ISSN 1330-9862
(FTB-1903)

original scientific paper

Medium Optimization for Producing Bioactive Exopolysaccharides by *Agaricus brasiliensis* S. Wasser *et al.* (=*A. blazei* Murrill ss. Heinem) in Submerged Culture

Chin-Hang Shu and Chun-Jun Xu*

Department of Chemical and Materials Engineering, National Central University, Jungli, Taoyuan, Taiwan

Received: August 16, 2006
Revised version: November 24, 2006
Accepted: December 9, 2006

Summary

The effects of media components, including carbon source, KH_2PO_4, and vitamin B_1, on the production and quality of *Agaricus brasiliensis* S. Wasser *et al.* (=*A. blazei* Murrill ss. Heinem) exopolysaccharides (ABEP) were studied in submerged culture. The quality of ABEP was characterized on the basis of their stimulation of the release of tumor necrosis factor-alpha (TNF-α) by macrophage cell line RAW 246.7 and their molecular mass distribution. Glucose was the best carbon source for the production of mycelial biomass and ABEP. The medium composition significantly affected both the relative content of β-(1-3)--glucan and the molecular mass (M_r) in the ABEP and, as a consequence, it also affected the biological activity of ABEP. A medium containing 3.0 g/L of KH_2PO_4 gave an ABEP of the highest biological activity (1440 pg of TNF-α/mL/5×10^4 cells), while a medium containing 10 mg/L of vitamin B_1 gave an ABEP with a biological activity of 1080 pg of TNF-α/mL/5×10^4 cells. In a bubble column bioreactor, an optimized medium gave a 1.35--fold increase in ABEP production with a 1.51-fold increase of its biological activity, when compared to the basic medium. This work demonstrates that the relative content of β-(1-3)--glucan in the ABEP is a useful reference indicator of biological activity.

Key words: *Agaricus brasiliensis*, *Agaricus blazei*, phosphate, exopolysaccharides, TNF-α, submerged culture

Introduction

Polysaccharides isolated from macrofungi, including edible and medicinal mushrooms, exhibit antitumor activity in animal models (*1*). Several commercial purified polysaccharides produced from macrofungi, such as schizophyllan, lentinan, and Krestin (polysaccharide Krestin, PSK), have passed through clinical trials in Japan and China. The antitumor activity of these polysaccharides arises from the fact that they stimulate T and B lymphocytes, monocytes and macrophages, causing these cells to secrete tumor necrosis factor-α (TNF-α) or interleukins in both cell culture and humans (*2*,*3*).

Such polysaccharides could potentially be extracted from the fruit bodies of macrofungi. However, despite of a long history of being a popular delicacy in many countries, cultivated mushrooms have been limited in modern biotechnological applications by the fact that they possess the ability to accumulate several toxic heavy metals, such as cadmium, lead, arsenic, copper, nickel, silver, chromium and mercury from contaminated soil (*4–6*). Thus, using a submerged culture to produce bioactive compounds of mushrooms in a well-controlled environment has become an attractive alternative.

The biological activities of polysaccharides depend on the chemical structure, the size of the polysaccharide

*Corresponding author; Phone: ++886 3 4227 151 ext. 34231; Fax: ++886 3 4263 749; E-mail: chinshu@ncu.edu.tw

backbone, the structure of the side chain groups and the degree of branching. The β-(1→3) backbone and the β-(1→6)-linked branches of polysaccharides are probably responsible for their antitumor activity (*2*,*7*,*8*). The biological properties of polysaccharides vary among microorganisms. For example, polysaccharides isolated from the fruiting body of *Agaricus brasiliensis* have stronger antitumor activity against Sarcoma 180 in mice than those from *Ganoderma lucidum*, *Lentinula edodes*, and *Coriolus versicolor* (*9*). Recently, polysaccharides isolated from mycelia of *A. brasiliensis* in submerged cultures have also shown antitumor activity (*10*).

The biological properties of polysaccharides that are produced in submerged culture depend strongly on the cultivation media and operating conditions (*11*). Unfortunately, the majority of studies of polysaccharide production by macrofungi in submerged culture are limited to characterizing how overall biomass and polysaccharide yields are affected by conventional operational parameters such as agitation rate (*12*,*13*), impeller designs (*14*), dissolved oxygen concentration (*15*), pH (*16*), and shear rates (*13*). Few workers have investigated the effect of such fermentation variables on the structure and biological activity of the polysaccharide produced.

Recently, the biological properties of polysaccharides have been correlated with their ability to stimulate release of tumor necrosis factor-alpha (TNF-α) by macrophage cell lines RAW 246.7 and J774 A.1 (*17*). Further, it seems that polysaccharides with high molecular mass have relatively high biological activities (*3*,*8*,*18*). These efforts have made it possible to monitor a submerged culture on the basis of the biological activity of ABEP by examining the molecular mass distribution of this polysaccharide and its stimulation of TNF-α release in macrophage cell line RAW 264.7 (*19*). We therefore have a useful system for evaluating the biological activity of polysaccharides produced by macrofungi.

In the present study, we have used these methods to evaluate the quality of the ABEP produced during submerged cultivation of *Agaricus brasiliensis*, with two main aims: firstly, to find a culture medium that gives a high productivity of high quality ABEP and, secondly, to correlate the quality of the ABEP produced, in terms of its biological activities, molecular mass distribution and β-glucan content, with the medium composition.

Materials and Methods

Microorganism

Agaricus brasiliensis ATCC 76739 was maintained on potato dextrose agar (PDA) slants. Slants were inoculated and incubated at 28 °C for 14 days and stored at 4 °C.

Culture conditions

Experiments were done using 250-mL flasks each containing 100 mL of medium, inoculated at 5 % (by volume), with the seed culture. The basic medium contained (in g per 1 L of distilled water): glucose 10, yeast extract 3, malt extract 3, polypeptone 5, $MgSO_4 \cdot 7H_2O$ 1. To study the effect of carbon source, glucose was substituted with sucrose, fructose, starch or lactose. The effect of the supplementation with KH_2PO_4 and vitamin B_1 were studied at six (0, 0.1, 0.5, 2, 3 and 5 g/L) and five (0, 10, 30, 50 and 100 mg/L) different concentrations, respectively.

The optimal medium for ABEP production suggested from these experiments contained (in g per 1 L of distilled water): glucose 10, yeast extract 3, malt extract 3, polypeptone 5, $MgSO_4 \cdot 7H_2O$ 1, KH_2PO_4 0.3, and 10 mg of vitamin B_1. Two media (basic and optimal medium) were evaluated in a 3-litre bubble column bioreactor (8 cm i.d. × 40 cm height) with a 2-litre working volume and operated at 28 °C, initial pH=5.2, and aeration at 0.15 vvm (*20*).

Analytical methods

Biomass concentration was determined by the dry mass method involving filtration of broth samples through preweighed filter discs (Whatman Ltd, Maidstone, UK). The filtrate was collected and stored at –20 °C for the measurement of residual glucose and exopolysaccharides. Residual glucose content was assayed by the dinitrosalicylic acid (DNS) method (*21*). The polysaccharide samples were pretreated by two filtration steps in series using an Amicon Ultra-15 centrifugal filter for 15 min at 6000 rpm, 20 °C and membrane filtration (relative molecular mass cutoff of 8 kDa). The polysaccharide concentration was determined by the phenol-sulfuric acid assay (*22*).

The molecular mass of polysaccharides was determined using a Shodex OHPak SB-804HQ gel permeation chromatography (GPC) system equipped with a GPC column and an RI detector (SFD, RI 2000). Polyethylene glycol (PEG) standards (Polymer Laboratories, Church Stretton, UK) with narrow polydispersity and with molecular mass ranging from 1.9 to 1260 kDa were used as the molecular mass standards. The relative amount of β-(1→3)-D-glucans in the polysaccharide were estimated by a fluorescence method (*23*).

Cytokine assays

The *in vitro* mouse macrophage RAW 264.7 cell line, purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan), where it is stored as BCRC 60001, was used in the biological activity assays. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM), which was supplemented with 10 % fetal bovine serum (FBS), 1 % (by volume) penicillin (100 U/cm^3), and 1 % (by volume) streptomycin (100 U/cm^3) (Gibco, BRL, Grand Island) in 24-well flat-bottomed plates (Sumitomo Baklite Co Ltd, Japan) until the cell density reached 5×10^4 cells per well in 1 cm^3 of culture medium. Distilled water was used as a control. Stimulation of the release of tumor necrosis factor-α (TNF-$\alpha_{polysaccharide}$-TNF-$\alpha_{control}$) was measured using ELISA kits according to the manufacturer's instructions as described elsewhere (*24*). Data are presented as a mean value of three independent experiments.

Results and Discussion

Effect of carbon source

Sucrose, lactose, glucose, starch, and fructose were tested to find a suitable carbon source for the growth and production of polysaccharides by *A. brasiliensis* ATCC 76739. Relatively high yields of biomass and polysaccharides were obtained with glucose and sucrose, while lactose supported the poorest growth (Table 1). The high specific polysaccharide yield obtained with glucose ($Y_{p/x}$= =0.48 g/g) was consistent with the yields obtained during the submerged cultivation of other macrofungi (*25–27*).

Table 1. Biomass, polysaccharide content and specific polysaccharide yield ($Y_{p/x}$) obtained in batch cultures of *Agaricus brasiliensis* supplemented with 1 % (by mass per volume) of different carbon sources in shaker flasks for 24 days

γ(carbon source) 10 g/L	γ(biomass) g/L	γ(polysaccharides) g/L	$Y_{p/x}$ g/g
Glucose	4.91±0.13	2.36±0.08	0.48
Fructose	4.17±0.21	1.98±0.11	0.47
Sucrose	4.97±0.18	2.24±0.06	0.45
Lactose	3.48±0.23	1.44±0.07	0.41
Starch	4.38±0.09	1.65±0.13	0.37

Fig. 1a shows the biological activity of the polysaccharides recovered from these cultivations in terms of their ability to stimulate the release of TNF-α by macrophages. Note that starch and α-cellulose did not stimulate TNF-α release by macrophages (<60 pg/mL/5×10^4 cells), but the exopolysaccharides of *A. brasiliensis* (ABEP) produced on the various carbon sources all stimulated high TNF-α release (≥800 pg/mL/5×10^4 cells). The ABEP produced with starch as the carbon source stimulated a relatively high TNF-α release of 1190 pg/mL/5×10^4 cells.

Since the literature claims that the β-(1-3)-linkage in polysaccharides is essential for antitumor activity and immunomodulation (*28*), the relative content of β-(1-3)--glucan in the polysaccharides was determined (Fig. 1a). The highest β-(1-3)-glucan content of the ABEP was 18 %, obtained with starch as the carbon source, and the lowest content was 7 %, obtained with lactose. The biological activity of polysaccharides showed a high correlation (R^2=0.93) with its β-(1-3)-glucan content. This observation was consistent with that of a previous study (*24*).

In order to elucidate the effects of carbon sources on the molecular mass distribution of polysaccharides, various ABEP preparations were characterized by gel permeation chromatography, giving the molecular mass distribution and the mass-average molecular mass (M_r) (Fig. 1b). The molecular mass within the ABEP samples is classified into three fractions: low molecular mass (LMM; 100–500 kDa), medium molecular mass (MMM; 500–1000 kDa), and high molecular mass (HMM; >1000 kDa).

The M_r of ABEP produced with various carbon sources ranged from 650 to 730 kDa. HMM was the major fraction of ABEP from the cultures that had glucose, sucrose, or fructose as the carbon source. On the other hand, HMM was the minor fraction of ABEP from the culture containing lactose, leading to a low M_r (650 kDa).

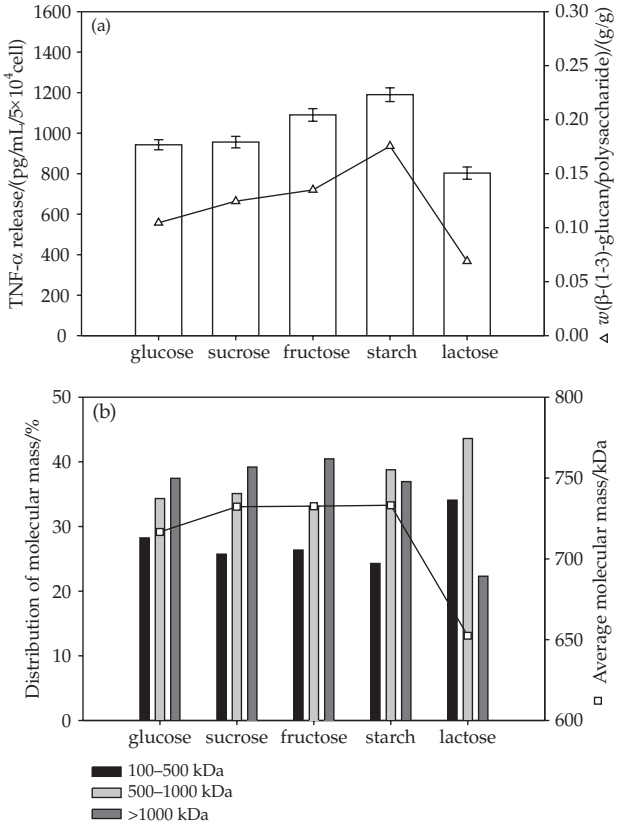

Fig. 1. (a) Biological activity (as TNF-α release capability) of exopolysaccharides from *Agaricus brasiliensis* grown on different carbon sources in shaker flasks for 24 days, and its correlations with the relative amount of β-(1-3)-glucan and (b) their molecular mass distribution and average molecular mass

Although low average molecular mass of the ABEP produced from lactose had a low biological activity in this study, there was a low correlation (R^2=0.70) between the biological activity of ABEP and its average molecular mass. Differences in biological activities of ABEP might depend on the degree of branching, molecular conformation, and the amount of β-glucan, besides the molecular mass (*19,29,30*).

Effect of phosphate supplementation

Both growth and polysaccharide production were affected when submerged cultures of *A. brasiliensis* were supplemented with KH_2PO_4 (Table 2). The highest biomass concentration after 24 days of culture, 5.41 g/L, was obtained with an initial KH_2PO_4 concentration of 2.0 g/L. This biomass concentration was about 10 % higher than that obtained in the culture without the added KH_2PO_4. In the case of ABEP, the highest concentration after 24 days of culture, 3.02 g/L, was obtained with an initial KH_2PO_4 concentration of 3.0 g/L. This ABEP concentration was over 20 % higher than that obtained in the culture without the added KH_2PO_4. Previous studies had reported that supplementation with inorganic phosphate enhanced biomass and exopolysaccharide yields in submerged culture of other macrofungi (*31,32*); however, lit-

Table 2. Biomass, polysaccharide content and specific polysaccharide yield ($Y_{p/x}$) obtained in batch cultures of *Agaricus brasiliensis* supplemented with six different concentrations of KH_2PO_4 in shaker flasks for 24 days

$\gamma(KH_2PO_4)$ g/L	γ(biomass) g/L	γ(polysaccharides) g/L	$Y_{p/x}$ g/g
0	4.91±0.13	2.36±0.08	0.48
0.1	5.03±0.11	2.48±0.05	0.49
0.5	5.10±0.15	2.54±0.06	0.50
2.0	5.41±0.14	2.86±0.07	0.53
3.0	5.35±0.15	3.02±0.09	0.56
5.0	4.83±0.11	2.39±0.10	0.49

tle attention has been paid to the influence of this supplementation on the biological activity.

Fig. 2a shows the biological activity, in terms of the stimulation of TNF-α release by macrophages, of the ABEP samples produced with different initial KH_2PO_4 concentrations. The highest biological activity (1440 pg of TNF-α/mL/5×10^4 cells) was obtained with the ABEP produced in the culture supplemented with 3.0 g/L of KH_2PO_4.

The content of β-(1-3)-glucan in the ABEP obtained from different KH_2PO_4 levels was also closely correlated with its biological activity (R^2=0.96) (Fig. 2a). On the other hand, there was little correlation (R^2=0.42) between the biological activity of ABEP and its M_r in the ABEP samples obtained in the KH_2PO_4 supplementation experiment (Fig. 2b).

Effect of vitamin B_1 supplementation

Although previous reports have shown that vitamins affect the growth of macrofungi and metabolite production (*33*,*34*), to our knowledge, this is the first study of the influence of a vitamin on the mycelial growth of *A. brasiliensis* and its exopolysaccharide production in submerged culture. When batch cultures of *A. brasiliensis* were supplemented with vitamin B_1, the highest biomass concentration at 24 days (5.17 g/L) was obtained with 10 mg/L of vitamin B_1, while the highest ABEP concentration (2.70 g/L), which corresponded to a specific product yield ($Y_{p/x}$) of 0.54 g/g, was obtained with 30 mg/L of vitamin B_1 (Table 3). The effects of B_1 supplementation were relatively weak, probably due to the fact that the medium used in this study contained complex nutrient sources (yeast extract, malt extract and polypeptone). On the other hand, the ABEP preparations obtained from these cultures did have significantly different biological activities. Again, the biological activity was highly correlated with the β-(1-3)-glucan content (R^2=0.95) (Fig. 3a). The average molecular mass of the ABEP also varied significantly with the level of vitamin B_1 supplementation (Fig. 3b). The M_r of ABEP showed a good correlation with its biological activity (R^2=0.85). In this experiment, the ABEP with the highest biological activity (1080 pg of TNF-α/mL/5×10^4 cells) and high M_r (743 kDa) was obtained with vitamin B_1 supplementation of 10 mg/L. For vitamin B_1 supplementation levels above 10 mg/L, both the M_r and the biological activity of ABEP decreased. As a result, the ABEP with the lowest biological activity (800 pg of TNF-α/mL/5×10^4 cells) and the lowest M_r (650 kDa) was obtained with 100 mg/L of vitamin B_1.

Exopolysaccharide production in a bubble column bioreactor

An optimal medium, supplemented with appropriate concentrations of vitamin B_1 and KH_2PO_4, as suggested from the results of the flask culture, was developed. The basic medium (*i.e.* without supplementations of vitamin B_1 and KH_2PO_4) was used as the control. Fig.

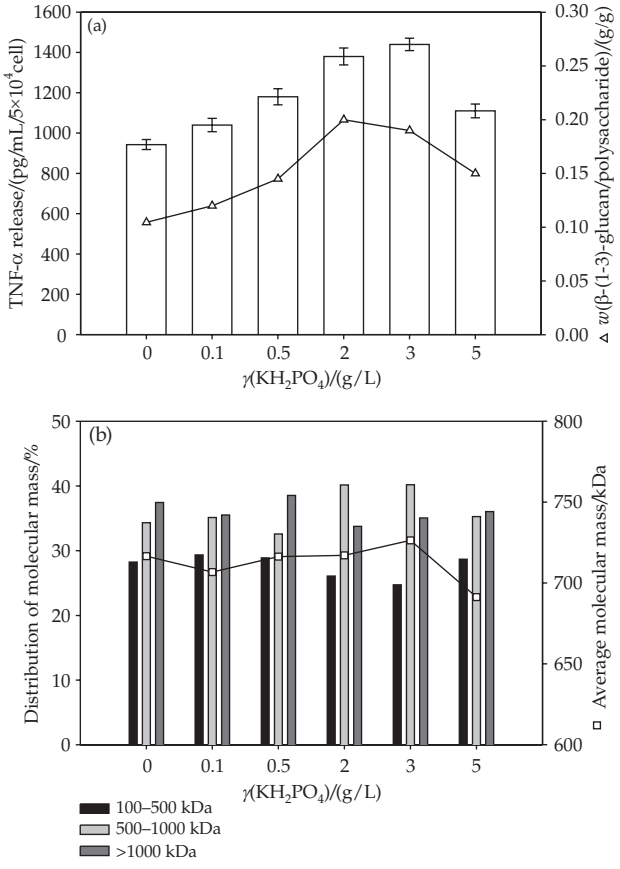

Fig. 2. (a) Biological activity (as TNF-α release capability) of exopolysaccharides from *Agaricus brasiliensis* grown on different concentrations of KH_2PO_4 in shaker flasks for 24 days, and its correlation with the relative amount of β-(1-3)-glucan and (b) their molecular mass distribution and average molecular mass

Table 3. Biomass, polysaccharide content and specific polysaccharide yield ($Y_{p/x}$) obtained in batch cultures of *Agaricus brasiliensis* supplemented with five different concentrations of vitamin B_1 in shaker flasks for 24 days

γ(vitamin B_1) mg/L	γ(biomass) g/L	γ(polysaccharides) g/L	$Y_{p/x}$ g/g
0	4.91±0.13	2.36±0.08	0.48
10	5.17±0.13	2.63±0.06	0.51
30	5.02±0.09	2.70±0.07	0.54
50	4.93±0.15	2.51±0.09	0.51
100	4.65±0.11	2.23±0.04	0.48

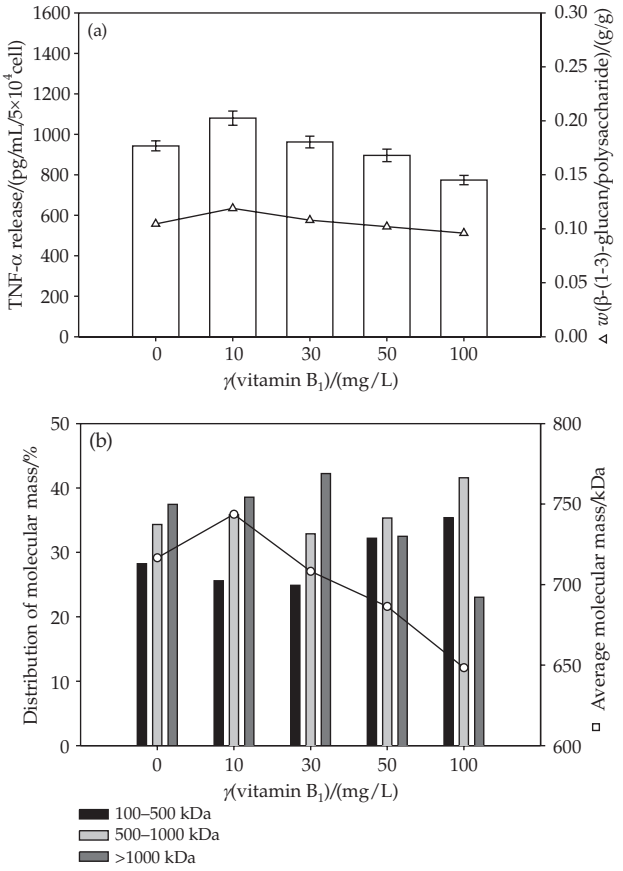

Fig. 3. (a) Biological activity (as TNF-α release capability) of exopolysaccharides from *Agaricus brasiliensis* grown on different concentration of vitamin B_1 in shaker flasks for 24 days, and its correlations with the relative amount of β-(1-3)-glucan and (b) their molecular mass distribution and average molecular mass

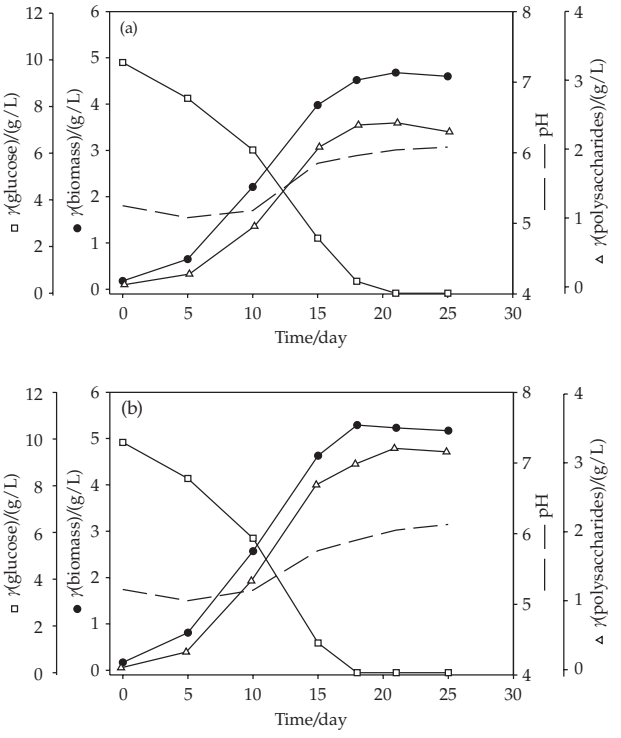

Fig. 4. Time course data of batch culture of *Agaricus brasiliensis* using (a) basic medium and (b) optimal medium in a 3-litre bubble column bioreactor

4 shows the results of fermentations carried out with these two media in batch cultures in a bubble column bioreactor. The maximum concentrations of biomass and polysaccharides in the optimal medium were 5.29 g/L after 18 days and 3.21 g/L after 21 days, respectively. The improvements in the maximum concentrations of biomass and polysaccharides resulting from the medium optimization were 13 and 35 %, respectively.

Fig. 5a shows the biological activities, on the basis of the stimulation of TNF-α release by macrophages, of the ABEP preparations produced from these two cultures. The ABEP produced with the optimal medium, in comparison with that produced with the basic medium, increased the TNF-α release by macrophages by 51 % (from 1036 to 1560 pg of TNF-α/mL/5×10^4 cells), and also increased the relative content of β-(1-3)-glucan from 12 to 21 %. In addition, the M_r of ABEP increased from 764 to 885 kDa as a result of higher fractions of MMM and HMM in ABEP from the optimal medium (Fig. 5b).

Conclusions

Supplementation of the growth medium for submerged culture of *A. brasiliensis* with different carbon sources, KH_2PO_4 or vitamin B_1 affects growth and the

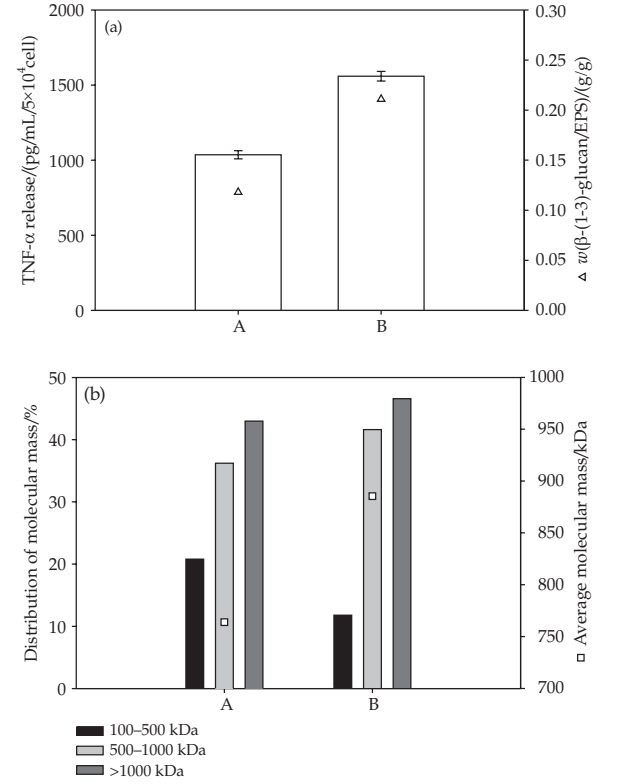

Fig. 5. (a) Biological activity (as TNF-α release capability) of exopolysaccharides from batch cultures of *Agaricus brasiliensis* grown on basic medium (A) and optimal medium (B) in a 3--litre bubble column bioreactor and its correlation with the relative amount of β-(1-3)-glucan and (b) their molecular mass distribution and average molecular mass

production of exopolysaccharides (ABEP). The biological activity and the molecular mass distribution of the ABEP produced are also affected. The relative content of β-(1-3)-glucan in ABEP showed strong correlations with its biological activity in studies of the effect of carbon source, KH_2PO_4 concentration and vitamin B_1 concentration. The average molecular mass of the ABEP also showed good correlations with its biological activity in the studies of the effect of carbon source and vitamin B_1 concentration, but the correlation was not good in the study of the effect of KH_2PO_4 concentration. We propose that the relative content of β-(1-3)-glucan in the ABEP can be used as an indicator of its biological activity. Medium optimization gave a 1.35-fold increase in ABEP production with a 1.51-fold increase in its biological activity in fermentations carried out in a bubble column bioreactor. These results provide valuable information for the production of bioactive polysaccharides of *A. brasiliensis* in submerged culture.

Acknowledgement

The authors would like to thank the Council of Agriculture of the Republic of China, Taiwan, for financially supporting this research under Contract No. COA 92 AG-3.1.3-FD-Z3.

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