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FUNGAL PROTEIN ESTIMATION OF *ASPERGILLUS ORYZAE* GROWING IN SOLID STATE CULTIVATION CONDITIONS

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ABSTRACT

The estimation of fungal protein is an essential parameter for nitrogen metabolism studies and evaluating the biological efficiency of fungal protein synthesis during fermented soybean meal processing. The aim of this study was to validate the fungal protein of *Aspergillus oryzae* SICC 3.302 measurement in solid state cultivation conditions using glucosamine content. Factors influencing the correlation between the glucosamine amount and fungal protein content, including incubation time, culture conditions (liquid or solid medium), carbon and nitrogen source, have been measured. Results suggest that the correlation of the glucosamine level and fungal protein content, regardless of incubation time and culture conditions, was a nearly constant. The influence of the medium composition, in particular the nitrogen source on the correlation has occurred. Collectively, these results indicated that glucosamine could be considered as reliable fungal protein indicator under the same nitrogen source.

Keywords: Glucosamine, fungal protein, *Aspergillus oryzae*, solid state cultivation.

INTRODUCTION

Soybean meal (SBM) is an important source of dietary protein in animal feed industry. However, variety of antinutritional factors, such as trypsin inhibitor, lectins and soya globulins, etc. contained in SBM limited the wide application of SBM in animal especially young animal feed (Dunsford *et al*., 1989; Li *et al*., 1990; Jiang *et al*., 2000). Fermentation with *Aspergillus oryzae,* a fungus widely used for hundreds of years in production of sake, miso, and soy sauce, could decrease antinutritional factors and improve the nutritional quality of SBM (Hong *et al*., 2004; Feng *et al*., 2007). Many biochemical reactions occurred during fermented SBM process involving substrate protein degradation and fungal protein synthesis, however, little is known about the method for distinguishing substrate protein and fungal protein when *A. oryzae* grow in solid state. The quantity of fungal protein is an essential parameter in nitrogen metabolism studies and for evaluating the biological efficiency of fungal protein synthesis during fermented SBM processing.

In the case of solid state fermentation, direct measurement of fungal protein is very difficult because fungi penetrate into and bind themselves tightly to the solid substrate particles (Desgranges *et al*., 1991). Many authors have described indirect methods to estimate biomass in solid state fermentation. These indirect methods are based on measuring the content of certain cell components such as glucosamine (Nahara *et al*., 1982; Sparringa and Owens, 1999; Wei *et al*., 2006), ergosterol (Nout *et al*., 1987; Wu *et al*., 2002), protein (Matcham *et al*., 1984; Córdova-López *et al*., 1996) and nucleic acids (Koliander *et al*., 1984; Wei *et al*., 2006), and measuring the biological activity like enzymatic activity (Barak and Chet, 1986) and respiration (Oriol *et al*., 1988), or measuring the consumption of some media components (Matcham *et al*., 1984). Meanwhile, previous studies have observed that the glucosamine content have a good correlations with sucrose consumption (Desgranges *et al*., 1991), amyloglucosidase and α-amylase level (Aidoo *et al*., 1981). These provide a possible indirect method to measure fungal protein, by determining the quantity of glucosamine in solid state cultivation conditions. However, less information is available on the correlation relationship between fungal protein and glucosamine level in solid state cultivation conditions.

Thus, the aim of the present work was to validate the fungal protein of *Aspergillus oryzae* measurement in solid state cultivation conditions using glucosamine content. As a fungal protein indicator, the relationship must ideally be constant throughout the fungal development. In the same way, culture conditions (liquid or solid medium) must not influence the relationship. If one of these two conditions is not fulfilled, this cell constituent cannot be considered as fungal protein indicator. In this study, agar plates covered with a membrane were used as a model system for solid state fermentation. This system prevents penetration of mycelium into the agar and allows the complete recovery of fungal biomass, so the content of glucosamine and fungal protein can be related to per gram dried biomass.

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MATERIALS AND METHODS

Microorganism

Aspergillus oryzae SICC 3.302, was maintained on potato dextrose agar (PDA) slants. After two days incubation at 30°C, the slants were stored at 4°C, for one month at most until use.

Preparation of spore suspension

Spore suspensions were prepared as previously (Córdova-López *et al*., 1996), the slants were suspended with 10ml 0.1% tween 80 sterile water and counted in blood counting chamber.

Submerged conditions

The liquid medium were formed by five carbon sources (glucose, sucrose, maltose, glycerol and sodium acetate) at 16g C/l, four nitrogen sources (bactopeptone, urea, $(NH_4)_2SO_4$ and $NaNO_3$) at 1.58g N/l and mineral element (0.1 g/l K_2HPO_4, 0.05 g/l KCl, 0.05 g/l $MgSO_4 \cdot 7H_2O$ and 0.001 g/l $FeSO_4$) in this experiment. Carbon, nitrogen and mineral element solutions were sterilized separately at 121°C for 30min.

The cultures were carried out in 250 ml Erlenmeyer flasks containing 95ml of different culture media. Each flask was inoculated with 5 ml of a spore suspension (Containing 10^6 spores ml^{-1}), and incubated at 30±0.5^{o}C for 24, 48, 72, 96 and 120h. All cultures were incubated on an orbital shaker at 120rpm. The fungal were harvested by filtering through a weighed filter (Millipore HVLP 04700, porosity 0.45μm) and the cake was washed with distilled water. Then, the dry weight is measured after drying in an oven at 65°C for 48h. The mycelium was grounded in a mortar, redried at 65°C for 24h and stored in a desiccator until analysed.

Solid state conditions

Because of the difficulty, or impossibility, of separating mycelium from solid state fermentation, the fungi was grown on agar medium to imitate the solid state conditions. The composition of agar medium was the same as the liquid medium except that each agar medium contained purified agar (15g/l). The sterilized and weighed filter (Millipore HVLP 04700, porosity 0.45μm) was placed on each agar plate, and it was inoculated 0.2 ml spore suspension (Containing 10^6 spores ml^{-1}). The agar plates were incubated at 30±0.5^{o}C in static conditions for 48h. The filter with the fungus was washed with distilled water and dried in an oven at 65°C for 48h. The pulverization and storage was the same as Submerged conditions.

Glucosamine determination

For fungal chitin hydrolysis into N-acetyl glucosamine, 20mg dried biomass was incubated with 2ml of H_2SO_4 (72%) in a test tube. After standing on a rotary shaker (130rpm) for 60min at 25°C, it was diluted with 3ml of distilled water and autoclaved at 121°C for 2h. The hydrolyzate was neutralized to pH 7.0 with 10M and then 0.5M NaOH using a pH meter, and diluted to 100ml. Finally, glucosamine was assayed by the colorimetric method described by Tsuji *et al*. (1969) and modified by Ride and Drysdale (1972). 1ml diluted hydrolysate was mixed with 1ml of $NaNO_2$ (5%) and 1ml of $KHSO_4$ (5%) in a centrifuge tube. After shaking occasionally for 15min, it was centrifuged at 3500rpm for 5min; 2ml of supernatant was mixed with 0.67ml of $NH_4SO_3NH_2$ (12.5%) and shaken for 3min. To the mixture was added 0.67ml of 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH; 0.5%, prepared daily), and then the mixture was boiled for 3min and immediately cooled to room temperature; 0.67ml of $FeCl_3$ (0.5%, prepared within 3 days) was added. After standing for 30min, the absorbance at 650nm was measured spectrophotometrically. The glucosamine content was calculated as milligrams per gram of fungal biomass according to the standard curve.

Protein assay

To determine the total protein of fungal cake, micro Kjeldahl method was used (AOAC, 1990). All reagents were of analytical grade, and determinations were conducted in triplicate unless stated otherwise. The total protein of fungal cake was expressed as milligrams per gram of fungal biomass.

RESULTS AND DISCUSSION

Effects of the age of the culture on fungal glucosamine and fungal protein content

Biomass, glucosamine and fungal protein were determined from *Aspergillus oryzae* grown in liquid medium (sucrose and $NaNO_3$) for 24, 48, 72, 96, 120h (Table 1). The fungal growth almost stopped after 48h cultivation and the biomass maintained at a relatively stable value. The glucosamine and fungal protein content of mycelium grown in liquid medium was 115 and 396 millgram per gram dried biomass respectively for 24h, and maintained at a nearly constant in the monitoring time. Scotti *et al*. (2001) and Desgranges *et al*. (1991) have obtained exactly the same results of the glucosamine respectively for *Cunninghamella elegans* and *Beauveria bassiana*. Moreover, the ratio of glucosamine and fungal protein was almost a constant too. It appears that the glucosamine, fungal protein content and the correlation of glucosamine and fungal protein of *Aspergillus oryzae* are constant during all cultivation time.

Effects of the culture conditions on fungal glucosamine and fungal protein content

To compare the culture condition on fungal glucosamine and fungal protein content, the nutrition was the same in submerged and solid state conditions, except containing

Table1. Evolution of glucosamine and fungal protein content during cultivation time.

Items	Cultivation time(hours)				
	24	48	72	96	120
Biomass(g)	0.17±0.02	0.60±0.06	0.65±0.02	0.64±0.06	0.65±0.06
Glucosamine (mg/g)[a]	115.07±2.27	119.98±3.27	116.92±8.37	117.69±3.35	119.29±4.09
Fungal protein (mg/g)[b]	396.18±17.85	391.43±8.34	398.22±6.49	400.92±3.97	398.33±9.61
a[c]	3.44±0.21	3.26±0.13	3.42±0.29	3.41±0.06	3.20±0.13

[a] Expresses in mg glucosamine per gram of biomass dry weight. [b] Expresses in mg fungal protein per gram of biomass dry weight. [c]Represents the correlation of fungal protein and glucosamine (expressed in mg fungal protein per millgram glucosamine). Data represents the means ± SEM, n=3.

Table 2. Effects of carbon source on glucosamine and protein content in submerged and in solid state conditions, with similar nutrients.

Culture conditions	Bactopeptone + carbon source	Glucosamine (mg/g)[a]	Fungal protein (mg/g)[b]	a[c]
Submerged conditions	Glucose	109.84±1.57	394.60±8.22	3.59±0.28
	Sucrose	109.85±12.42	395.39±9.42	3.60±0.07
	Maltose	111.80±2.81	401.09±2.73	3.59±0.07
	Glycerol	111.77±5.90	400.62±6.26	3.59±0.17
	Sodium Acetate	115.53±11.74	393.52±15.90	3.40±0.11
Solid state conditions	Glucose	84.10±3.43	305.03±11.09	3.63±0.27
	Sucrose	85.07±1.31	306.64±9.74	3.60±0.07
	Maltose	85.30±0.18	307.61±15.34	3.61±0.17
	Glycerol	84.32±0.59	296.62±48.25	3.52±0.57
	Sodium Acetate	86.70±2.71	309.25±5.92	3.57±0.10

[a] Expresses in mg glucosamine per gram of biomass dry weight. [b] Expresses in mg fungal protein per gram of biomass dry weight. [c]Represents the correlation of fungal protein and glucosamine (expressed in mg fungal protein per millgram glucosamine). Data represents the means ± SEM, n=3.

Table 3. Effects of nitrogen source on glucosamine and protein content in submerged and in solid state conditions, with similar nutrients.

Culture conditions	Sucrose + nitrogen source	Glucosamine (mg/g)[a]	Fungal protein (mg/g)[b]	a[c]
Submerged conditions	Bactopeptone	109.85±2.42	395.39±1.42	3.60±0.07
	Sodium nitrate	111.11±18.82	392.80±29.64	3.54±0.32
	Ammonium Sulfate	136.44±2.88	336.96±51.00	2.47±0.33
	Urea	135.10±5.74	321.39±36.31	2.39±0.36
Solid state conditions	Bactopeptone	85.07±1.31	306.64±9.74	3.60±0.07
	Sodium nitrate	84.99±1.37	303.75±20.43	3.57±0.24
	Ammonium Sulfate	93.11±5.56	226.30±13.31	2.43±0.13
	Urea	93.21±7.08	233.68±22.77	2.53±0.41

[a] Expresses in mg glucosamine per gram of biomass dry weight. [b] Expresses in mg fungal protein per gram of biomass dry weight. [c]Represents the correlation of fungal protein and glucosamine (expressed in mg fungal protein per millgram glucosamine). Data represents the means ± SEM, n=3.

15g/l agar in solid state condition. As shown in table 2 and table 3, the glucosamine and fungal protein content of mycelium grown in submerged medium were higher than those obtained in solid state conditions on the corresponding medium. But, the correlation of glucosamine and fungal protein were very close between submerged (3.57mg fungal protein per millgram glucosamine) and solid state conditions (3.59mg fungal protein per millgram glucosamine) on the corresponding medium. Contrary to the results obtained by Ride and Drysdale (1971) on *Fusarium oxysporum*, our results permit to deduce that for *Aspergillus oryzae*, the glucosamine content is not influenced by the culture conditions. Scotti *et al.* (2001) reported the same result on *Cunninghamella elegans*.

Effects of the medium composition on fungal glucosamine and fungal protein content

To show the influence of the medium composition on glucosamine and fungal protein content, two experiments were carried out in submerged and solid state conditions.

The results (Table 2) show that the glucosamine, fungal protein content and the ratio of glucosamine and fungal protein were not influenced by carbon sources, whether in submerged or solid state condition. Thus, *Aspergillus oryzae* cultivated on bactopeptone and different carbon sources had the same glucosamine (117.76mg glucosamine per gram of biomass dry weight) and fungal protein content (397.05mg fungal protein per gram of biomass dry weight) in submerged condition, whereas it contained only a mean value of 85.10mg glucosamine per gram of biomass dry weight and 305.03mg fungal protein per gram of biomass dry weight in solid state condition. Moreover, the correlation of glucosamine and fungal protein were almost the same value.

Consequently, within different carbon source, the glucosamine, fungal protein content can be considered as a good indicator of the biomass growth. Furthermore the glucosamine amount can be considered as a good indicator of fungal protein.

The glucosamine, fungal protein content and the ratio of glucosamine and fungal protein, grown on media containing different nitrogen sources and sucrose as carbon sources, were determined. As shown in table 3, the glucosamine, fungal protein content and the ratio of glucosamine and fungal protein varied according to the carbon source.

The results can be divided in two categories in submerged and solid state condition, respectively. Lowest glucosamine content and highest fungal protein content and the ratio of glucosamine and fungal protein values were obtained with bactopeptone and sodium nitrate, with a mean value of 110.48 and 85.03mg glucosamine per gram of biomass dry weight, 394.10 and 305.20mg fungal protein per gram of biomass dry weight and 3.57 and 3.59 mg fungal protein per millgram glucosamine in submerged and solid state condition respectively. Ammonium sulfate and urea media give around 135.77 and 93.16mg glucosamine per gram of biomass dry weight, 329.18 and 230.00mg fungal protein per gram of biomass dry weight and 2.43 and 2.48mg fungal protein per millgram glucosamine in submerged and solid state condition respectively.

Obviously, the glucosamine amount can be considered as a good indicator of the fungal protein within each category of carbon source, whatever the culture conditions may be. Our results can be compared with those results obtained by Ride and Drysdale (1971) concerning *Fusarium oxysporum* and Desgranges *et al*. (1991) with *Beauveria bassiana*. Indeed, glucosamine content of *Beauveria bassiana* varied according to the medium composition, between 43 and 71mg of glucosamine per gram of fungal dry weight. But in this paper, comparison of glucosamine content was done for media having different compositions, as well for carbon and nitrogen sources as concentrations. Moreover, these results also show that glucosamine amount can be very different according to the strain.

CONCLUSION

The results of the experiments show that the glucosamine measurement of *Aspergillus oryzae* can be considered as a reliable indirect method for estimating fungal protein. In fact, the correlation of glucosamine and fungal protein content is constant whatever the age of the cultivation and culture conditions may be. On the other hand, the correlation appears to depend on the medium composition. Indeed, it was affected by the nitrogen source, but not by the carbon source. However, when the medium is well defined, this study demonstrates that the established results permit a good estimation of the fungal protein.

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