spray drying processes. The control group was treated with PBS.

Among the groups treated with intra-cellular polysaccharides extracts submitted to drying processes, only *IPS freeze* at 20 mg/ml showed some stimulative effect over macrophages proliferation. This can mean that the IPS extracts present little immunomodulator action on macrophages, or the concentrations used were very low.

For extra-cellular polysaccharides extracts, the ones submitted to freeze drying process showed little or no stimulative effect on the proliferation of macrophages *in vitro* (14.50% of stimulation at 20 mg/ml) (Table 9). On the other hand, the extracts submitted to vacuum drying and spray drying (for all three inlet temperatures tested) processes, obtained great values of stimulation, with emphasis on *EPS spray 180°C*, *EPS vacuum* and *EPS spray 240ºC*, all at 20 mg/ml (63.25%, 51.98% and 49.00% of stimulation, respectively) (Table 9). These data can indicate that the increase of extracts concentration was not inhibitory over the macrophages proliferation, at least until the maximum concentration used (20 mg/ml). It is difficult to compare these results with other works because, in most studies, other protocols are used. However, these results were better than showed by LIMA et al. (2006) that using the same procedure and testing vegetal extracts from *Siolmatra brasiliensis*, at 5 mg/ml, obtained 36% of macrophages stimulation.

β-glucan binds to macrophages and activates them to phagocytose tumour cells, that have been "tagged" for destruction by antibodies and small serum proteins (complement proteins), which bind to the tumour cell, this process is called opsonisation. Macrophage attachment to tagged (opsonised) tumour cells is primed by beta-glucan, which optimises binding to the complement proteins on the tumour cell, thus enhancing phagocytosis of tumour cells (BROWN and GORDON, 2001).

The reduction of tetrazolium salts (MTT) is now recognized as a safe, accurate alternative to radiometric testing. Among the applications for the method are drug sensitivity, cytotoxicity, response to growth factors, and cell activation (VAN DE LOOSDRECHT et al., 1994; MOSMANN, 1983).

Table 9. Percentage of mice macrophages stimulation *in vitro* by treatments with extracts of *Agaricus blazei* extra and intra-cellular polysaccharides after submitted to freeze, vacuum and spray drying processes

Treatments		Macrophages stimulation (%)
Extracts	**Concentrations**	
EPS freeze	20 mg/ml	14,50
	10 mg/ml	0,76
	5 mg/ml	-4,71
EPS vacuum	20 mg/ml	51,98
	10 mg/ml	34,47
	5 mg/ml	26,56
EPS spray 120ºC	20 mg/ml	24,96
	10 mg/ml	2,08
	5 mg/ml	30,94
EPS spray 180ºC	20 mg/ml	63,25
	10 mg/ml	24,69
	5 mg/ml	22,09
EPS spray 240ºC	20 mg/ml	49,00
	10 mg/ml	39,01
	5 mg/ml	25,81
IPS freeze	20 mg/ml	37,40
	10 mg/ml	-7,88
	5 mg/ml	-41,86
IPS vacuum	20 mg/ml	-65,62
	10 mg/ml	-71,49
	5 mg/ml	-44,52
IPS spray 180ºC	20 mg/ml	5,40
	10 mg/ml	-0,95
	5 mg/ml	-19,90

5 CONCLUSION

The goals of this work were cultivation of *Agaricus blazei* by submerged fermentation and extracting the intra-cellular and extra-cellular polysaccharides. In a subsequent step, the polysaccharides extracts were submitted to three drying methods: freeze, vacuum and spray drying. Finally, it was analyzed some parameters of each method of drying, the chemical composition of resultant powders, and their *in vitro* stimulant action on proliferation of Wistar mice macrophages.

The drying methods tested showed good results in efficiency and yield, around 90% and 95%, respectively, except spray drying process, but these parameters can be improved by addition of adjuvants, like gum arabic. The values of humidity contents and water activity on polysaccharides extracts submitted to drying were satisfactory, allowing the use of these methods of drying for the production of this product in the pharmaceutical industry, because the values obtained were below the minimum necessary for the development of microorganisms and the triggering of unwanted chemical reactions.

With respect to chemical composition of the extracts submitted to drying, there was a decrease in the levels of proteins and total sugars, and an increase in reducing sugars contents. Such results are not positive because it can cause a decrease in the biological effect of the product.

In tests of immunomodulator action, all extra-cellular polysaccharides extracts showed some level of stimulation of mice macrophages *in vitro*, more than 20% of macrophages stimulation; and the intra-cellular polysaccharides extracts, in general, had not the same biological effect.

This work was an initial study to evaluate the influence of the drying method on some characteristics and properties of the resulting product. Further studies should be made in order to evaluate other parameters that are possibly modified during the process of drying, as well as evaluation of other biological effects, such as antitumor activity and antioxidant.

6 BIBLIOGRAPHY

ALDEN, M.; TORKINGTON, P.; STRUTT, A.C.R. Control and instrumentation of a Fluid Bed Dryer using Temperature Difference Technique: Development of a working model. **Powder Technology**, 54, p. 15-25, 1988.

AOAC - Association of Official Analytical Chemists. **Official Methods of Analysis**. 15 ed. Washington: AOAC, 1990. 1298p.

BAIRD, R.M.; BLOOMFIELD, S.F. Microbial quality assurance in cosmetic, toiletries & non-sterile pharmaceuticals. New York: Taylor & Francis, 258 p, 1996.

BEUCHAT, L. R. Microbial stability as affected by water activity. **Cereal Foods World**, v. 26, n.7, p. 345-349, 1981.

BEUCHAT, L. R. Influence of water activity on growth, metabolic activities and survival of yeast´s and molds. **Journal of Food Protection,** v. 46, p. 135-141, 1983.

BROWN, G.D. and GORDON, S. 2001. A new receptor for beta-glucans. **Nature**, 413: 36-7.

CHANG, H. L.; CHAO, G. R. ; CHEN, C. C. ; MAU, J. L. Non-volatile taste components of *Agaricus blazei*, *Antrodia camphorata* and *Cordyceps militaris* mycelia. **Food Chemistry**, 74, p. 203-207, 2001.

CHRISTENSEN, C. M.; KAUFMANN, H. H. MICROFLORA. In: Christensen, C. M. Storage of cereal grain and their products. **St. Paul: American Association of Cereal Chemists**, p. 158-192, 1974.

COULTATE, T. P. Food – The Chemistry of its Components. **Series of the Royal Society of Chemistry Paperbacks**, 3rd edition. London, Royal Society of Chemistry, p. 321-339, 1996.

CUI, F. J.; LI, Y.; XU, Z. H. ; XU, H. Y. ; SUN, K. ; TAO, W. Y. Optimization of the medium composition for production of mycelial biomass and exopolymer by *Grifola frondosa* GF 9801 using response surface methodology. **Bioresource Technology** 97, p. 1209-1216, 2006.

DI PIERO, R. M. Potencial dos cogumelos *Lentinula edodes* (Shiitake) e *Agaricus blazei* (Cogumelo-do-sol) no controle de doenças em plantas de pepino, maracujá e tomate, e a purificação parcial de compostos biologicamente ativos. Master Dissertation. Universidade de São Paulo, 2003.

DIAS, E. S.; ABE, C.; SCHAWAN, R. F. Truths and myths about the mushroom *Agaricus blazei*. **Sci. Agric**. v. 61, p. 545-549, 2004.

DONG, Q.; YAO, J.; YANG, X. T.; FANG, J. N. Structural characterization of water-soluble β-D-glucan from fruiting bodies of *Agaricus blazei* Murr. **Carbohydrate Research,** 337, p. 1417-1421, 2002.

DU, Y.; GU, G.; HUA, Y.; WEI,G. ; YE, X. ; YU,G. Synthesis and anti-tumor activities of glucan derivatives. **Tetrahedron**, 60, p. 6345-6345-6351, 2004.

DUBOIS, M.; GILLES, K. A.; HAMILTON, J. K.; REBERS, P. A.; SMITH, F. Colorimetric method for determination of sugar and related substances. **Analytical Chemistry**, Washington, v. 28, n. 3, p. 350-356, 1956.

FAN, L.; SOCCOL, A. T.; PANDEY. A.; SOCCOL, C. R. Effect of nutrional and environmental conditions on the production of exo-polysaccharide of *Agaricus brasiliensis* by submerged fermentation and its antitumor activity. **LWT**, 2005.

GAVA, A. J. Princípios de tecnologia de alimentos. São Paulo, Nobel, p.239-240, c.7, 1978.

GERN, J. C. Production and characterization of extra-cellular polysaccharide from submerged fermentation by *Agaricus brasiliensis*. Master BIODEV Dissertation. Universidade Federal do Paraná, 2005.

HAYASHI, H.; KUMAZAWA, E.; SAEKI, Y.; ISHIOKA, Y. Continuous vacuum dryer for energy saving. **Drying Technol**, 1 (2), p. 275-284, 1983.

HONG, J. H.; CHOI, Y. H. Physico-chemical properties of protein-bound polysaccharide from *Agaricus blazei* Murill prepared by ultrafiltration and spray drying process**. International Journal of Food Science and Technology**, 42, p. 1–8, 2007

HUANG, Q.; JIN, Y.; ZHANG, L.; CHEUNG, P. C. K.; KENNEDY, J. F. Structure, molecular size and antitumor activities of polysaccharides from *Poria cocos* mycelia produced in fermenter. **Carbohydrate Polymers**, 70, p. 324-333, 2007.

IAMFES - International Association of Milk, Food and Environmental Sanitarians. **Guia de Procedimentos do Método de Análise de Perigos em Pontos Críticos de Controle – APPCC**. Cítara Editora, São Paulo, 1991.

KING, V. A.; LIU, C. F; LIU, Y. J. Chlorophyll stability in spinach dehydrated by freeze-drying and controlled low-temperature vacuum dehydration. **Food Research International**, 34, 167–175, 2001.

KODAMA, N.; MURATA, Y.; PHARM, B.; ASAKAWA, A.; INUI, A.; HAYASHI, M.; SAKAI, N.; NANBA, H. Maitake D-fraction enhances antitumor effects and reduces immunosuppression by mitomycin-C in tumor-bearing mice. **Nutrition** 21, p. 624-629, 2005.

LAI, L. S.; YANG, D. H. Rheological properties of the hot-water extracted polysaccharides in Ling-Zhi (*Ganoderma lucidum*). **Food Hydrocolloids** 21, p. 739–746, 2007

LEE, B. C.; BAE, J. T.; PYO, H. B.; CHOE, T. B.; KIM, S. W.; HWANG, H. J.; YUM, J. W. Biological activities of polysaccharides produce from submerged culture of the edibles Basidiomycete *Grifola frondosa*. **Enzyme and Microbial,** 32, p. 574-581, 2003.

LIMA, A. P.; PEREIRA, F. C.; VILANOVA-COSTA, C. A. S. T.; RIBEIRO, A. S. B. B.; SILVEIRA-LACERDA, E. P. Avaliação da atividade antitumoral e citotóxica da planta *Siolmatra brasiliensis*. **Revista Eletrônica de Farmácia**, Suplemento Vol 3 (2), 44-46, 2006.

LIN, H.; SHE, Y. H.; CASSILETH, B. R.; SIROTNAK, F.; CUNNINGHAM, R. S. Maitake beta-glucan MD-fraction enhances bone marrow colony formation and reduces doxorubicin toxicity in vitro. **Int Immunopharmacol**, 4(1), p. 91–99, 2004.

LOWRY, O. H.; ROSBROUGH, N. J.; FARR, A. L.; RANDALL, R. J. Protein measurement with the Folin-Phenol reagents. **J. Biol. Chem**, 193, p. 265-275,1951.

LULL, C.; WICHERS, H. J.; SAVELKOUL, H. F. J. Antiinflammatory and Immunomodulating Properties of Fungal Metabolites. **Mediators of Inflammation,** 2, p. 63-80, 2005.

LUO, X.; Xu, X.; Yu, M.; Yang, Z.; Zheng, L. Characterisation and immunostimulatory activity of an α-(1-6)-D-glucan from the cultured *Armillariella tabescens* mycelia. **Food Chemistry,** 111, p. 357–363, 2008.

MASTERS, K. Spray Drying Handbook, 5th ed**.; Longman Scientific & Technical: Essex**, p. 158-189, 1991.

METHACANON, P.; MADLA, S.; KIRTIKARA, K.; PRASITSIL, M. Structural elucidation of bioactive fungi-derived polymers. **Carbohydrate Polymers,** v. 60, p.199-203, 2005.

MIZUNO, T.; HAGIWARA, T.; NAKAMURA, T.; ITO, H.; SHIMURA, K.; SUMIYA, T.; ASAKURA, A. Antitumor activity and some properties of water-soluble polysaccharides from "Himematsutake", the fruiting body of *Agaricus blazei* Murill. **Agric. Biol. Chem**. V. 54, p. 2889-2896, 1990a.

MIZUNO, M., INAGAKI, R., KANAO, T., HAGIWARA, T., NAKAMURA, T., ITO, H., SHIMURA, K., SUMIYA, T., ASAKURA, A.. Antitumor activity and some properties of water-insoluble heteroglycans from ''Himematsutake", the fruiting body of *Agaricus blazei* Murrill. **Agric. Bio., Chem.** 54 (11), 2897–2905, 1990b.

MIZUNO, T., SAITO, H., NISHITOBA, T. & KAWAGISHI, H. Antitumor-active substances from mushrooms. **Food Reviews International**, 11, 23–61, 1995.

MIZUNO, T. Development of antitumor polysaccharides from mushroom fungi. **Foods Food Ingred J Jpn,** 167:69–85, 1996

MIZUNO, M., MORIMOTO, M., MINATO, K., TSUCHIDA, H. Polysaccharides from *Agaricus blazei* stimulate lymphocyte T-cell subsets in mice. **Biotechnol. Biochem**. 62 (3), 434–437, 1998.

MIZUNO, M., MINATO, K., ITO, H., KAWADE, M., HIROFUMI, T., TSUCHIDA, H. Anti-tumour polysaccharides from the mycelium of liquid cultured *Agaricus blazei*. **Biochem. Mol. Biol**. Int. 47 (4), 707–714, 1999.

MOSMANN, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. **J Immunol Methods**. v. 65, n. 1-2, p. 55-63, 1983.

MUJUMDAR, A. S. An overview of innovation in industrial drying: current status and R&D needs. **Transp Porous Med**, v. 66, p. 3–18, 2007.

NAKAJIMA, A.; ISHIDA, T.; KOGA, M.; TAKEUCHI, T.; MAZDA, O.; TAKEUCHI, M. Effect of hot water extract from *Agaricus blazei* Murril on antibody-producing cells in mice. **International Immunopharmacology**, v. 2, p. 1205-1211, 2002.

PINTO, T.J.A.; KANEKO, T.M.; OHARA, M.T. Controle biológico de qualidade de produtos farmacêuticos, correlatos e cosméticos. São Paulo: Atheneu, 309 p, 2000.

RATHANANAND, M.; KUMAR, D. S.; SHIRWAIKAR, A.; KUMAR, R.; KUMAR, D. S.; PRASAD, R. S. Preparation of mucoadhesive microspheres for nasal delivery by spray drying. **Indian Journal of Pharmaceutical Sciences**, v. 69, p. 651-657, 2007.

RATTI, C. Hot air and freeze-drying of high-value foods: a review. **Journal of Food Engineering**, v. 49, 311–319, 2001.

RUBEL, R.; LEIFA, L.; PANDEY, A.; LORQUIN, J.; SOCCOL, C. R. Influence of different carbon sources on biomass and exopolysaccharide production by *Ganoderma lucidum* in submerged fermentation. **In: International Congresses In Food Industries**, Clermont Ferrand, France, Abstract, 2004.

SERRANO, M. A. Determination of an empirical correlation for the mass transfer coefficients of pharmaceutical powders in a Fluid Bed Dryer. Master of Science Dissertation. University of Puerto Rico, 2004.

SINGH, R. P.; HELDMAN, D. R. Food dehydration. **Introduction to Food Engineering**, 2nd ed, p. 415–442. 1993.

SMITH, J. E.; SULLIVAN, R.; ROWAN, N. The role of polysaccharide derived from medicinal mushrooms in cancer treatment programs: current perspectives (review). **International Journal of Medicinal Mushrooms**, 5, 217-234, 2003.

SNOWMAN, J. W. Freeze dryers. **Industrial Drying of Foods**. p. 134–155, 1997.

SOUTHGATE, D.A.T. **Determination of food carbohydrates**. London. Ed. Applied Science Publishers LTD, p. 177, 1976.

TAKAKU, T., KIMURA, Y., OKUDA, H. Isolation of an antitumor compound from *Agaricus blazei* Murril and its mechanism of action. **Journal of Nutrition**, v. 131, 5, p.1409-1413, 2001.

VALDUGA, A. T.; BATTESTINI, V; FINZER, J, R, D. Drying of maté extract in spray dryer. **Ciênc. Tecnol. Aliment,** Campinas, v.23, no.2, 2003.

VAN DE LOOSDRECHT AA, BEELEN RH, OSSENKOPPELE GJ, BROEKHOVEN MG, LANGENHUIJSEN MM. A tetrazolium-based colorimetric MTT assay to quantitate human monocyte mediated cytotoxicity against leukemic cells from cell lines and patients with acute myeloid leukemia. **J. Immunol Methods**. 174(1-2): 311-320, 1994.

WASSER, S. P., AND A. L. WEIS. Therapeutic effects of substances occuring in higher Basidiomycetes mushrooms: a modern perspective. Review Crit. **Rev. Immunol,** 19, p. 65-95, 1999.

WASSER, S.P. Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. **Appl. Microbiol. Biotechnol**., v. 60, p. 258-274, 2002.

XIAO, J-H; CHEN, D-X.; WAN, W-H.; HU, X-J.; QI, Y.; LIANG, Z-Q. Enhanced simultaneous production of mycelia and intracellular polysaccharide in submerged cultivation of *Cordyceps jiangxiensis* using desirability functions. **Process Biochemistry**, 2006.

YOSHIYUKI, A., NAOHITO, O., MASUMI, O., SHOZO, O. & TOSHIRO, Y. Change of biological activities of (1–3)-b-D-glucan from *Grifola frondosa* upon molecular weight reduction by heat treatment. **Chemical & Pharmaceutical Bulletin**, 38, 477–481, 1990.

ZOU, X. Effects of Zn supplementation on the growth, amino acid composition, polysaccharide yields and anti-tumour activity of *Agaricus brasiliensis*. **World Journal of Microbiology & Biotechnology**, 21, p. 261–264, 2005.