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ORIGINAL ARTICLE

Study on the effects of different culture conditions on the morphology of *Agaricus blazei* and the relationship between morphology and biomass or EPS production

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Abstract *Agaricus blazei* Murill has recently been shown to have strong immunomodulatory and anti-cancer properties. Two factors contribute to the development of particular morphological forms, and meanwhile fungal morphology directly or indirectly influences the productivity of fungal fermentations. Therefore, the present study was undertaken in order to optimize submerged culture of *A. blazei* for the highest amount of biomass and EPS production, and to evaluate the relationship between culture conditions, morphology and productivity of this mushroom in submerged culture. Different carbon and nitrogen sources at different pH were applied in submerged cultured *A. blazei* Murill. Morphological parameters (circularity, compactness and mean diameter) were determined, and the relationships between pellet morphology, biomass and EPS productivity were evaluated, applying different statistical tests. According to our results, starch and yeast extract were the best carbon and nitrogen sources for both mycelia biomass and EPS production. Small feather-like fluffy and loosely packed pellets with a small compacted central core can be introduced as desirable morphological features of *A. blazei* producing the highest biomass and EPS in these media.

Keywords *Agaricus blazei* · Fungi · Optimization · Submerged culture

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Introduction

Fungi and mushrooms are fascinating natural sources for obtaining bioactive materials, especially immunomodulatory polysaccharides, that have been applied in some valuable medicinal and complementary preparations. The usual commercial method for production of mushrooms is cultivating the fruit body on solid state media such as woody materials, straw and different composts. Production of mushrooms in submerged cultures has recently become attractive because of the potential for higher mycelial production in a compact space and in a shorter time with fewer chances for contamination. Moreover, by optimization of nutritional requirements, pH, and other physicochemical conditions, the desired production of exopolysaccharides and other pharmaceutical compounds can be obtained (Kim et al. 2002).

Some studies have reported that mushrooms are able to grow in a variety of morphological forms in submerged culture, and that different morphological properties of fungi and mushrooms may be affected by changing culture conditions like pH, temperature, culture constituents, shear stress, inoculums volume, etc. For fungi and mushrooms, these morphological forms can vary from dispersed mycelial filaments to dense pellets which are interwoven mycelial masses (Kossen 2000). On the other hand, there are some reports showing that different morphological forms produce different amounts of secondary metabolites.

Although adequate characterization of fungal morphology in the early stage of cultivation has been assumed to be a prerequisite for such studies, no simple relationship between morphology and productivity has been found (Grimm et al. 2005)

Agaricus blazei belongs to the basidiomycetes and is a native of Brazil. In traditional medicine, it has been used to help against a variety of diseases, including cancer, arteriosclerosis, diabetes and hepatitis (Grinde et al. 2006). Some recent reports have reported strong immunomodulating properties for *A. blazei* (Hetland et al. 2008). There are several reports on the anti-cancer properties, and on the anti-tumor (Ellertsen et al. 2006; Lee et al. 2003; Park et al. 2001; Takimoto et al. 2004) and anti-virus activities (Sorimachi et al. 2001; Grinde et al. 2006), of this mushroom. Two studies have shown that *A. blazei* extracts may also be used as an adjuvant to vaccines (Chen and Shao 2006; Jenneman et al. 1999). Although in recent years several studies have reported on the effects of different culture conditions on fungal morphology and metabolite production for some medicinal mushrooms (Cui et al. 1998; Park et al. 1999; Cho et al. 2002; Hwang et al. 2004), according to the best of our knowledge, there is no report on the effects of different culture conditions on the morphology of *A. blazei*, or on any relationship between morphology and biomass or EPS production.

In the present study, applying a one-factor-at a-time method, the effects of carbon and nitrogen sources, and initial pH on the morphology of *A. blazei* and the relationship between morphology and biomass and EPS production were investigated.

Materials and methods

Microorganism and media

A strain of *A. blazei* Murill (strain DPPH 131) was provided from the Medical Science Culture Collection, School of Pharmacy, Shaheed Beheshti University, Iran. The stock culture was maintained on malt extract agar slants and was sub-cultured every 2 months. The slants were incubated at 25°C for 7 days. The inoculum cultures were grown in 500-mL flasks containing 100 mL of basal medium containing glucose 10 g/L, yeast extract 3 g/L, malt extract 10 g/L, and mycological peptone 1 g/L. The flasks were kept at 25°C in a rotary shaker-incubator at 120 rpm for 7 days. All treatments were carried out in 500-mL flasks containing 100 mL of the medium at 25°C for 14 days after inoculating with 3% (v/v) of the inoculum cultures. All experiments were performed at least in triplicate.

Carbon sources

Six different carbon sources, i.e., galactose, lactose, starch, maltose, mannitol and glucose were tested. Fermentation medium contained 10 and 4 g/L of carbon source and yeast extract, respectively.

Nitrogen sources

Five different sources of complex and simple nitrogen were used, i.e., urea, ammonium chloride, yeast extract, phyton peptone and mycological peptone. The fermentation condition was as above but the nitrogen source was replaced at an equivalent concentration of experimental source. Thus, the concentrations were: urea 4%, ammonium chloride 4%, yeast extract 4%, mycological peptone 4%, and phyton peptone 4%. Glucose (10 g/L) was used as carbon source.

Initial pH

The effect of initial pH 4.0–8.0 was studied. Other culture conditions were the same as the above-mentioned basal medium.

Analytical methods

The fermentation broth was centrifuged at 10,000 *g* for 25 min and the resulting supernatant was filtered through a 0.45-μm membrane filter (Millipore) and mixed with four volumes of absolute ethanol, stirred vigorously and left overnight at 4°C. The EPS was precipitated by centrifugation at 10,000 *g* for 25 min and was lyophilized and measured gravimetrically. Mycelium dry weight was also measured after repeated washing of the mycelial pellet with distilled water and drying at 70°C overnight.

Characterization of the morphology

Samples were fixed with an equal volume of fixative (13 mL of 40% formaldehyde, 5 mL of glacial acetic acid and 200 mL of 50% ethanol). Aliquots (0.1 mL) of each fixed sample was transferred to a slide, air-dried, and then stained with lacto-phenol cotton blue (ingredients per liter: phenol 200 g, cotton blue 0.5 g , glycerol 400 mL, lactic acid 200 mL, deionized water 200 mL). Observation and characterization of pellets were carried out using a microscope (BH2; Olympus, Japan) equipped with camera. For each sample, the size of 25 pellets was characterized by measuring the area and perimeter of the pellet core and the maximum diameter of the pellet, using ocular micrometer. The morphology of the pellets was characterized by their mean diameter, circularity and compactness. Circularity or shape factor was estimated as the ratio of the Fieret's

minimum diameter to the Fieret's maximum diameter of the pellets (Park et al. 2002). The compactness was estimated as the ratio of the projected area of the hyphae in a clump to the projected convex area of that clump, the latter being the area after filling internal voids and concavities in the clump's external perimeter.

Statistical analysis of the data

Data from morphological characterization were analyzed by SPSS version 15 (SPSS, Chicago, IL, USA). The mean values of each shape parameter were compared by applying one-way ANOVA and post hoc LSD multiple comparison tests with a 0.05 significance level. The nonparametric Kruskal–Wallis *H* test and Mann–Whitney *U* test were applied to compare variable between groups and to determine whether the EPS and biomass production under different culture conditions were statistically different from each other, considering a p value of ≤ 0.05.

Results and discussion

Morphology

Although *A. blazei* is one of the most important medicinal and commercial mushrooms, there are few publications on the effect of different culture conditions on mycelial biomass and EPS production by it (Shu et al. 2004; Hamedi et al. 2007). According to best of our knowledge, however,

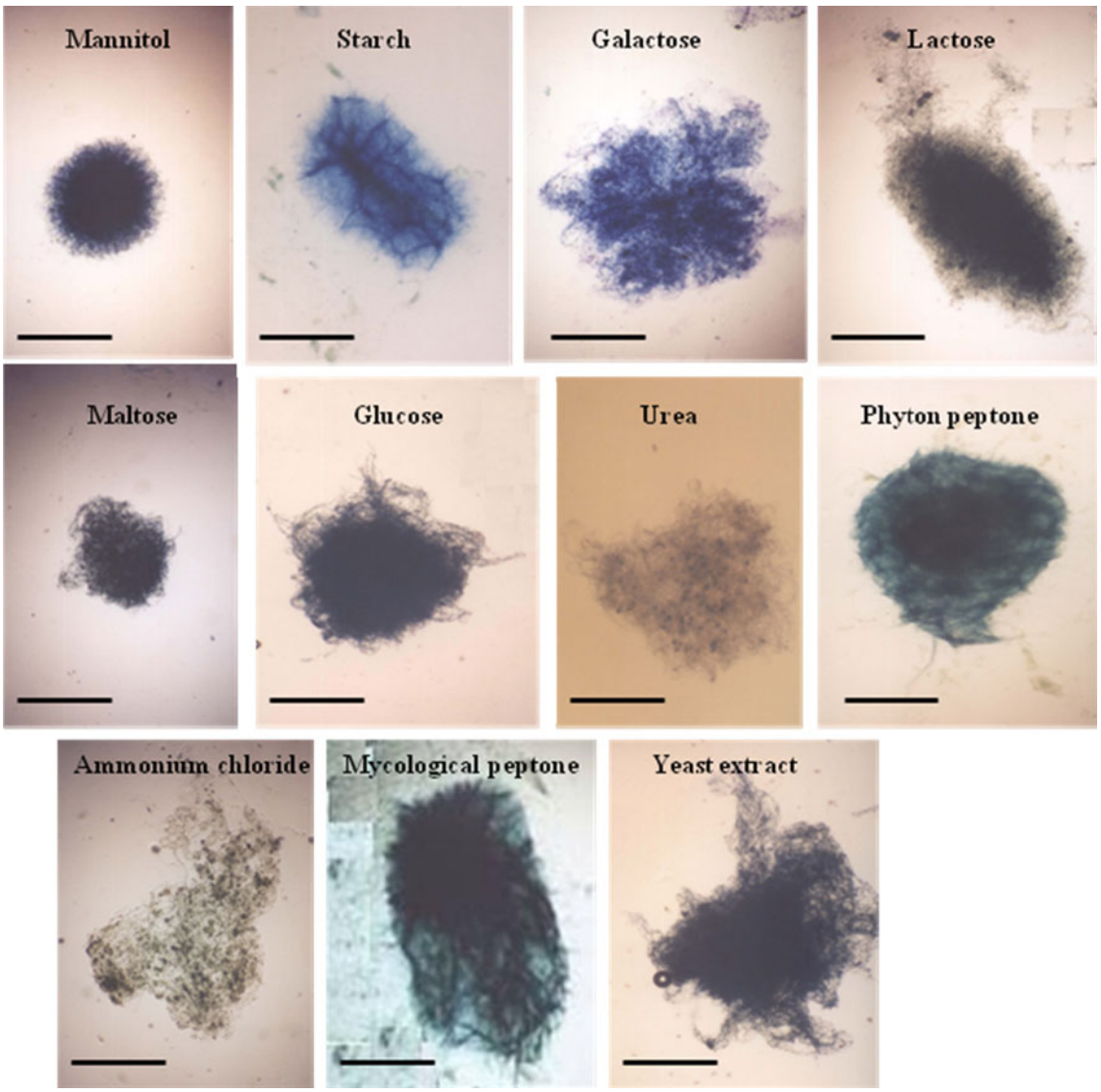

Fig. 1 The typical pellet morphology of *A. blazei* grown 14 days in submerged cultures with different carbon and nitrogen sources. At least 25 pellets were stained with lacto-phenol cotton blue and then studied by microscope. *Bars* 60 μm

there is no report on the effects of different culture conditions on morphology as well as a relationship between morphology and biomass or EPS production by *A. blazei*. One of the important parameters which have important effects on biomass and metabolite production in fermentation medium is pellet morphology (Park et al. 2002). Among different morphological properties of pellet-forming fungi mostly mean diameter, hairiness, core area, mean area, compactness and circularity of the pellet have been considered (Jimenez-Tobom et al. 1997; Park et al. 2001; Sinha et al. 2001; Hwang et al. 2004). In the present study, the pellet morphology was characterized with respect to pellet mean diameter, circularity and compactness of a pellet. Pellets, fungal fragments and clumps were differentiated by their grayness level. The pellets were stained with lactophenol-cotton blue, where lacto-phenol serves as a mounting fluid; meanwhile, cotton blue stains the chitin present in the cell walls of mycelium (Parija and Prabhakar 1995). Organisms suspended in the stain were killed due to the presence of phenol. The high concentration of the phenol inhibits lysis of the cells by deactivating lytic cellular enzymes. The method allowed for staining andfor determining the core pellets and the outer hairy regions accurately. Some instances of pellet morphology in different culture conditions are shown in Fig. 1.

Effects of different carbon sources

Figure 2 represents mean values of circularity, compactness, mean diameter, EPS and biomass production against different carbon sources used in this investigation. As shown in Fig. 2, there was no significant difference between different groups in circularity with an exception when starch was supplied as a carbon source. The starch-supplied group also showed significant differences in compactness, EPS and biomass production with other groups.

Table 1 shows the results of one-way ANOVA analysis on the image data analysis emphasis on the mentioned results. As shown in Table 1, no significant difference was observed between the circularity of the groups, but significant differences were observed between groups in their compactness and mean diameter.

Multiple comparisons by post hoc LSD test (data not shown) revealed that only starch supply causes significant impacts ($p<0.05$) on the compactness of colonies compared to those of other treatments. Starch-supplied groups also showed significant differences ($p<0.05$) in the circularity and mean diameter of their pellets only in those groups which were supplied with mannitol and lactose, respectively. Mean diameter of pellets in glucose supplied group was significantly different with those of the rest treatments. Interestingly, there were lots of conidia on the mycelia grown in mannitol- and maltose-supplied cultures (Fig. 3).

Kruskal–-Wallis *H* test revealed that EPS and biomass production between different groups with different carbon sources was significantly different ($p<0.05$). Mann–Whitney *U* tests showed that EPS and biomass production only in submerged cultures containing starch as carbon source were significantly more than other groups. In our previous work (Hamedi et al. 2007), starch was the best carbon source (among examined carbon sources) for EPS and biomass production by *A. blazei* Murill. There are some other reports on the effects of carbon sources on EPS and biomass production by *A. blazei* Murill (Shu et al. 2004), but they may not be conclusive due to lacking statistical evaluation. Collectively, although different carbon sources in submerged

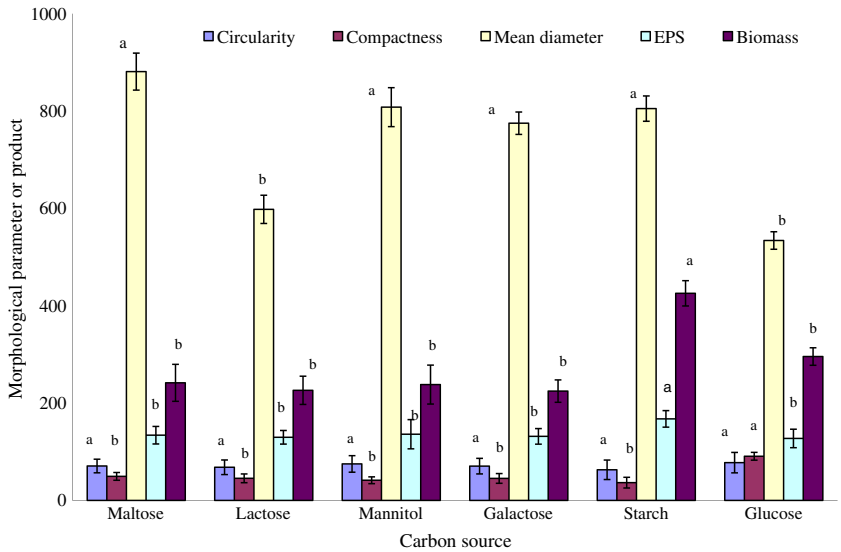

Fig. 2 Effects of different carbon sources on morphological parameters of *A. blazei* and biomass and EPS productivity after 14 days. The values of circularity and compactness are in percent, mean diameter in μm and biomass and EPS production in mg/100 mL. All values are means of at least 25 samples ± SD. Different *letters* show significant differences between groups at $p \leq 0.05$

Table 1 One-way ANOVA results of morphological parameters of submerged cultured *A. blazei* grown in with different carbon sources, after 14 days; 25 pellets in each group were analyzed

	Relationship	Sum of squares	*df*	Mean square	*F*
Circularity	Between groups	3,329.233 ns	5	665.874	1.819
	Within groups	52,709.360	144	366.037	
	Total	56,038.593	149		
Compactness	Between groups	45,711.013***	5	9,142.203	35.133
	Within groups	37,471.280	144	260.217	
	Total	83,182.293	149		
Mean diameter	Between groups	231.400***	5	46.280	8.222
	Within groups	807.319	144	5.606	
	Total	1,038.719	149		

df Degrees of freedom, *F* value is the ratio of the mean squares, *ns* not significant

***Significant at $p \leq 0.001$

culture are able to change the morphology of *A. blazei* pellets, only starch has statistically significant effects on morphology parameters. Interestingly, lower compactness and circularity of pellets consuming starch as their carbon source were favorable for producing greater amounts of EPS and biomass by *A. blazei*. This may be of relevance for the small feather-like fluffy and loosely packed pellets with a small compacted central core produced by starch-supplied *A. blazei* in submerged culture (Fig. 1b), since nutrient uptake can be accomplished more easily in such pellets compared to the compacted ones.

This is in agreement with the results reported by Park et al. (1999) for *Mortierella alpina*. They suggested that small sized feather-like mycelial clump of *Mortierella alpina* is favorable to produce higher amount of arachidonic acid. In contrast, the study on *Cordyceps militaris* morphological forms by Kim et al. (2003) suggested that larger and more compact pellets were more productive. Considering different reports on productive fungal morphology may suggest that for production of each metabolite the desirable morphology depends on the nature of the organism (Hwang et al. 2004).

Effect of different nitrogen sources

Figure 4 represents mean values of circularity compactness, mean diameter, EPS and biomass production against different nitrogen sources used in this investigation. As shown in Fig. 4, morphological parameters were significantly different between pellets grown in submerged cultures with different nitrogen sources. This is evidenced by analysis of the morphological features of different groups by one-way ANOVA (Table 2).

Multiple comparisons by post hoc LSD test (data not shown) revealed that compactness and circularity of colonies in complex nitrogen sources (yeast extract, phyton peptone) were significantly different from those colonies in simple nitrogen sources (urea and ammonium chloride). However, no difference was observed between either yeast extract and phyton peptone supplies or

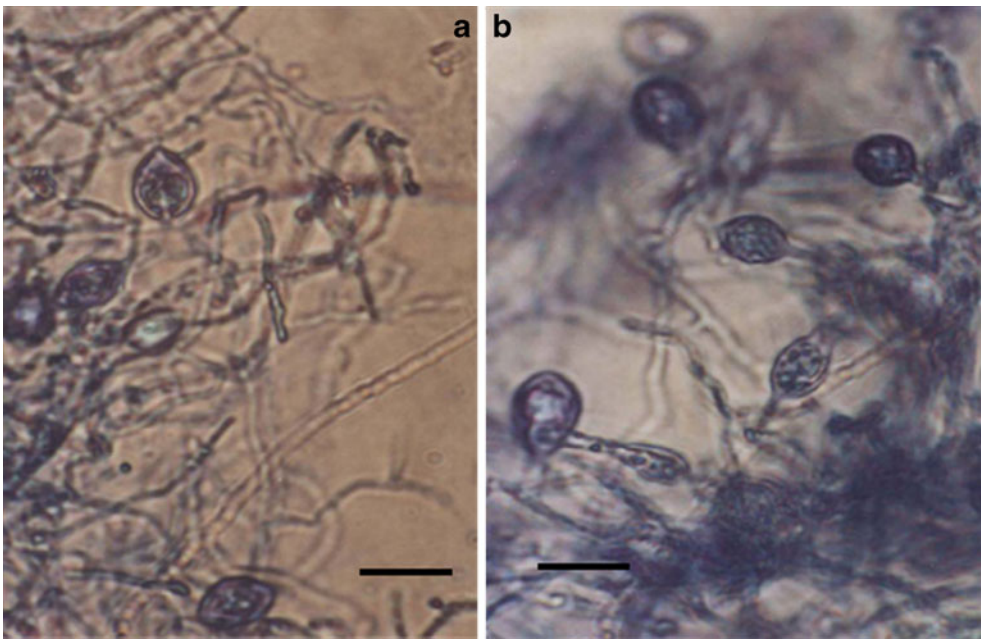

Fig. 3 Conidia production on *A. blazei* mycelia 14 days grown in mannitol (**a**) and maltose (**b**) supplied cultures. At least 25 pellets were stained with lacto phenol cotton blue and then studied by microscope. *Bars* 15 μm

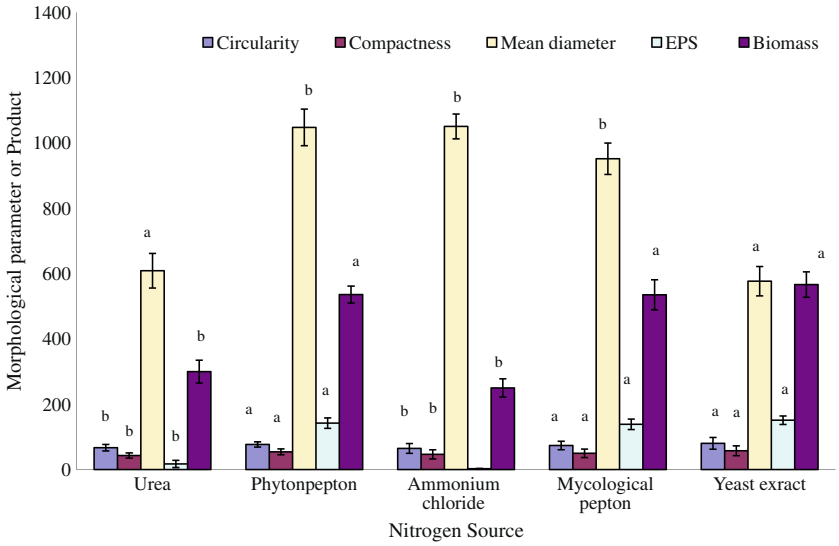

Fig. 4 Effects of different nitrogen sources on morphological parameters of *A. blazei* and biomass and EPS productivity after 14 days. The values of circularity and compactness are in percent, mean diameter in μm and biomass and EPS production in mg/100 mL. All values are means of at least 25 samples ± SD. Different *letters* show significant differences between groups at $p \leq 0.05$

between urea and ammonium chloride. Yeast extract was significantly different with the other groups in mean diameter.

Kruskal–Wallis *H* test revealed that EPS and biomass production between submerged cultures with different nitrogen sources was significantly different ($p < 0.05$). Mann–Whitney *U* tests showed that EPS and biomass production of submerged culture containing complex nitrogen sources (yeast extract, phyton peptone and mycological peptone) were significantly higher than those of submerged cultures containing simple nitrogen sources (urea and ammonium chloride). The obtained results indicated that complex nitrogen sources are generally better than simple nitrogen sources for fungi and mycelia production. Similarly, other researchers have also reported that complex nitrogen sources are better for EPS and biomass production in different mushrooms (Chen et al. 2008; Hamedi et al. 2007; Lee et al. 2004). *A. blazei* exhibit loose compacted filamentous pellets in submerged culture with simple nitrogen sources (ammonium chloride and urea; Fig. 1), while pellets consuming complex nitrogen sources had a compacted central core with fluffy loosely packed filamentous outer zones. In pellets which were grown in submerged culture containing yeast extract, the hyphal length was critically longer in the outer zone and made starfish-like pellets. Interestingly, pellets which were grown in submerged culture containing complex nitrogen sources had significantly more compactness and circularity than those that consumed simple nitrogen sources. Teng et al. (2009) also studied the effect of nitrogen source on morphology and antibiotic production by *Rhizopus chinensis* and reported that, in submerged culture containing different nitrogen compounds, morphological parameters such as hyphal length and degree of branching can be significantly affected. They also reported that the highest antibiotic production was achieved with pellets which were fluffy with a compact core and a loose outer zone. It seems that a fluffy and loose outer zone may be more desirable for nutrient and oxygen uptake and thus more EPS and biomass production.

Effect of different initial pH

The pH of the medium is a very important but often neglected environmental factor. Different points on a pH–growth plot may be interpreted in terms of effects on

Table 2 One-way ANOVA results of morphological parameters of *A. blazei* grown in submerged cultures with different nitrogen sources for 14 days; 25 pellets in each group were analyzed

	Relationship	Sum of squares	*df*	Mean square	*F*
Circularity	Between groups	4,291.822***	4	1,072.956	1.819
	Within groups	36,153.410	120	301.278	
	Total	40,445.232	124		
Compactness	Between groups	3,382.997***	4	845.749	4.315
	Within groups	23,517.803	120	195.982	
	Total	26,900.800	124		
Mean diameter	Between groups	553.087***	4	138.272	22.076
	Within groups	730.760	120	6.090	
	Total	128.848	124		

df degrees of freedom, *F* the ratio of the mean squares

***Significant at $p \leq 0.001$

Table 3 One-way ANOVA results of morphological parameters of *A. blazei* grown in submerged cultures with different initial pH for 14 days; 25 pellets in each group were analyzed

	Relationship	Sum of squares	*df*	Mean square	*F*
Circularity	Between groups	56,331.760 ns	4	14,082.940	0.899
	Within groups	1,487,544.8	95	15,658.366	
	Total	1,543,876.5	99		
Compactness	Between groups	1,714.900***	4	428.725	3.951
	Within groups	10,307.850	95	195.982	
	Total	12,022.750	99		
Mean diameter	Between groups	65.802***	4	138.272	22.706
	Within groups	114.125	95	6.090	
	Total	179.927	99		

df Degrees of freedom, *F* the ratio of the mean squares, *ns* not significant

***Significant at $p \leq 0.005$

transport of nutrients, nutrients solubility, enzyme reactions or surface phenomena. The composition of the medium can affect the initial pH and the extent and direction of pH drifts during growth of the fungus. Table 3 represents the results of the analysis of morphological parameters in different pH by one-way ANOVA. Indeed, there was no significant difference between different groups in circularity while compactness and mean diameter were significantly different ($p<0.005$). The phenomenon of pellet formation is strongly influenced by the pH (Gerlach et al. 1998). Carrying out batch cultivations of *Aspergillus oryzae* in a stirred tank reactor, Carlsen et al. (1996) found that, at pH values below 2.5, the mycelium grew heavily vacuolated and the cells appeared swollen, resulting in poor growth. At pH 3.0–3.5, freely dispersed hyphal elements resulted, while at pH 4.0–5.0, the broth consisted of both pellets and freely dispersed hyphae. It was hypothesized that, in general, the tendency of the mycelium to form pellets increases as the pH value of the culture rises (Papagianni 2004). In the present study, initial pH had no significant influence on pellets compactness, but those pellets grown in submerged culture with initial pH 8 showed biomass significantly bigger than those grown in pH 4 (Fig. 5).

Only mean diameters of pellets grown in submerged culture with the initial pH 5 were significantly different from other groups (Fig. 5). Surprisingly, Kruskal–Wallis *H* test revealed that EPS and biomass production in submerged cultures with different initial pH were not significantly different.

In our previous work (Hamedi et al. 2007), EPS and biomass production were the highest in submerged culture with initial pH of 7. In optimization studies, we found that initial pH did not have significant effect on EPS production by *A. blazei*. However, Shu et al. (2004) reported that pH

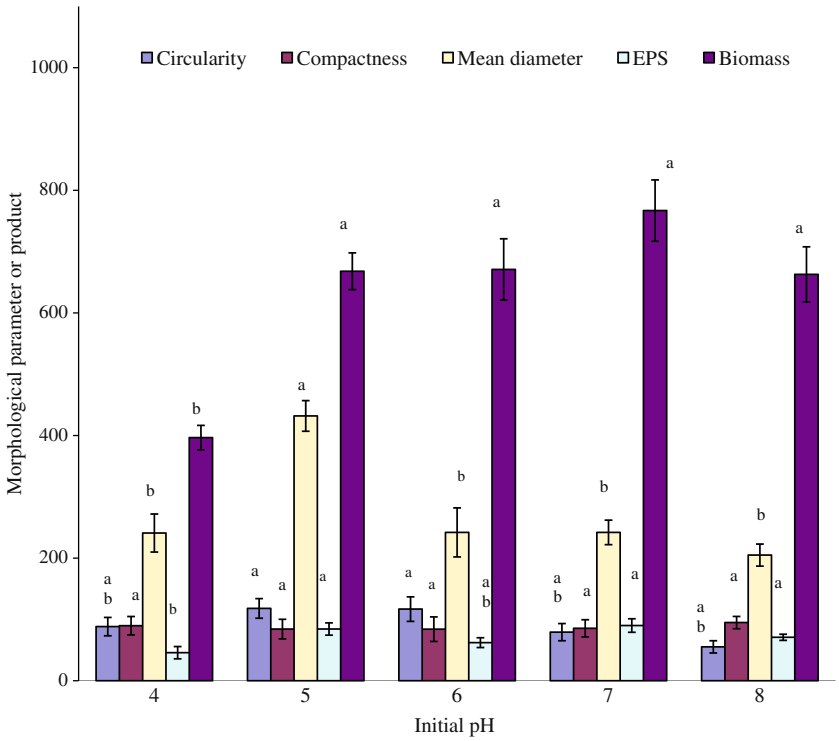

Fig. 5 Effects of initial pH on morphological parameters of *A. blazei* and biomass and EPS productivity after 14 days. The values of circularity and compactness are in percent, mean diameter in μm and biomass and EPS production in mg/100 mL. All values are means of at least 25 samples ± SD. Different *letters* show significant differences between groups at $p \leq 0.05$

affected EPS production. This different result could be due to different statistical methods and a different strain of the fungi. On the other hand, the composition of the medium can affect the initial pH, and the extent and direction of pH during growth of the fungus. One way to minimize the pH changes during growth in submerged culture is to apply different buffer systems, but the ions required to stabilize the pH can also have some effects on biological activities of the fungi growing in the submerged culture, as well as the other physicochemical properties of such cultures (Papagianni 2004).

Conclusion

In the present study, the effects of culture conditions on morphological parameters (circularity, compactness and mean diameter) of *A. blazei* and relationships between pellet morphology, biomass and EPS productivity were investigated statistically, and it was revealed that different culture conditions have significant effects on *A. blazei* pellet morphology as well as on its biomass and EPS productivity. When different carbon sources were applied, significant increases in EPS and biomass production were accompanied by significantly lower compactness and circularity. However, when different nitrogen sources were supplied, higher compactness and circularity were favorable for significant increases in EPS and biomass production. This might be due to different culture compositions (especially different carbon sources) in these studies. Overall, in starch-supplied cultures (with yeast extract as nitrogen source), *A. blazei* produced small feather-like fluffy and loosely packed pellets with a small compacted central core which produced the highest biomass and EPS production, and this may be the desirable pellet morphology for these objectives. It was revealed that some carbon sources (mannitol and maltose) can stimulate conidia production and asexual reproduction of *A. blazei*, which needs to be further investigated in future studies.

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