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Production and characterization of extra-cellular polysaccharide from submerged fermentation by *Agaricus brasiliensis*

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SUMMARY

1. Introduction

Human quest for innovation is a non-ending phenomenon. The concern of health in this regard has been of prime importance. Consumers are deeply concerned about how their health care is managed, administered and priced. They are frustrated with the expensive, high-tech disease-treatement approach predominant in modern medicine. The consumer is seeking complementary or alternative beneficial products that make nutraceutical articularly appeling.

Researches have shown that these diseases can be cured or controlled by the consumption of certain mushrooms as a functional food or through the use of extracted biologically active compounds from them. Mushrooms are potential sources of these new substances (activators) and have recently become more attractive directly for purpose of drug development (OHNO et al., 2001).

In fact, human has utilized high fungi for treatment of disease, recovery and strengthening of immune system for a long time. For example, *Ganoderma lucidum* was used as medicine and health food in the Far-East for more than 2000 years. Modern technology has elucidated the principles of these high fungi, which was β-glucan. Recently, more β-glucan compounds were clinically proved, like lentinan (*Lentinus edodes*), sonifilan – SPG (*Schizophyllum commune*), GRN (*Grifola frondosa*) (FAN, 2002).

The antitumor polysaccharides of fruiting bodies of *A blazei* have been well studied in the last two decades, in which 1,3-β-glucan with 1,6-β-linkage branch and β-glucan-protein complex were isolated and shown to possess strong antitumor activity.

The polysaccharide-protein complex (ATOM) from the mycelium of *A blazei* was isolated and its antitumor activities against four kinds of established mouse tumors were examined (ITO *et al.*, 1997).

1.1. Mushrooms- *Agaricus brasiliensis*

Higher Basidiomycetes such as *Lentinus edodes* (Shiitake), *Ganoderma lucidum* (Reishi), *Inonotus obliquus* (Chaga) and many others (WASSER, 2002) are subject of great interest because their nutritional value and pharmacological properties.

Among the mushrooms species of higher Basidiomycetes, *Agaricus blazei* Murril has received attention due to its bioactive compounds.

Agaricus blazei was first recognized as a novel species in 1945 by an American mycologist, W. A. Murrill, according to the specimen collected by Mr. R. W. Blaze from his open lawn in Gainesville, Florida on April, 1944. (FAN, 2002).

It was no doubt that the pioneering Japanese mycologists first "blazed" the path for its cultivation and deserved credit for bringing this species forward, which was resulted from its re-discovery by Brazilian farmer -Takatoshi Furumoto. It was reported that in 1965 Furumoto found this mushroom in the hill regions of the Atlantic Rainforest in the district of Piedade, south of São Paulo State, Brazil, which was well known to the local people (COLAUTO et al. 2002; TAKAKU et al. 2001; BARBISAN et al, 2003; FAN, 2002). He accredited this local famous mushroom and then sent the spore to Japan, Iwade Research Institute of Mycology, which was established in 1963 in Tsu, Mie Prefecture.

Agaricus blazei Murril, popularly known as the sun mushroom and as "Himematsutake" or "Agarikusutake" in Japan to where this mushroom is exported since 1965.

Analysis of data on cultivated mushroom originating from Brazil, and study of type material of *A. blazei* Murril shows dramatic differences between them. On the basis of existing differences the correct name for widely cultivated mushroom was proposed as new for science species *Agaricus brasiliensis. A. blazei* is the North American endemic not cultivated species known from three localities – one in Florida and two in South Carolina (WASSER, 2002).

1.1.1. Common names

With the distribution, this mushroom gained more names, besides the scientific name *Agaricus brasiliensis*. In Brazil, it is called as "Mushroom of God" (Cogumelo de Deus) because of magic like function known to local people, or "Mushroom of Sun" (cogumelo do Sol) as the tropical habitat. In Japan, the Kawariharatake and *Agaricus heterosistes* Heinem et Gooss were used until the agreement confirmed by scientists in the meeting of Japanese Scientific Society held in 1982. Now Himematsutake is used as commercial name in Japanese market, besides "*Agaricus* of Brazil". In the United States, the Royal Sun Agaricus, Murrill's Agaricus or ABM, King Agaricus, or Almond Portobello are used for this mushroom (FAN, 2001). In China, Jisongrong is used according to the Japanese name – Himematsutake.

1.1.2. Taxonomy

A taxonomic position is given to *Agaricus blazei* in
http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=79798. This classification is presented below:

Kingdom: Fungi
Phylum: Basidiomycota
Class: Homobasidiomycetes
Order: Agaricales
Family: Agaricaceae
Genus: Agaricus
Specie: *Agaricus blazei*

However WASSER et al., 2002 studied deeply the most of the known cultivars originate from Brazil and described it as a new species - *Agaricus brasiliensis*. Because taxonomics problems this literature review will use the term *Agaricus blazei*.

Figure 1. Fruiting bodies and spores of *A. blazei* Murrill

1.1.3. Economic aspects

This mushroom is consumed by the population as a food, but mainly as tea. It has become the center of a US$ 600 million industry in Japan since 1995 (FAN, 2002).

The commercial product principally is dried fruiting body. It has been reported that 100-300 tons of the dried fruiting body of *A. brasiliensis* is produced every year in Japan and is used by 300000-500000 persons for cancer prevention and/or as an adjuvant to cancer chemotherapy drugs after the removal of a malignant tumor (TAKAKU *et al.* 2001). It is estimated in Brazil that there are about 20 tons of dried fruiting body produced in the 1996/1997. Of all, more than 90% were exported to Japan (dried). In China, it was estimated that more than 500 tons of dried mushrooms are produced every year.

1.1.4. Chemistry and nutritional value of fruiting body

The raw fruiting body of this mushroom contains 85-87% water, like other *Agaricus* species. Dry mushroom is rich in protein as well as carbohydrate. It contains 40-45% crude protein (N x 6.25), 38-45% carbohydrates (soluble non-nitrogenous substances), 6-8% fiber, 5-7% crude ash, and 3-4% crude lipid. The vitamin content (in mg/100g) on dry weight basis is 0.3 B_1, 3.2 B_2, and 49.2 niacin. It also contains a relatively large amount of

ergosterol (0.1-0.2%), which is converted into vitamin D_2 via pre-vitamin D_2 after exposure to light and cooking (FAN, 2002). (Table 1).

Table 1. Nutritional components and polysaccharide content of *A blazei* fruiting body

Components	Content (%, on dry basis)	Components	Content (on dry basis)
Crude protein	42.64	P	11.59 mg/g
Crude fat	2.78	B1	0.37 mg/100g
Total carbohydrate	46.58	B2	3.66 mg/100g
Crude fiber	7.53	Niacin	21.90 mg/100g
Polysaccharide	2.41	Ergosterol	135..57 mg/100g
Ash	8.57		

* Zhong et al., 1999 in FAN, 2002.

1.1.5. Properties of *A. blazei*

1.1.5.1. Chemoprotection and Antimutagenic Effect

In order to evaluate the effect of *A. blazei* on DNA and anticarcinogenic activity *in vivo* and *in vitro* studies were carried out.

BELLINI *et al*. (2004) examined the anticlastogenic effect of *Agaricus blazei* in Chinese hamster ovary cells, CHO-k1, using methyl methanesulphonate (MMS) as the DNA damage inducing agent. They showed that aqueous solutions obtained from a mixture of lineages and single lineages of the mushroom are anticlastogenic, suggesting the presence of a chemoprotective agent(s).

The results found by MENOLLI *et al* (2001), showed that an aqueous solution obtained from a mixture of lineages of the mushroom reduced the frequencies of micronuclei induced by MMS in culture Chinese hamster V79 cells. The results suggested that these mushroom exhibited antimutagenic activities that might contributed to an anticarcinogenic effect.

BARBISAN *et al.* (2002) reported that 2-week treatment with aqueous extract from *Agaricus blazei* (like a tea) before the initiation of rat liver carcinogenesis by diethylnitrosamine (DEN) exerted a liver-protective effect as seen by decreased serum transaminase levels and regenerative cell proliferation and early liver pre-neoplasia development 48 h after the iniciation procedure. The autors concluded that aqueous extract of *A. blazei* has a hepatoprotective effect on liver toxicity and on the initiation step of hepatocarcinogenesis in an environment of moderate toxicity.

1.1.5.2. Antioxidant effect

IZAWA *et al.* (2004) used thiredoxin-deficient yeast system to screen for antioxidant activity in mushrooms, and found that the edible mushroom *Agaricus blazei* Murril is an excellent source of antioxidants.

1.1.6. Polysaccharides from *A. blazei*

Mushrooms represent an unlimited source of polysaccharides with antitumor and immunostimulating properties. These polysaccharides are of different chemical composition, with most belonging to the group of β-glucans; these have β-(1→3) linkages in the main chain of the glucan and additional β-(1→6) branch points that are needed for their antitumor action.

IKEGAWA *et al.*, in 1969, published one of the first scientific reports on antitumor activities of the extract obtained by hot water from fruiting bodies of some edible fungi belonging to the *Polyporaceae* family, which showed marked host-mediated anti-tumor activity against cancers like Sarcoma 180 grafted on animals, was identified as polysaccharides of β-D-glucans, which produced D-glucose with acid hydrolysis (WASSER, 2002).

The immunostimulating activity and antitumor action of *A.blazei* extracts were investigated in different laboratory models, including Sarcoma 180 and Meth-A fibrosarcoma tumor-bearing mice (KAWAGISHI et al. 1989, 1990; MIZUNO et al. 1990a,

1998). MIZUNO *et al.*, 1989 first reported the extraction and purification methodology of polysaccharide of *A blazei* fruiting body in English, in which about seventeen water-soluble polysaccharide fractions were isolated, seven were demonstrated to have antitumor activity.

KAWAGISHI *et al.* (1989) and MIZUNO *et al* (1990) isolated water-insoluble polysaccharides from the fruiting body of *A. blazei*. Antitumor activities and physic-chemical properties of the fractions also were analyzed. The polysaccharide-containing materials were extracted from the fruiting bodies of A. blazei with aqueous ammonium oxalate and sodium hydroxide. They found a remarkable antitumor activity in a glycoprotein, FIII-2-b. MIZUNO *et al* (1990) reported that this polysaccharide portion of this polysaccharide-protein complex (polysaccharide, 50,2% and protein, 43,3% each on a weight basis) consisted of β-(1→6)-glucan.

Not only fruit bodies but also cultured mycelia of *A. blazei* are a source of antitumor polysaccharides. ITO *et al.* (1997) isolated polysaccharide compound from hot water extract of mycelium. It was identified as a glucomannan-protein complex and denominated as ATOM (antitumor organic substance Mie). The authors examined the tumor growth suppression by the i.p. or p.o. administration, using the tumor (Meth A fibrosacoma, Shionogi carcinoma 42) and allogeneic tumor (Sarcoma 180 solid, Ehrich ascites carcinoma- EAC), four kinds of established mouse tumors. ATOM was highly effective on subcutaneously administration and the dose of oral administration was much higher than that of i.p as to reach the same level of inhibition, but it had advantage of no injection suffering. Thus the tumor growth-inhibitory effect of ATOM is apparently due immunological host-mediated mechanisms.

A liquid medium filtrate separated from mycelium after submerged cultivation of *A. blazei* contained manana-protein complex (AB-FP) with a molecular weight of 10^5-10^7 Da and a small amount of glucose, galactose and ribose. The yield of AB-FP was 575mg/l liquid medium filtrate, and it possesses significant antitumor activity (in WASSER, 2002).

According to FAN (2002), two extra-cellular polysaccharide (EPS) of *Agaricus brasiliensis* were obtained in the submerged fermentation; one was soluble in water and the other was insoluble. The extra-cellular polysaccharide that was soluble in water was conjugated with protein.The EPS soluble in water had 5.14% protein and the EPS insoluble

in water possessed 14.93% protein. The molecular weight of the EPS soluble in water was 2.09×10^6 Da.

The EPS of *A blazei* produced from submerged fermentation showed strong inhibition against Sarcoma 180 in mice, reaching 72.19% inhibition compared with control group. Furthermore, 50% of mice in the test group demonstrated total tumor regression (FAN, 2003).

Thus, antitumor polysaccharides investigated in *A. blazei* fruit body, culture mycelia, or produced extra-cellularly in a culture medium have different chemical structures, but all have interesting properties, mainly antitumor effect.

1.2. Submerged cultivation of Mushrooms

Because it usually takes several months to cultivate the fruiting body of the fungi and it is difficult to control the product quality during its cultivation, submerged fermentation for producing the antitumor principle fell in human scope. However the literature about submerged cultivation of *A. blazei* is very limited.

KAWAGOE *et al*. (2004) cultivated *A. blazei* mycelium in bubble column. The fermenter was made with a glass pipe that had a diameter of 63 mm and a height of 1,5 m, and its total volume was 4,7 m^3. They investigated the effects of cultivation temperature, pH, composition of culture medium, aeration rate and time of seed cultivation on the cell growth characteristics. The best medium was: glucose (10-20 g/L); $MgSO_4 \cdot 7H_2O$ (0,9 g/L); KH_2PO_4 (0,9 g/L); yeast extract/peptone (equi-mass mixture)(5g/L). They also showed a promoting effect of sulphite pulping waste on the growth of *A. blazei* at a concentration of 5g/L. The optimal pH and temperature seem to be around 4.5 and 30°C, respectively. The found a $Y_{x/s}$ ~1 that means that amounts of glucose consumed was converted approximately to an equal mass of cell.

FAN (2002) studied the optimization of the media for producing extra-cellular polysaccharide using *Agaricus brasiliensis* LPB 03. Cultures were prepared in 250-mL Erlenmeyer flasks containing 50 mL medium. The parameters evaluated were temperature, initial pH, agitation/stationery, inoculation rate, concentration of glucose, peptone,

K$_2$HPO$_4$·3H$_2$O, MgSO$_4$·7H$_2$O. He concluded that for the EPS production, glucose at 20g/l, yeast extract 4 g/l, K$_2$HPO$_4$ 0.6 g/l and MgSO$_4$ 0.3 g/l were most suitable. The temperature of 30°C was best for the EPS production. Best initial pH was 5.7 for the biomass and 6.1 for the EPS production. In the growth kinetics, the quantity of extra-cellular polysaccharide reached at the peak (8.634mg/50ml) on 8th day and remained more or less same till 12th day of fermentation. This suggested that the fermentation should be terminated before 10 day's culture if the objective was the production of extra-cellular polysaccharide.

1.3. Aeration rate on fungi growth and EPS production

Recently, XU and YUN (2004) reported a result concerning EPS production by *Paecilomyces tenuipes* C240 and its quality under different aeration rates. They found that 3,5 vvm was the best level of aeration for mycelial growth, but for the EPS production was not significative difference between 2,5 and 3,5 vvm. They found that an air flow rate of 0.5 vvm was not good for biomass and EPS production. They also showed that different aeration rates produced EPS with different composition. KIM *et al.* (2005), reported a study with DOC (dissolved oxygen concentration) controlled in batch cultures of *A. blazei*. They changed agitation speed and aeration rate to maintain a constant DOC and found that when the DOC was controlled at level up of 20% with an agitation speed range of 100-450 rpm, the EPS production of 5,7 g/L is 2.2-fold higher than when the DOC was not controlled.

1.4. Effect of pH control on EPS and biomass production by mushroom

The pH of fermentation broth is a key factor for biomass and metabolites production by the microorganisms and the pH control of the media has a great influence on the microbial exopolysaccharide formation (YANG and LIAU, 1998).

CHIN-HANG *et al.*, (2004), studied the effects of culture pH on the production, molecular weight distribution and the bioactivity of polysaccharides produced by *Agaricus*

blazei were evaluated by four pH-controlled batch cultures. As the culture pH of each batch was controlled from 4.0 to 7.0, the maximum polysaccharide concentration increased from 561 to 1.252 mg /L, but the average molecular weight of the polysaccharides decreased monotonically from 1080 kDa to 600 kDa. KIM *et al.*, (2004) also studied the pH control in *A. blazei* submerged culture. He reported that the maximum EPS of 3.69 g/L and biomass production of 7.38 g/L were achieved with the controlled pH at 5.0.

1.5. Addition of Mineral Sources in fungi cultivation

Micro and macroelements have an important role in microrganism metabolism, mainly as enzymes co-factors (JONATHAN and FASIDI, 2001). KIM, *et al.* 2002, reported a study about EPS production and mycelial growth of *Paecilomyces sinclairii* in submerged fermentation with different mineral sources. They found that the maximum mycelial growth and polysaccharide production was achieved in the medium containing potassium, followed by manganese and iron. JONATHAN and FASIDI (2001) showed that for EPS and biomass production by *Lentinus subnudus* , Mg and Zn are the best minerals, while *S. commune* needs, mainly, Mg and Fe.

KIM *et al.*, 2004, reported a study about mineral sources in *A. blazei* growth and EPS production. He found a remarkable EPS improvement when he added Mn at 500 mg/L at the medium. Zinc also presented an important effect in EPS production. His basal media already contained iron at 100 mg/L.

1.6. Plant oils and fatty acid effect on fungi cultivation

In a submerged fermentation, it is commum the foam formation because aeration and agitation during the process. In order to avoid the foam formation without any decrease in EPS production, antifoam agents must be studied and some plant oils can be employed in a large-scale submerged process.

YANG *et al.*, (2000) studied the fatty acids and plant oils addition effect on submerged fermentation of *Ganderma lucidum*. They found that the stimulation or inhibition

for biomass an EPS production depends on types and levels of fatty acids. Oleic acid at level of 1.5 g/L led to an increase in biomassa and palmitic acid enhanced the EPS production. However, linoleic acid supressed mycelial growth and EPS production. No literature about the oils effects in *A. brasiliensis* cultivation was found.

2. Objectives

The research in submerged culture of *A. brasiliensis* aims to achieve the development and the application of the process for polysaccharide and biomass production in large scale bioreactors that might be commercially profitable.

However, there are much subjects to be studied and would be impossible to approach everything in this work.

So, the aim of this study will be, increase the knowledge about submerged cultivation of *A. brasiliensis* in bioreactor, exopolysaccharide production and its characterization.

3. Materials and Methods

3.1. Strain of *A. brasiliensis* LPB 03

The strain of *A. brasiliensis* LPB 03 was isolated by FAN LEIFA (2000) in Brazil. The strain was maintained on potato-dextrose agar (PDA) medium at room temperature. The sub-culturing was made at each three months. The inoculum was made with 5 agar block (5mm in diameter each) in 250 ml Erlenmeyer flasks containing 50 ml medium (pH 6 ,0) and incubated at 30°C for 10 days, 120 rpm. The medium was prepared with glucose 20 g/l, yeast extract 4g/l, K_2HPO_4 0.6 g/l and $MgSO_4$ 0.3 g/l (FAN, 2002). This media was called basal media.

3.2. Inoculum

For the second stage inoculum, was used 5 mL of the first inoculum (section 2.1) in 45 mL media and incubated at 30°C, in shaker, for 7 days, 120 rpm. After this period, this fermented broth was filtered using a screen with 2.0 mm pores size, so this biomass was triturated and washed with 150 mL of water deionized. This mycelial suspension was used for the fermentation studies.

3.3. Analysis of biomass and extra-cellular polysaccharide

Biomass was determined by dry weight estimation. The culture was filtered using Whatman 1 filter paper, washed with 20 mL of water deionized and dried at 60°C for 48 hours. The filtrate was concentrated in a rotary evaporator at 55°C under low pressure and the extra-cellular polysaccharides was precipitated with four volum of 95% ethanol keeping, previously, over-night in the freezer. This mixture was left overnight at -10°C. The precipitated EPS was centrifuged at 6000 rpm and washed twice with 95% alcohol and acetone, respectively. The concentration of polysaccharide was determined by phenol-sulfuric method (DUBOIS *et al*, 1956), utilizing glucose as standard.

3.4. Analysis of residual Sugar

The residual sugar was measured by SOMOGY-NELSON (1945) using glucose in the standard curve.

3.5. Study of Plant Oils Effect

It was tested for biomass and EPS production seven plant oils at two level of concentration 1% (w/v) and 2% (w/v). This experiment was carried out in 125 mL shake flasks containing 50 mL of basal media, with only 2 g/L of yeast extract, at 30°C, 150 rpm for seven days. The plant oils tested were: canola, corn, sunflower, soybean, olive, rice and cotton. The plant oil fatty acid composition, with the exception of cotton oil, are showed in table 2. These datas were obtained with the plant oils supplier.

TABLE 2. Composition of the seven kinds of oils used showing the diferent concentrations of fatty acid contents.

Fatty Acid	Canola	Sunflower	Corn	Soy	Olive	Rice
Saturated Fatty Acids						
14:0 Miristic	0.1 %	0.1 %	0 %	0.1 %	0 %	0.29 %
16:0 Palmitic	4.2 %	5.6 %	12.9 %	11.6 %	12.99 %	19.53 %
18:0 Estearic	2.3 %	4 %	2.2 %	3.8 %	1.78 %	1.01 %
20:0 Araquidic	0.7 %	0.3 %	0.6 %	0.5 %	0 %	2.53 %
22:0 Behenic	0.4 %	0.9 %	0.2 %	0.4 %	0 %	0 %
24:0 Lignoceric	0.2 %	0.4 %	0.2 %	0.2 %	0 %	0 %
Mono-unaturated Fatty Acids						
16:1 Palmitoleic	0.5 %	0.2 %	0.3 %	0.3 %	1.63 %	0.34 %
18:1 Oleic	62.1 %	26 %	33.4 %	24.1 %	70.94 %	38.83 %
20: 1 Gadoleic	1.3 %	0.2%	0.3 %	0.3 %	0 %	0 %
22: 1 Erucic	0.2 %	0 %	0 %	0 %	0 %	0 %
Poli-unsaturated Fatty Acids						
18:2 Linoleic	19.3 %	62.2 %	48.9 %	52.9 %	11.14 %	36.62 %
18:3 Linolenic	8.3 %	0.1 %	1 %	5.8 %	1.22 %	0.84 %
Other Fatty Acids	0.4 %	0 %	0 %	0 %	0.3 %	0.01 %

Source: Bunge Foods S.A. (2004).

3.6. Effect of mineral Sources

The influence of some mineral sources was tested in this work. This experiment was carried out in 125 mL shake flasks containing 50 mL of basal media at 30°C, 120 rpm for seven days. Five elements were added separately to the basal media at two levels, 50 mg/L and 300 mg/L. These mineral sources were: $FeCl_3$, $MnSO_4.H_2O$, $CaCl_2.2H_2O$, $CuSO_4.H_2O$, $ZnSO_4.7H_2O$. The samples with basal media only were called control.

3.7. Stirred-tank Fermentor Scale Study

A first kinectic was done to determine the time with the maximum production of EPS to carry out the further experiments. The medium was prepared with glucose 20 g/l, yeast extract 4g/l, K_2HPO_4 0.6 g/l and $MgSO_4$ 0.3 g/l (FAN, 2002). Inoculation rate will be 40 ml of suspension of mycelium per liter (inoculation rate of 4%). The experiment was conducted at 30°C (EPS optimized conditions by FAN, 2002). The air flow rate used was 1 vvm (air volum/broth volum/min) and an agitation of 100 rpm. This study was done in a 2 L bioreactor (INCELTECH) with 1.5 L of useful volum. The samples were taken each 12 hours during 9 days.

3.8. Stirred-tank Fermentor Scale Study and Effect of pH control on biomass and polysaccharide production

The medium was prepared with glucose 20 g/l, yeast extract 4g/l, K_2HPO_4 0.6 g/l and $MgSO_4$ 0.3 g/l (FAN, 2002). Inoculation rate will be 40 ml of suspension of mycelium per liter (inoculation rate of 4%). The experiment was conducted at 30°C (EPS optimized conditions by FAN, 2002). The air flow rate used was 1 vvm (air volum/broth volum/min) and an agitation of 100 rpm. This study was done in a 2 L bioreactor (INCELTECH) with 1.5 L of useful volum.

To control pH during the fermentation period, it was used H_3PO_4 2N and NaOH 2N. To avoid the excessive oscilation of the peristaltic pumps a dead band of 0.05 was used.

The pH was controlled at 5.0, 6.0 and 7.0. The samples were taken each 12 hours during 5 days.

Figure 2. Stirred-tank fermentor, INCELTECH, 2L, used for the pH control studies.

3.9. Study Aeration Rate Effect on Biomass and Exopolysaccharide Production in Stirred-tank Fermentor Scale

In order to investigate the most favourable aeration rate on mycelial growth and polysaccharide production, *A. brasiliensis* was cultivated with three levels of air flow, 0.5 vvm , 1.0 vvm and 2.0 vvm. This set of experiments were done in a 8L bioreactor (MARUBISHI, CO) with 4 L of useful volum. It was utilized the basal medium with no control of pH. The experiments were carried out at 30°C, 100 rpm. The samples were collected each 12 hours until 120 hours.

Figure 3. Stirred-tank fermentor, MARUBISHI, 8L, used for study of different aeration rates.

3.10. EPS Composition Study

The EPS analyzed it was produced in basal media. It was done the monosaccharide composition study.

3.11. Acid Hydrolise

Twenty milligrams of EPS was solubilized in water, hydrolysed with 1M trifluoroacetic acid (TFA) for 5 h at 100 °C. After the hydrolise, the TFA was completely evaporated at room temperature (ADAMS, 1969).

3.12. Reduction

The resulting monosaccharides were reduced with NaBH$_4$ (WOLFROM and THOMPSON, 1963) at room temperature for 3 hours.

The hydretos ions (H$^-$) supplied by reducing agent (NaBH$_4$) reduce the carbonila groupments of monosaccharides extremity redutor, originating alditols. The excess of the reductor agent is decomposed and Na$^+$ are removed by cationic resin in the acid form (Lewatit S-100). The solution was filtered and evaporated until the complete dry. The boric acid was removed by methanol addition to the system, promoting the tetramethil boretus formation, volatile compound eliminated in a rotary evaporator at 40°C.

3.13. Acetilation

The resultants alditols were acetilated (WOLFROM and THOMPSON, 1963b) by the addition of 0.5 mL of piridin and 0.5 mL of acetic anidrid, in a closed hydrolise tube for 24 hours at room temperature. Ice was added to stop the reaction by degradation of the excess of acetic anidride. The alditols acetate were extracted with chloroform (about 3 mL) and the piridin excess was complexed with CuSO$_4$ 5% in water, resulting in piridin sulphate, soluble in water, being separated of chloroform phase and eliminated by sucessives washing with destilated water. The chloroform phase with the alditol acetates was collected and, after the chloroform evaporation, the sample was analysed by GC-MS.

3.14. Gas Chromatography – Mass Spectrometry (GC-MS)

It was used a liquid-gas chomatograph VARIAN, model 3300, acopled to a mass spectrometer FINNINGAM TRAP, model 410, using a capilar column OV-225. Helium was utilized as array gas with a flux of 1 mL/min with initial temperature of 50°C and followed by a gradient of 1°C/min until 230°C (HARRIS, 1994).

4. Results and Discussion

4.1. Oils Effect

Growth and EPS production by *Agaricus brasiliensis* was significantly enhanced (around 1.5-fold for biomass and around 5-fold for EPS) in the media that contained rice oil at 2% (w/v) with 8.56 g/L of mycelial yield and 275.01 mg/L of EPS, while the control achieved a maximum biomass and EPS production of 4.68 g/L and 61.85 mg/L, respectively. The maximum EPS production, 281 mg/L, was observed with olive oil 2%. This was closely followed by olive oil at 1%, 268.32 mg/L and cotton oil at 2%, 217.61 mg/L. The results with plant oils are represented in the figures 4 and 5.
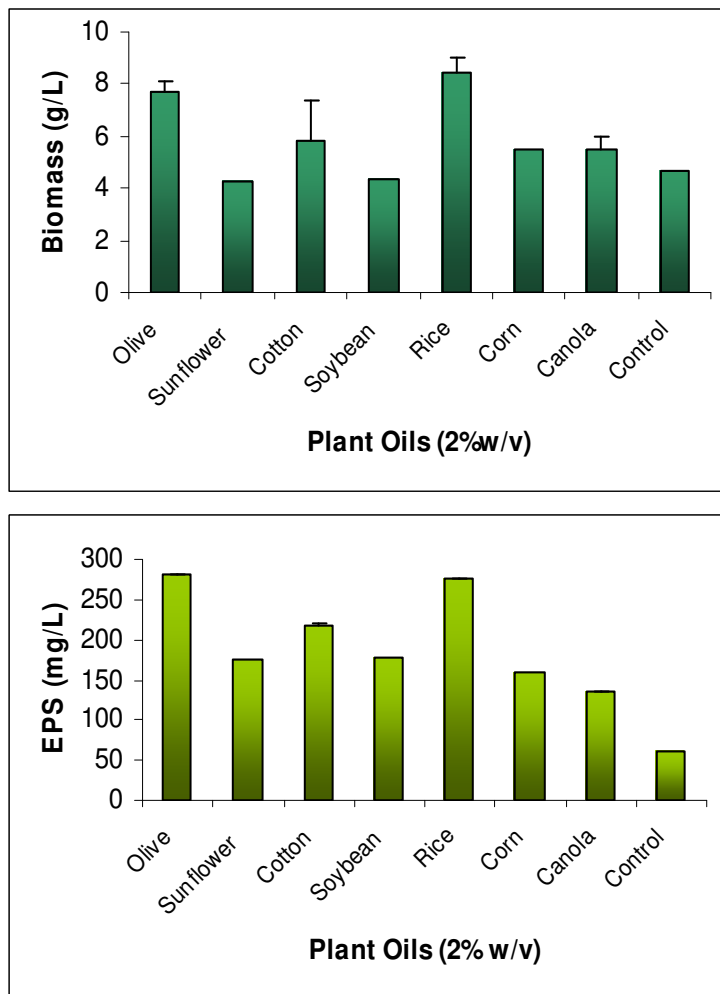
Figure 4. Biomass and EPS production by *A. brasiliensis* LPB 03 with 1% (w/v) of different plant oils.
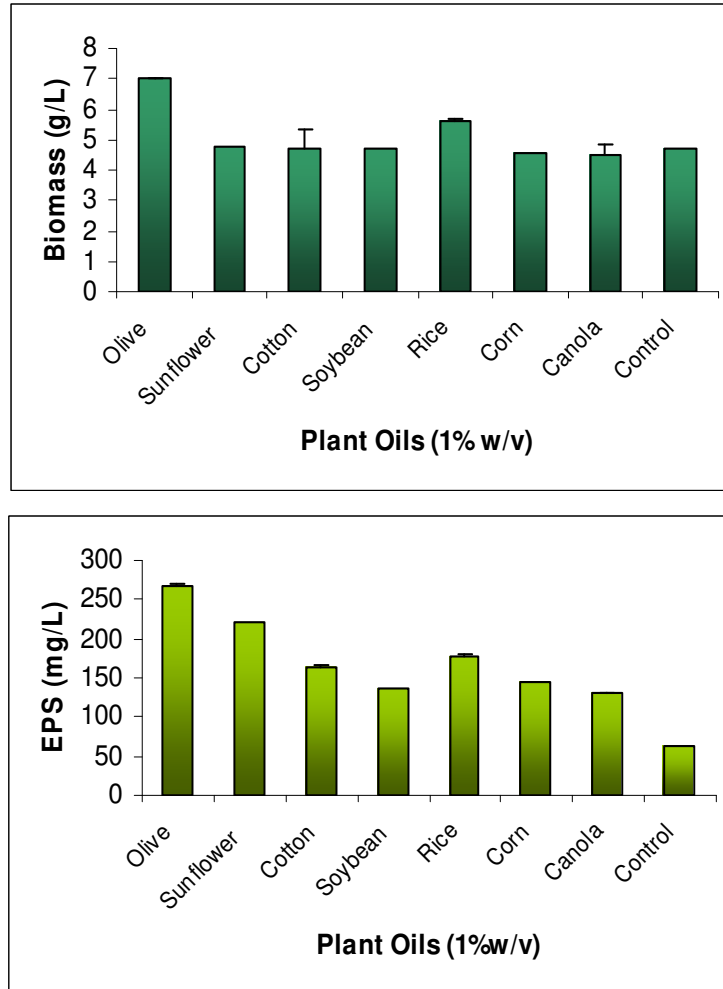
Figure 5. Biomass and EPS production in shake flasks by *A. brasiliensis* LPB 03 with 2% (w/v) of different plant oils.

Rice and olive oils contain the highest levels of palmitic acid (saturated acid) 19.53% and 12.99 %, respectively and their stimulatory effect might be attributed to this fact. Olive oil has oleic acid as the main component (70%) and the rice oil also has a high oleic oil concentration, so this oil may also contribute the stimulation.

Although all oils were beneficial to cell growth and led to the increase of EPS production, soybean oil at the two different concentrations gave rise to relatively lower mycelial growth and exopolysaccharide production. This oil is the richest in linoleic acid that might not has a very good effect. The oleic acid stimulatory effect and linoleic acid inhibition effect were observed by YANG *et al*. (2000), in *Ganoderma lucidum* submerged cultivation.

The mechanism of stimulation has been suggested as the lipids may be partially incorporated in the cell membrane thereby facilitating immediate uptake of nutrients from the culture medium or the lipids affecting, directly the level of synthesis of the enzymes involved in polysaccharide production (YANG et al., 2000).

4.2. Mineral Sources Effetcs on Biomass and EPS production

Among the mineral sources, the highest EPS yield (264.22 mg/L) was obtained on media containing Fe^{3+} at 50 mg/L, an increase of around 15% when compared with the control with 225.27 mg/L of polysaccharide. Mn^{2+} at 300 mg/L and Ca^{2+} at 50 mg/L led to a high EPS production with 253,88 mg/L and 248.94mg/L, respectively.
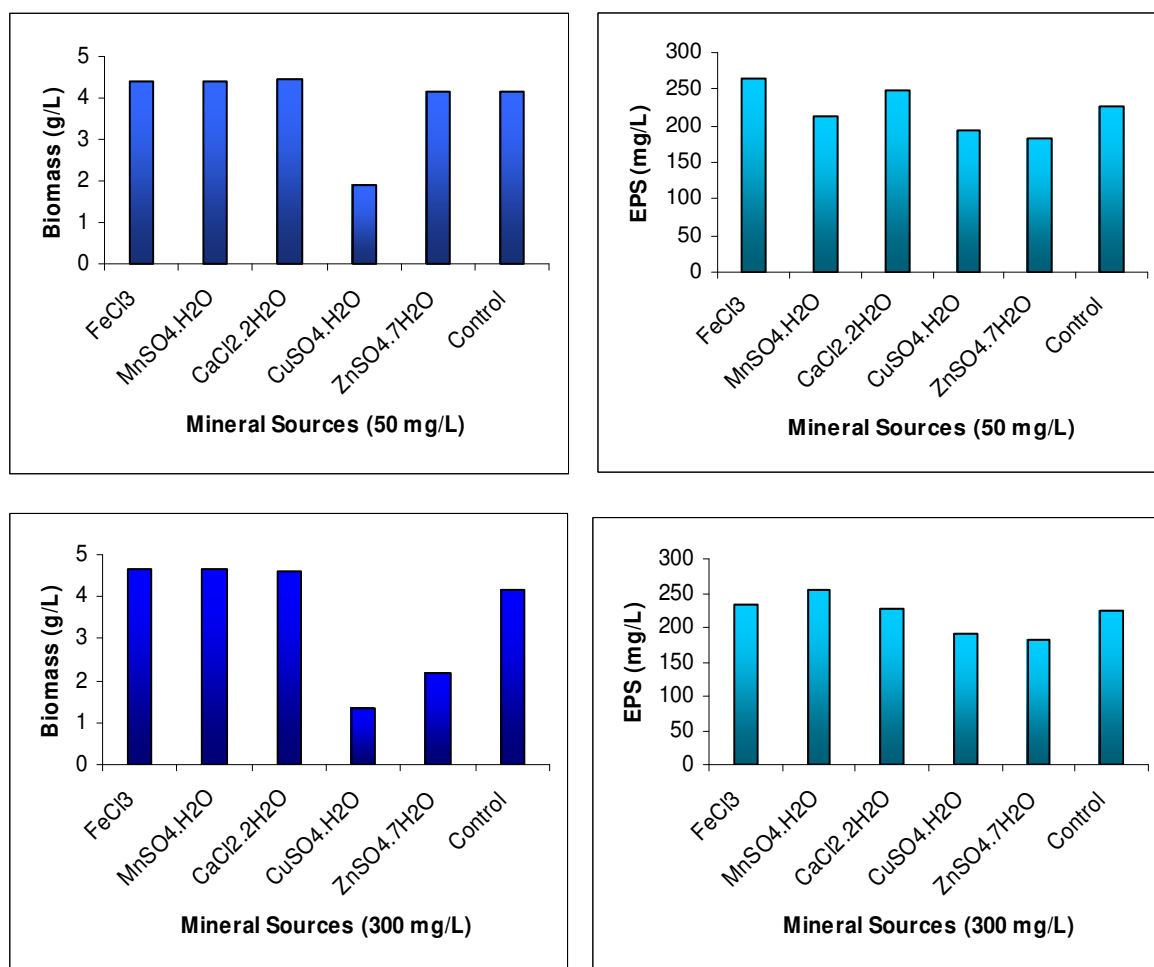
Figure 6. Effect of different mineral sources in flasks at two level (50 mg/L and 300 mg/L) on mycelial growth and EPS production by *A. brasiliensis*.

In the media containing Cu^{2+} and $Zn^{2+,}$ it was noted that the cells formed higher pellets. It was known that the morphological form of the culture has influence in metabolites production by fungi. It is possible that the pellet form promoted by these minerals can influence in EPS production, however, a depression on biomass production was observed. This depression might imply a toxic effect of these minerals in these concentrations.

4.3. Kinectics of production of biomass and exopolysaccharide in bioreactor

This kinectics aimed to know the maximum EPS production time in bioreactor. Figure 7 and 8 shows the typical profiles for substrate consumption, mycelial growth and polysaccharide production.
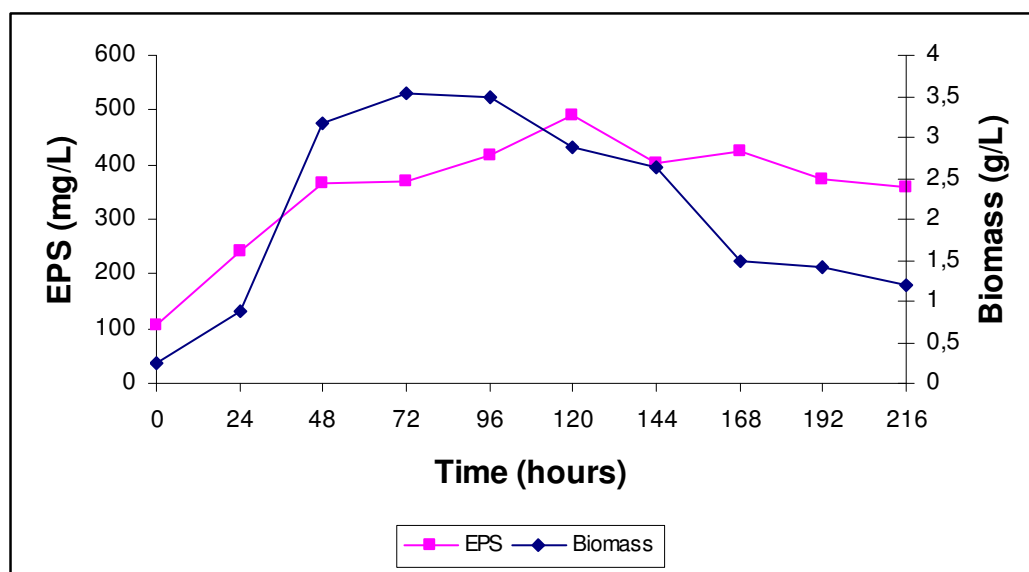

Figure 7. Kinectics of biomass and EPS production in basal media, in 2L bioreactor (INCELTECH) by *A. brasiliensis* LPB 03.
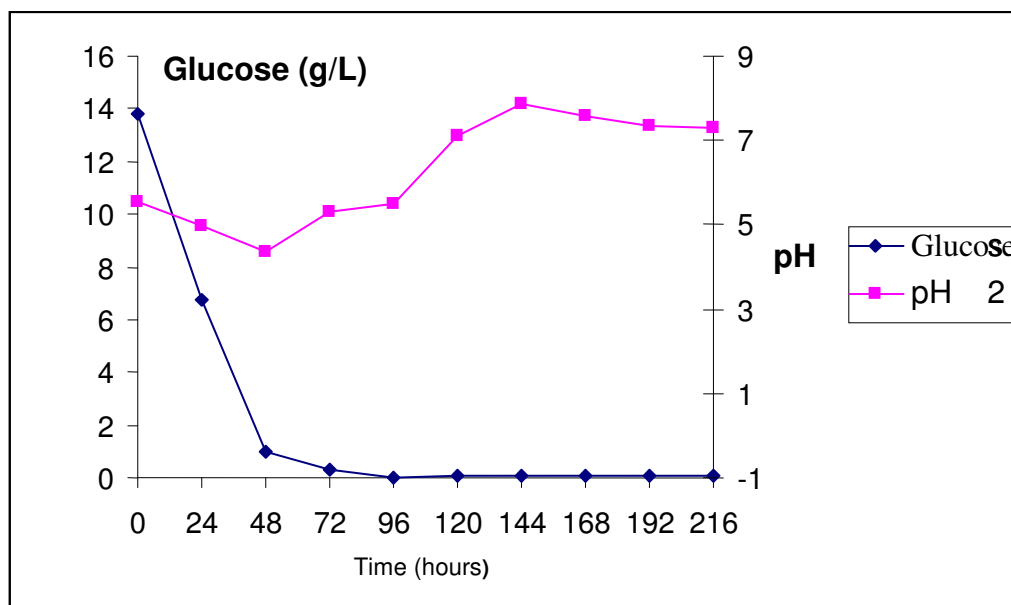
Figure 8. Broth media pH and glucose consumptiom with basal media, in 2L bioreactor (INCELTECH), by *A. brasiliensis* LPB 03.

Complete sugar consumption was observed at 96 hours. The highest biomass concentration (3.55 g/L) was observed at 96 hours, then it decreased, probably because of cells autolysis. EPS started to be produced around 12 hours of cultivation and accumulated. The maximum EPS concentration was observed at 120 hours with 409.12 mg/L, 78% higher than the concentration obtained in shake flasks, and remained almost constant until the 8th day of cultivation, then it decreased. Because this, the optimization fermentations in bioreactor were suspended on the 5th day.

The pH of the broth, firstly adjusted to 5.62, decreased until 4.37 on 2nd day associated with the glucose consumption and start to increase with the biomass and EPS production, achieved 7.84 on 6th day. The initial pH decrease is, possible, due to the acid organic production with glucose consumption.

4.4. Aeration rate effect in bioreactor

The maximum mycelial yield (5.35 g/L) and EPS production (1235.96 mg/L) were obtained with 2.0 vvm. However, no obvious difference was noted between 0.5 vvm with 305.36 mg/L of EPS and 3.05 g/L biomass yield and 1.0 vvm with 355.75 mg/L of EPS

yield and 3.51 g/L of mycelial yield, respectively. The EPS productivity with 2.0 vvm increased about 6-fold when compared with other conditions. The growth of *A. brasiliensis* at different aeration rates is illustrated in Fig 9.
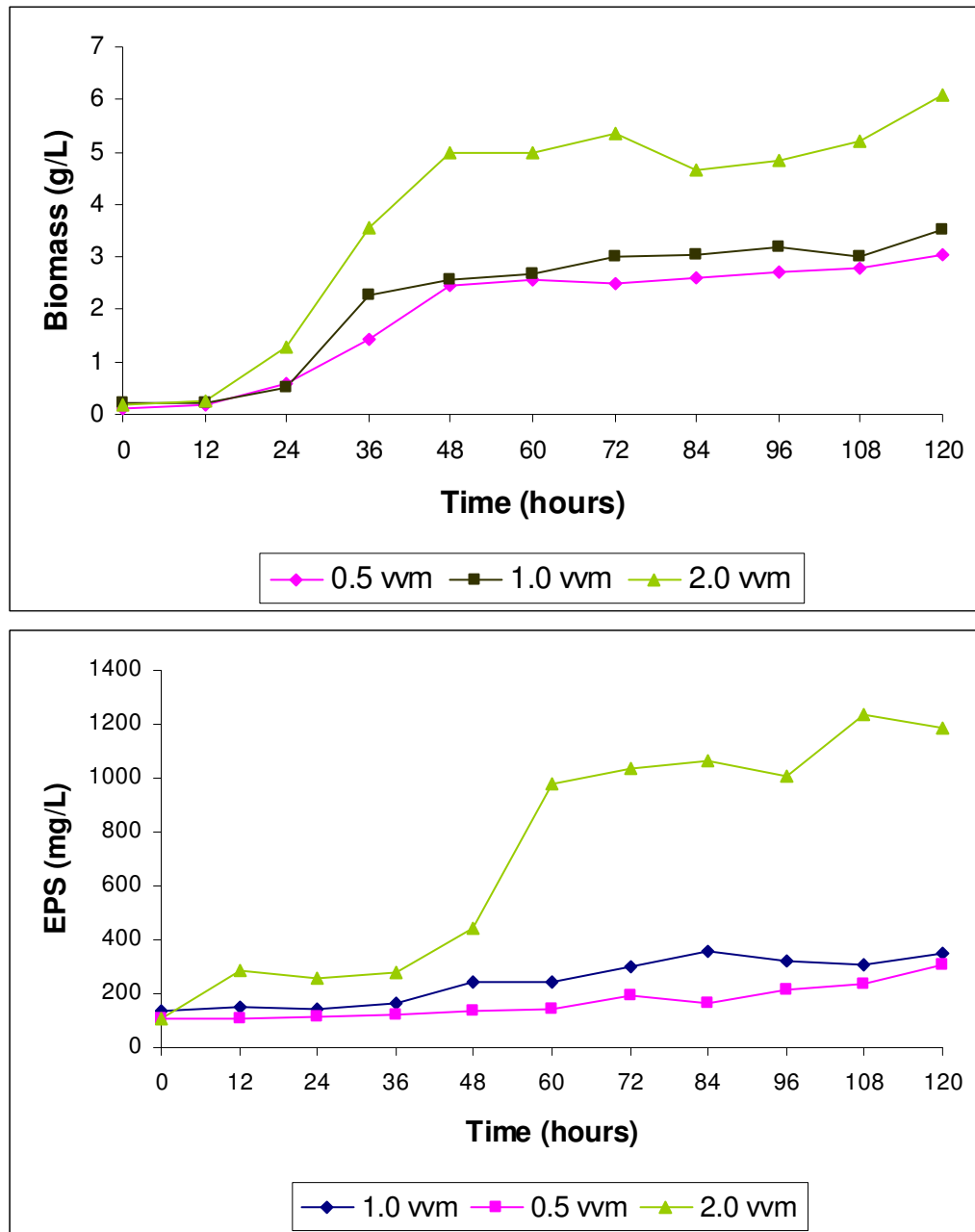

Figure 9. Different aeration rates on EPS and biomass production by *A. brasiliensis* LPB 03.

This result is similar to that showed by XU and YUN (2004) that showed that higher aeration (3.5 vvm) resulted in a higher EPS productivity by *Paecilomyces tenuipes.* KIM *et al.* (2004), also reported that an intensive aeration rate and agitation (20% controlled DOC) is important to EPS production in *A. blazei*.

The results suggest that oxygen availability is desirable to *A. brasiliensis* in their submerged culture for EPS production. Aeration also results in better homogeneity of the broth media. This condition helps to maintain constant the nutrients concentration in all points of the bioreactor and can contribute with the EPS production.

A better mixing caused by the fluid agitation could be favourable to EPS removal of fungi wall, enhancing its secretion (KIM *et al.*, 2005).

4.5. Controlled pH effect on biomass and EPS production in bioreactor

The maximum mycelial biomass was highest (5.40 g/L) at 96 hours of fermentation when the pH was regulated at 6.00. However, the optimum pH value control for bioreactor culture of *A. brasiliensis* LPB 03 was 7.00 with 901.31 mg/L of EPS yield at 84 hours of fermentation.
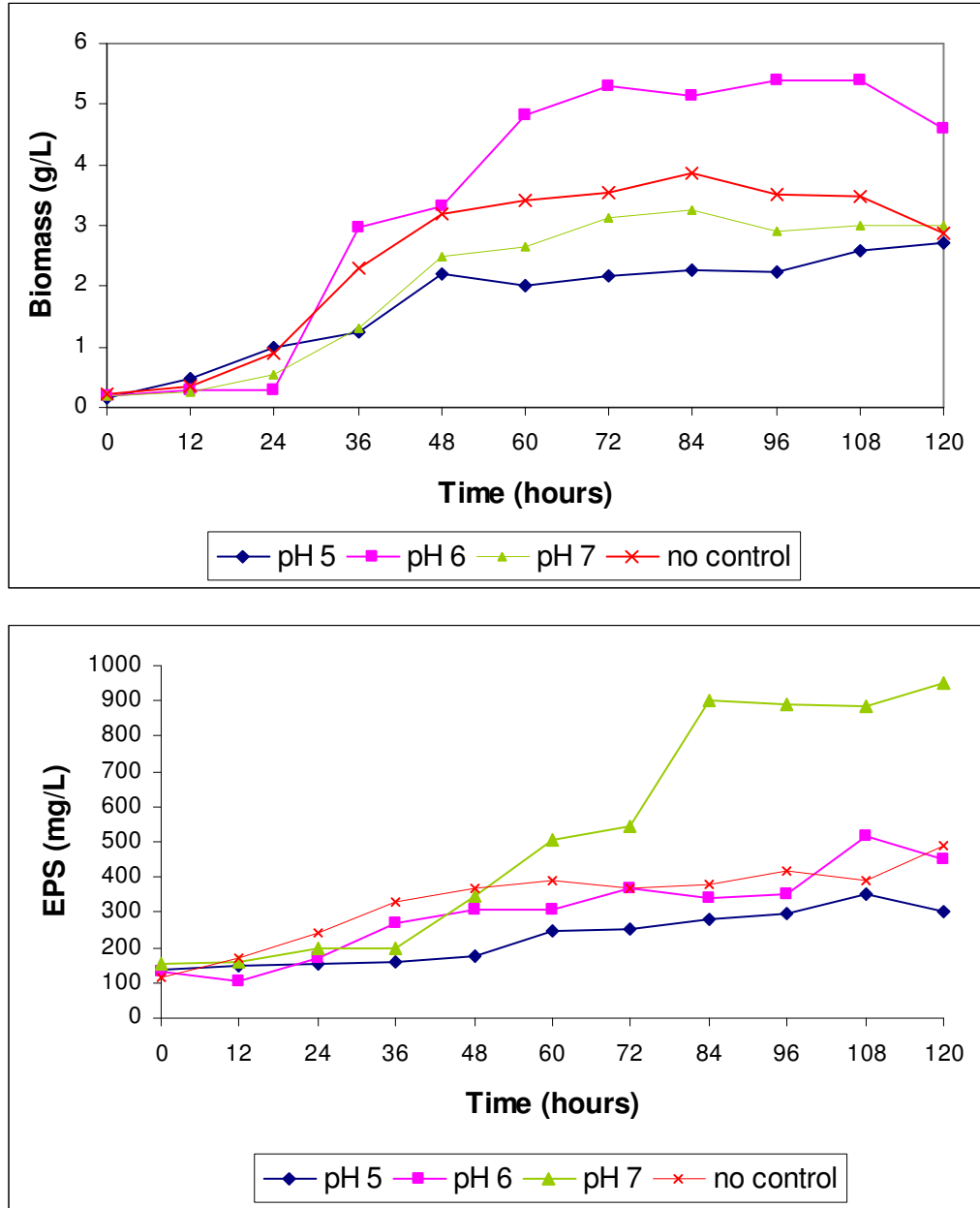
Figure 10. Controlled effect of pH on biomass and EPS production by *A brasiliensis* LPB 03.

However, when the fermentation was carried out with pH control (5.0), both mycelial growth and EPS production indicated lower values than those in uncontrolled pH.

As previously described, the pH of the culture media with no control declined from 5.62 to 4.37 during exponential growth phase, and then returned to around 7.00 (best pH verified in this work) at 120 hours of the culture. This observation is basically in accordance

with the data described by KIM *et al.*, (2004), however, the end fermentation pH with no control and the best control value of pH in his work was 5.0 both for biomass and EPS production.

Many investigators claimed that the pH is a critical factor in biomass accumulation and metabolite formation. In general, cells can only grow within a certain pH range, and metabolite formation is also often affected by pH. FANG and ZHONG, (2002) reported that lowering the initial pH from 6.5 to 3.5 gradually led to a higher production of EPS and higher specific production of intracellular polysaccharide in *Ganoderma lucidum.*

4.6. EPS monosaccharide composition

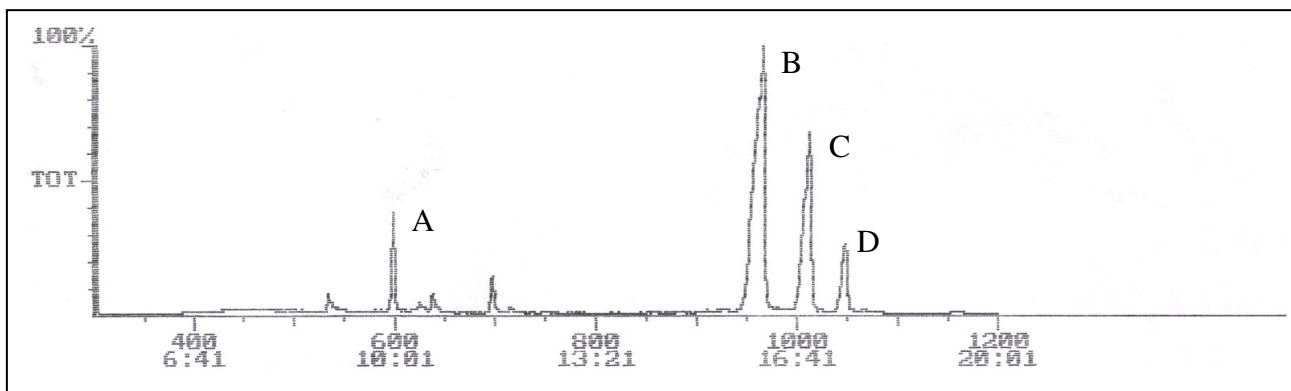The EPS was composed by 57,68% of manose, 28,17 % of galactose, 8,35% of glucose and 5,80% of rhamnose.

Figure 11. GC-Chromatrogram of EPS

GC-Chromatrogram and retention times. A - rhamnose, B – manose, C – galactose and D – glucose.

5. Conclusion

The goal of this work was enhancing the EPS and biomass production by *A. brasiliensis* LPB 03 in submerged fermentation in bioreactor.

The main results are summarized below:

- The best mineral source are Fe^{3+} at 50 mg/L, Mn^{2+} at 300 mg/L and Ca^{2+} at 50 mg/L with 264.22 mg/L, 253.88 mg/L and 248.84 mg/L of polysaccharide yield compared with the control with 225 mg/L ;

- Olive and rice oils at 2% were the plant oils most suitable for EPS production with 281 mg/L and 275 mg/L, respectively, followed by cotton oil at 2% with 217 mg/L;

- An aeration rate of 2.00 vvm and pH controlled at 7.00 were the best conditions to produce EPS in stirred-tank fermentor with 1235.96 mg/L and 901.31 mg/L, 7.72-fold and 5.63-fold, respectively, higher than the production obtained by FAN, 2002.

- The EPS was composed of by 57.68% of manose, 28.17 % of galactose, 8.35% of glucose and 5,80% of rhamnose.

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