

*Full Length Research Paper*

# Polysaccharides production by submerged fermentation of *Coprinus comatus* and their inhibitory effects on non-enzymatic glycosylation

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**Bioactive polysaccharides produced by fungi, especially by medicinal mushrooms, have attracted great interest. Polysaccharides from *Coprinus comatus* have been reported to possess hypoglycemic activity. In the present study, the effects of carbon source, initial glucose concentration and initial peptone level on cell growth and polysaccharide production were examined in the submerged fermentation system of *C. comatus*. Maltose was found to be disadvantageous for cell growth, but favorable for extracellular polysaccharide (EPS) production. Glucose was beneficial for both cell growth and production of intracellular polysaccharide (IPS), which was markedly improved when a high initial glucose concentration was used. The highest maximal EPS production level (0.89 g/l) was obtained with an initial glucose concentration of 40 g/l. Cell growth was positively related to increases in initial peptone concentration and polysaccharide production was maximized at 10 g/l of peptone. Finally, high inhibitory activity of *Coprinus* polysaccharides (both EPS and IPS) on non-enzymatic glycosylation (NEG) was demonstrated for the first time. The findings in this study can be applied to improve the performance of *C. comatus* cultures in the production of bioactive metabolites on a bioreactor scale and provide a foundation for further investigation into medicinally active compounds derived from *C. comatus*.**

**Key words:** *Coprinus comatus*, polysaccharide, submerged fermentation, non-enzymatic glycosylation.

## INTRODUCTION

Medicinal mushrooms are abundant sources of a wide range of useful natural products as well as new compounds with interesting biological and pharmacological activities (Lorenzen and Anke, 1998). Bioactive polysaccharides produced by fungi, especially by edible and medicinal mushrooms, such as *Ganoderma lucidum*, *Grifola frondosa*, *Agaricus blazei* Murill, *Cordyceps militaris*, *Laetiporus sulphureus*, have attracted great interest (Hwang and Yun, 2010; Kim et al., 2010; Kodama et al., 2003; Liu et al., 2010; Zhao et al., 2010). In order to improve the production of fungal

bioactive polysaccharides, investigators have worked to optimize the fermentation conditions and medium composition of solid-state and submerged fermentation systems (Hwang and Yun, 2010; Kim et al., 2002; Kim et al., 2010; Kwon et al., 2009; Tang and Zhong, 2002). Traditionally, edible and medicinal mushrooms have been produced in solid cultures using composts or lignocellulosic wastes, such as straw or wood, a process that usually takes several months to produce fruiting bodies (Solomons, 1975).

However, recently, investigators have found that growing mushroom mycelia by submerged fermentation in a defined medium is a rapid alternative method for obtaining fungal biomass of consistent quality (Kwon et al., 2009; Yang and Liao, 1998). In addition, polysaccharides that have synergistic bioactive effects with

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mycelia can be produced simultaneously with this method (Kim et al., 2002; Kim et al., 2002). *Coprinus comatus* (popularly known as shaggy ink cap), of the order *Coprinaceae* and family *Coprinus*, is a delicious and nutritious edible mushroom that is highly valued for its physiological benefits. Polysaccharides from *C. comatus* have been reported to have antidiabetic, antitumor, antimutagenic and hepatoprotective effect (Ding et al., 2010; Lee et al., 1999; Li et al., 2010; Yamac et al., 2009). However, so far, little is known concerning production of *C. comatus* polysaccharides during fermentation. Carbohydrates and nitrogen are the major nutrient sources for *C. comatus* in culture.

The influences of carbon source and initial peptone concentration on the production of biomass and polysaccharide by *C. comatus* in submerged fermentation have yet to be resolved. The aims of the present study were three-fold. First, we examined the effects of carbohydrate source and initial peptone concentration on final cell density and polysaccharide productivity because carbohydrates are important carbon and energy sources for higher fungi and nitrogen source is related to cell growth and biomass density in microorganism cultures. Secondly, we studied the relationship between pellet morphology and polysaccharide production. Finally, *C. comatus* polysaccharide inhibition of NEG a medicinal application was evaluated. The information obtained in this study may be fundamental to the development of large scale fermentation of *C. comatus*, particularly for polysaccharide production.

## MATERIALS AND METHODS

### Strain, medium and flask culture

*C. comatus* CC0312 were screened and collected at the School of Biotechnology, Jiangnan University (Wuxi, China) were used in this study. The specimens were maintained on potato-dextrose-agar (PDA) slants. The slants were incubated at 25°C for 7 d and then stored at 4°C for about 4 weeks. The seed culture medium consisted of the following components: glucose 30 g/l, yeast extracts 4 g/l, corn steep liquor 3 g/l, KH<sub>2</sub>PO<sub>4</sub> 2 g/l and MgSO<sub>4</sub>·7H<sub>2</sub>O 3 g/l. For preparation of the inoculum, five ~5-mm pieces of *C. comatus* CC0312 mycelia were transferred from a slant into each 250 ml flask containing 50 ml seed medium. The flasks were then placed in a rotary shaker incubator at 150 rpm and 25°C for 7 d. All fermentation cultures were carried out in 500-ml flasks containing 150 ml fermentation medium in accordance with the experimental design. The initial pH of the medium was ~5.0 and it was inoculated with ten percent (v/v) of the seed culture, then followed by incubation at 25°C on a rotary shaker (150 rpm).

### Manipulations of carbohydrate source and initial peptone concentration

The effects of carbohydrate source on fungus cultures were studied by using various carbohydrate sources, namely sucrose, glucose, maltose and fructose. The fermentation medium was composed of 10 g/l of peptone, 2 g/l of KH<sub>2</sub>PO<sub>4</sub>, 3 g/l of MgSO<sub>4</sub>·7H<sub>2</sub>O and 30 g/l of the experimentally specified sugar (sucrose, glucose, maltose, or

fructose). A range of concentrations of glucose were further tested to obtain information regarding optimal cell growth and product accumulation. The initial peptone concentrations tested were 5, 7.5, 10 and 15 g/l. The fermentation medium was composed of 40 g/l of glucose, 2 g/l of KH<sub>2</sub>PO<sub>4</sub>, 3 g/l of MgSO<sub>4</sub>·7H<sub>2</sub>O and a range of experimentally specified peptone concentrations were investigated. The medium was inoculated as described above. Multiple flasks were run at the same time and three flasks were sacrificed at each sampling point.

### Analytical methods

Fermented broth samples were collected at each experimental time point over the course of fermentation. Dry cell weight (DCW) was obtained by centrifuging a sample at 4500 rpm (2355 g) and 20°C for 20 min, washing the pelleted cells three times with distilled water and drying at 105°C for sufficient time to obtain a constant weight. Meanwhile, the supernatant was used for EPS preparation and measurement according to the method described by Wu et al. (2006). Residual sugar content was determined by the phenol-sulfuric acid method (Dubois et al., 1956). For analysis of IPS content, the mycelia biomass was submerged in hot water for 4 h at 100°C. Then, extracted samples were centrifuged at 4500 rpm (2355 g) and 20°C for 20 min and the supernatant were used for IPS preparation and measurement following the same procedure used for EPS analysis above. Polysaccharide productivity was calculated according to the following formula: (maximum polysaccharide production – initial polysaccharide amount) / culture time. Using a series of testing sieves, pellets from each sample were separated according to size. The size distribution frequency of pellets was based on the ratio of DCW. Average growth rate was calculated as: (maximum DCW – initial DCW)/initial DCW/culture time.

### EPS and IPS inhibition of non-enzymatic glycosylation (NEG)

EPS and IPS were lyophilized and then dissolved in 0.2 mol/l phosphate buffer (pH 7.4) at several concentrations. Metformin, an antidiabetic drug (Shenzhen Zhonglian pharmaceutical Company Limited, China), was used as a reference standard and also dissolved in phosphate buffer. *In vitro* NEG inhibition tests were performed as described previously (Ding et al., 2010).

## RESULTS AND DISCUSSION

### Effects of carbon source

Fermentation of *C. comatus* cultures with glucose (7 d), fructose (9 d) and sucrose (8 d) produced maximum cell densities of 10.25 ± 0.93, 9.64 ± 0.18 and 9.06 ± 0.83 g DCW/l, respectively (Table 1). Almost all of the sugar content had been utilized at the end of fermentation period when these three carbon sources were used. However, when maltose was used, a lower final cell density was obtained and substantial residual sugar (5.1 g/l) remained in the medium at the end of fermentation (day 9). As shown in Table 1, the best cell yield on sugar and the shorter culture time were achieved when glucose was used for the fermentation of *C. comatus*. These data indicate that of the four sugars tested, glucose was the best candidate for the cell growth of *C. comatus* by

**Table 1.** Effects of carbon source on cell growth and production of IPS and EPS<sup>a</sup>.

Carbon source	Glucose	Maltose	Fructose	Sucrose
DCW (g/l)	10.25 ± 0.93 (day 7) <sup>b</sup>	7.22 ± 0.35 (day 9)	9.64 ± 0.18 (day 9)	9.06 ± 0.83 (day 8)
Cell yield on sugar (g DCW/g sugar)	0.35 ± 0.01	0.29 ± 0.01	0.33 ± 0.00	0.31 ± 0.00
Maximum IPS content (mg/g DCW)	47.80 ± 3.10	43.20 ± 1.00	43.70 ± 5.20	39.20 ± 0.80
Maximum IPS production (g/l)	0.48 ± 0.02	0.31 ± 0.00	0.40 ± 0.05	0.35 ± 0.03
IPS productivity (mg/l/d)	69.30 ± 0.70	34.80 ± 0.50	46.00 ± 1.60	44.30 ± 1.30
Maximum EPS production (g/l)	0.57 ± 0.02	0.59 ± 0.03	0.42 ± 0.02	0.41 ± 0.00
EPS productivity (mg/l/d)	78.60 ± 2.20	63.30 ± 1.80	44.40 ± 1.00	48.80 ± 2.50

a Standard deviations were calculated from three independent samples; b culture time (days).

submerged fermentation. The maximal EPS production was obtained when maltose was used as the carbon source (Table 1).

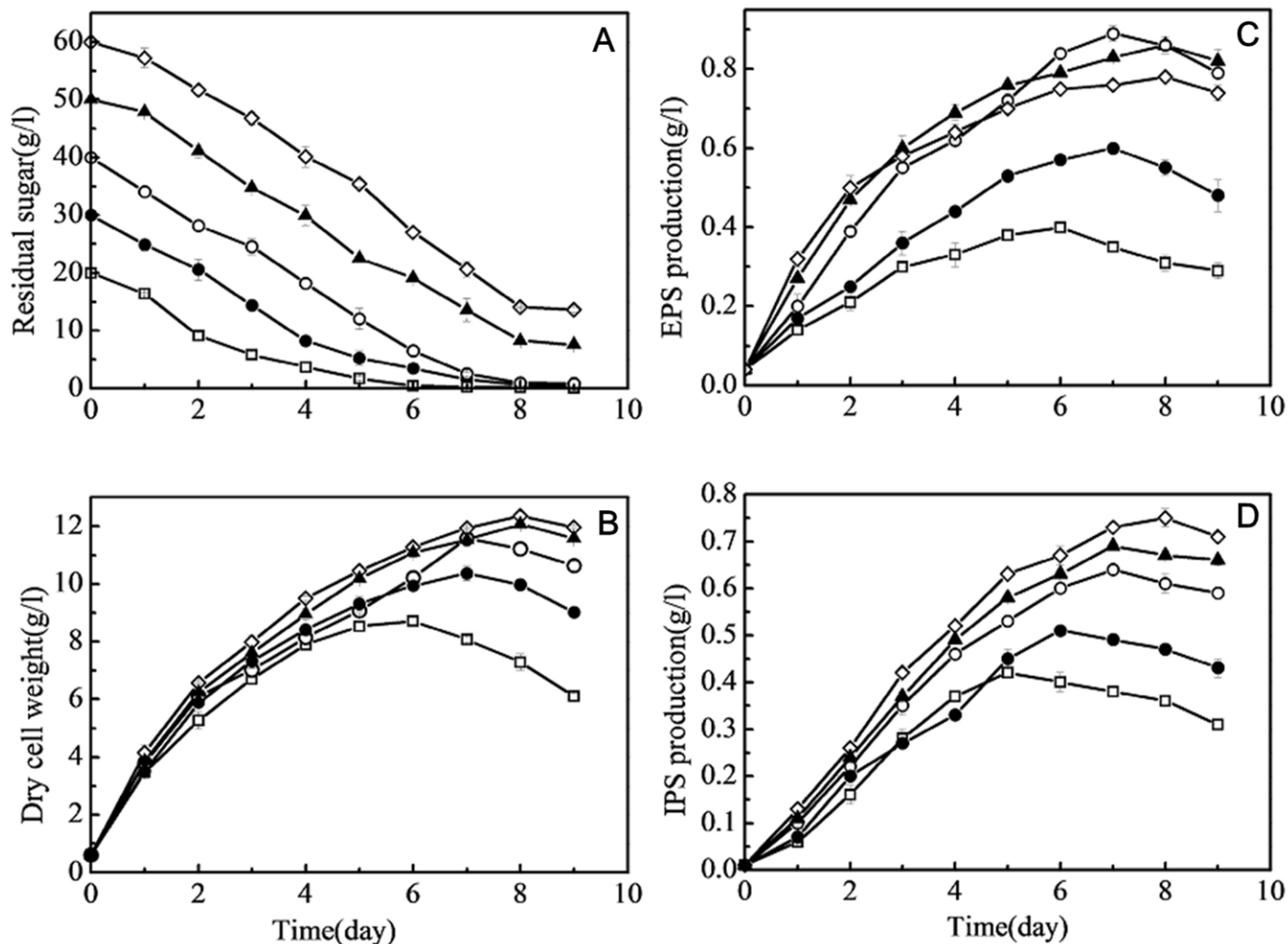
Similar patterns have been observed in culture studies of various edible mushrooms, such as *Ganoderma lucidum* (Tang and Zhong, 2002), *Pleurotus nebrodensis* (Le et al., 2007), *Agrocybe cylindracea* (Kim et al., 2005). It was indicated in Table 1 that maltose is favorable for EPS production, but not so favorable for cell growth. Many small-size pellets were observed in maltose-supplied cultures; these small-size pellets may allow for better biosynthesis of polysaccharide, relative to larger pellets. In addition, the use of glucose as the carbon source yielded the highest maximal production of IPS as well as the highest overall IPS productivity. From the above results, it can be concluded that of the carbon sources tested here, maltose yields the best EPS production, but it is the worst carbon source for cell growth. Although the production of EPS was a little lower with glucose than with maltose use of glucose was favorable for cell growth as well as IPS production.

### Effects of initial glucose concentration

The effects of initial glucose concentration on DCW and polysaccharide production were investigated with the aim of maximizing these variables. As shown in Figure 1B, the maximum dry cell mass with an initial glucose level of 20 g/l (6 d), 30 g/l (7 d), 40 g/l (7 d), 50 g/l (8 d), and 60 g/l (8 d) was 8.72 ± 0.11, 10.36 ± 0.25, 11.58 ± 0.06, 12.07 ± 0.02 and 12.36 ± 0.14 g DCW/l, respectively. Correspondingly, the cultures average growth rates ranged from 2.18 per day to 2.54 per day, with the maximum rate being observed with an initial concentration of 40 g/l. A higher glucose concentration was generally favorable for production of more biomass in the fermentation of *C. comatus*, results which are similar to those obtained previously with lactose in the fermentation of *G. lucidum* (Tang and Zhong, 2002). On the contrary, in cultures of *Aspergillus niger* and *G. lucidum* (Fang and Zhong, 2002; Papagianni et al., 1999), average growth rate has been reported to decrease with

increases in initial glucose concentration. These observations suggest that response to initial sugar concentration varies across different culture systems. Thus, detailed studies should be performed for each species and system of interest. Glucose consumption as a function of initial glucose concentration is summarized in Figure 1A. Under an initial glucose level of 20, 30 and 40 g/l, the sugar in the medium was nearly exhausted at day 6, day 7 and day 7, respectively. However, with an initial glucose concentration of 50 and 60 g/l, 8.23 and 14.09 g/l of residual sugar remained in the medium at day 8, respectively. The cell yields relative to initial glucose concentration used were 0.45 ± 0.02, 0.36 ± 0.01, 0.31 ± 0.00, 0.29 ± 0.01 and 0.27 ± 0.02 g DCW/g glucose with initial glucose concentrations of 20, 30, 40, 50 and 60 g/l, respectively (Figure 1B). Thus there was a clear trend of decreasing cell yield with increasing initial glucose level. Maximal EPS production values were 0.40 ± 0.01, 0.60 ± 0.00, 0.89 ± 0.02, 0.86 ± 0.02 and 0.78 ± 0.00 g/l with initial glucose concentrations of 20 g/l (6 d), 30 g/l (7 d), 40 g/l (7 d), 50 g/l (8 d), and 60 g/l (8 d), respectively (Figure 1C). Correspondingly, overall EPS productivity values were 60.1 ± 2.4, 80.0 ± 1.5, 121.4 ± 1.8, 102.5 ± 2.1 and 92.5 ± 3.0 mg/l/d and the EPS yields relative to initial glucose concentration were 18.4, 19.7, 22.7, 19.6 and 16.1 g DCW/g sugar, respectively. Together, these results suggest that an initial glucose concentration of ~40 g/l is optimal for EPS production.

As shown in Figure 1D, IPS production peaked on days 5, 6, 7, 7 and 8 with initial glucose concentrations of 20, 30, 40, 50 and 60 g/l, respectively; those respective peak values were 0.42 ± 0.00, 0.51 ± 0.01, 0.64 ± 0.01, 0.69 ± 0.00, and 0.75 ± 0.02 g/l and the corresponding IPS productivity values were 82.0 ± 3.8, 83.4 ± 1.3, 90.0 ± 2.4, 97.1 ± 1.0 and 105.0 ± 1.8 mg/l/d. Higher initial glucose levels generally increased IPS accumulation. The highest IPS content levels at initial glucose concentrations of 20, 30, 40, 50 and 60 g/l were 50.7, 51.3, 58.8, 59.8 and 61.1 mg/g DCW, respectively. Fungal morphology is an important parameter that affects the physical properties of the fermentation broth, which are closely related to rheological behavior. Higher fungi usually form pellets during their fermentation process and



**Figure 1.** Effect of initial glucose concentration on glucose consumption; **A**, cell growth; **B**, EPS; **C** and IPS; **D**. Symbols for initial glucose concentration (g/l): 20 ( $\square$ ), 30 ( $\bullet$ ), 40 ( $\circ$ ), 50 ( $\blacktriangle$ ), and 60 ( $\diamond$ ). Error bars indicate standard deviations from three independent samples.

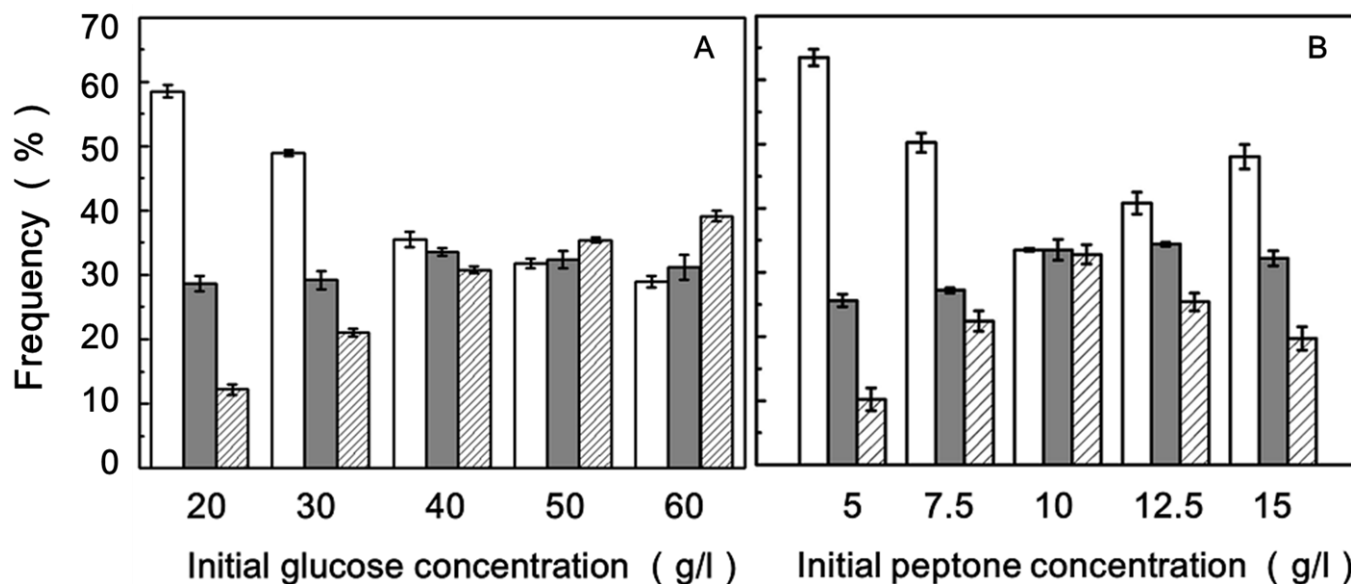
mycelial morphology and pellet size are related to polysaccharide production (Fang et al., 2002; Fang and Zhong, 2002; Lee et al., 2004). Higher initial glucose concentration was found to produce smaller sized pellets in our study (Figure 2A); this trend might be related to osmotic pressure (Fang and Zhong, 2002; Zhang et al., 1995). With initial glucose levels in the range of 20 to 60 g/l, as used here, the osmotic pressure is estimated to be in the range of 2.67 to 8.19 atm (Zhang et al., 1995). Furthermore, smaller pellets were associated with greater biosynthesis of IPS (Table 2), a finding which fits well with prior findings in *G. lucidum* cultures (Fang and Zhong, 2002).

#### Effects of initial peptone concentration

As shown in Table 3, maximum DCW rose with increases

in initial peptone level. It suggested that a higher peptone concentration can yield a higher cell density in the fermentation of *C. comatus*. Maximal EPS production (0.65 g/l) and the greatest overall EPS production (0.90 g/l) were obtained with an initial peptone concentration of 10 g/l (Table 3).

In order to examine the relationship between pellet size and metabolite production, pellet size distribution was measured again (Figure 2B). Changes in peptone concentration substantially affected pellet size without greatly affect osmotic pressure (data not shown), indicating that peptone concentration effects on pellet size cannot be attributed to osmotic pressure alone. The percentage of pellets that were <1.6 mm in diameter was maximal in those cultures with the highest production levels of EPS and IPS with 10 g/l of initial peptone, indicating that smaller pellet size may be favorable for EPS biosynthesis.



**Figure 2.** Effect of initial glucose; **A** and peptone; **B** concentration on the pellet size of *C. comatus* on day 8 and on day 7, respectively. Symbols for pellet diameter: >2.0 mm (white bar), 1.6 to 2.0 mm (gray bar) and ≤1.6 mm (hatched bar). Error bars indicate standard deviations from three independent samples.

**Table 2.** Effect of pellet size on IPS content<sup>a</sup>.

Diameter of pellet (mm)	>2.0	1.6~2.0	<1.6
IPS content (mg/g DCW)	47.90 ± 2.30	58.50 ± 1.80	71.70 ± 2.50

<sup>a</sup> Standard deviation was calculated from three independent samples.

**Table 3.** Effects of initial peptone concentration on cell growth and production of IPS and EPS<sup>a</sup>.

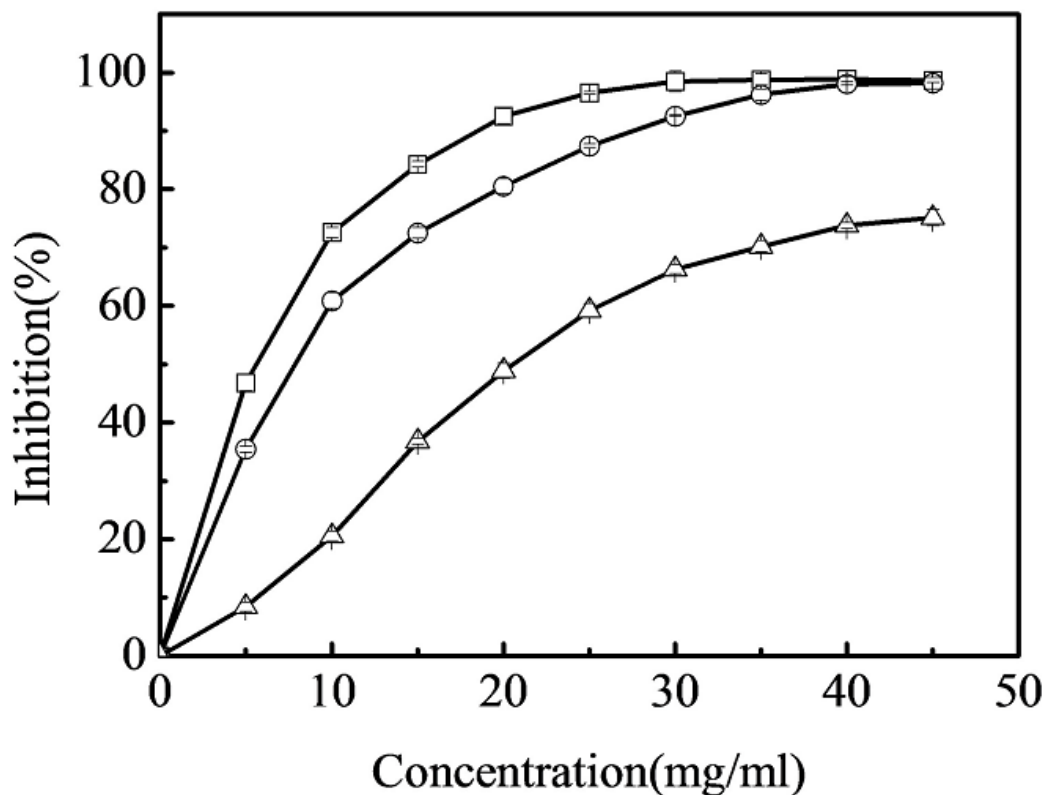
Initial peptone concentration (g/l)	5	7.5	10	12.5	15
DCW (g/l)	10.17 ± 0.38	10.98 ± 0.24	11.49 ± 0.15	12.21 ± 0.18	12.49 ± 0.83
	(day 8) <sup>b</sup>	(day 7)	(day 7)	(day 7)	(day 7)
Maximum EPS production (g/l)	0.67 ± 0.02	0.79 ± 0.03	0.90 ± 0.03	0.82 ± 0.02	0.73 ± 0.05
Maximum IPS content (mg/g DCW)	47.20 ± 1.30	51.60 ± 2.40	58.60 ± 1.20	51.10 ± 1.50	47.40 ± 0.90
Maximum IPS production (g/l)	0.47 ± 0.02	0.56 ± 0.00	0.65 ± 0.02	0.61 ± 0.01	0.58 ± 0.01

<sup>a</sup> Standard deviation was calculated from three independent samples; <sup>b</sup> culture time (days).

### Effects of polysaccharides on NEG

Our polysaccharides and metformin inhibition of NEG data are summarized in Figure 3. *C. comatus* polysaccharides markedly inhibited the formation of advanced glycation end products (AGEs) *in vitro*. In the concentration range of 0 to 10 mg/ml, EPS and IPS mediated inhibition of NEG increased more sharply than that mediated by metformin. A 98% NEG inhibition level was reached when ≥30 mg/ml of EPS or ≥40 mg/ml of IPS was used. However, at the concentration 42 mg/ml,

metformin's inhibition rate on NEG was <75%. To our knowledge, this is the first report to describe the NEG inhibition effects of *C. comatus* polysaccharides. NEG reactions between sugars and the amino groups of proteins can produce AGEs (Hasegawa et al., 2002) and thereby contribute to diabetic complications and play an important role in diabetic micro- and macro-angiopathy (Zhang et al., 1995; Zhang and Cui, 2007). Given reports indicating that inhibiting NEG can attenuate increases in blood glucose concentration (Zhang and Cui, 2007; Zhang and Zhang, 2006), the inhibitory effects of EPS



**Figure 3.** Polysaccharide and metformin inhibition of NEG. Symbols for inhibitors: EPS ( $\square$ ), IPS ( $\circ$ ), and metformin ( $\triangle$ ). Error bars indicate standard deviations from three independent samples.

and IPS on NEG might explain, at least in part, the hypoglycemic activity of *C. comatus* polysaccharides.

In conclusion, this present work demonstrated the effects of carbon source, initial glucose concentration and initial peptone level on *C. comatus* cell growth and *C. comatus* production of EPS and IPS. The experiments herein showed that metabolite production by *C. comatus* is affected by pellet size and demonstrated, for the first time, inhibition of NEG by *C. comatus* polysaccharides. The present results are applicable to the optimization of *C. comatus* cultures for use in the highly efficient production of bioactive metabolites (polysaccharides) on a bioreactor scale. They also provide a foundation for further investigation into medicinally active ingredients in *C. comatus* cultures.

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