The Influence of Culture Conditions on Fungal Pellet Formation by Submerged Fermentation of *Cordyceps sinensis* (*Paecilomyces hepiali*) - Cs 4

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Abstract: The main aim of this study was to establish the optimal submerged culture conditions of a strain belonging to *Cordyceps sinensis (Paecilomyces hepiali)* - Cs 4. The effects of culture media composition (carbon, nitrogen and mineral sources) as well as other important physical and chemical factors that could affect the submerged fermentation (such as pH levels, temperature, agitation speed) on mycelia pellet formation and fungal biomass production were investigated. The morphological features of mycelia were comparatively analyzed using digital microscopic images by which the roughness and compactness of the fungal pellets, as well as their total number, were used as the best culture indicators of the optimal submerged fermentation procedure.

Key words: *Cordyceps sinensis, Paecilomyces hepiali,* culture conditions, fungal pellets formation, biomass production, submerged fermentation

1 Introduction

Cordyceps sinensis (Berk.) Sacc. Link is a famous mushroom species from the Family Claviceptaceae; (Ascomycetes), having the following common names: *Cordyceps,* caterpillar fungus, dong zhong chang cao, dongchongxiacao (China), semitake (Japan). At the same time, inside its natural habitats, *Cordyceps sinensis* is parasitic on the larvae of moths, especially bat moths *(Hepialis species).* More recently, *C. sinensis (Paecilomyces hepiali)* Cs-4, which belongs to the Ascomycetes, has been cultivated *in vitro* by submerged fermentations in order to produce fungal pellets which contain exo-polysaccharides for medicinal purposes due to their beneficial physiological activities upon human health.[1] Although many investigators have attempted to find out the optimal conditions for submerged fermentations in order to increase the fungal pellets formation as well as fungal biomass production from several fungi, to the best of our knowledge the nutritional requirements and environmental conditions for submerged culture of *C. sinensis (P. hepiali)* Cs-4 have not yet been demonstrated.

2 Materials and Methods

2.1 Micro-organisms and culture media

C. sinensis (P. hepiali) Cs-4 was used as a pure strain from the culture collection of Hangzhou Bioer Technology, Co. The stock cultures were maintained on potato-dextrose agar (PDA) slants. Slants were incubated at 25°C for 5-7 days and then stored at 4°C. The seed culture was grown in a 250-ml flask containing 100 ml of PMP medium (2% potato-dextrose broth, 1% malt extract, 0.1% peptone) at 23°C on rotary shaker incubators operated at 150 rev min^{-1} for 7-12 days.

2.2 Inoculum preparation

C. sinensis Cs-4 was initially grown on PDA medium in Petri dishes, and then transferred to the seed culture medium by punching out 5 mm of the agar plate culture with a sterilized cutter. The seed culture was grown in a 250-ml flask containing 100 ml of the nutritive culture medium of PDB medium (2% potato-dextrose broth) at 25°C on a rotary shaker incubator (150 rev min^{-1} for 5-7 days). The flask culture experiments were performed in a 250-ml flask containing 100 ml of culture media after inoculating with 5% (v/v) of the seed culture.

2.3 Experimental conditions

The fermentation was achieved by inoculating 100 ml of culture medium with 3-5% (v/v) of the seed culture and then cultivated at 23-25°C in rotary shake flasks of 250 ml. The experiments of fermentations were conducted under the following conditions: temperature, 25°C; agitation speed, 120-180 rev min^{-1}; initial pH, 4.5-5.5. The seed culture was transferred to the fermentation medium and cultivated for 7-12 d. All experiments were performed at least in triplicate.

Samples collected at various intervals from shake flasks were centrifuged at 12000 g for 15 min, and the resulting supernatant was filtered through a membrane filter (Millipore, 0.45 μm). Dry weight of mycelium was measured after repeated washing of the mycelial pellet with distilled water and drying at 70°C overnight to a constant weight.

3 Results and Discussion

3.1 Effect of carbon source

In order to find a suitable carbon source for the mycelia growth and consequently for fungal pellets formation the cells of *C. sinensis* Cs-4 were cultivated for 7-12 days in different nutritive culture media containing various carbon sources, and each carbon source was added to the basal medium at a concentration level of 1.5% (w/v). When the cells were grown in the sucrose medium, both the mycelia pellets formation and fungal biomass production were the highest among those tested (Table 1). Maltose also significantly increased mycelia growth and fungal pellet formation.

Table 1. Effect of carbon source on mycelia growth of *C.sinensis* in shake cultures

Carbon source (1.5%, w/v)	Biomass (dry wt, g/l)	No. of fungal pellets per flask	Final pH
Glucose	6.46±0.10	41±0.05	5.5
Maltose	5.21±0.15	35±0.12	5.8
Sucrose	7.28±0.35	55±0.03	5.1
Xylose	4.95±0.28	28±0.07	5.3

Cultures were grown for 12 days at 25°C and an initial pH of 5.5. Data are the means± S. D. of triplicate determinations.

3.2 Effect of nitrogen source

To investigate the effect of nitrogen sources on mycelia growth and fungal pellet production, cells were cultivated in media containing various nitrogen sources, where each nitrogen source was added to the basal medium at a concentration level of 10 g/l. Among five nitrogen sources examined, rice bran was the most efficient for mycelia growth and fungal pellets formation (Table 2). At the same time, malt extract was one of the best nitrogen sources for a high mycelia growth and fungal pellet formation. Peptone, tryptone and yeast extract are also known as efficient nitrogen sources for fungal pellet formation and exo-polysaccharides production by using liquid cultures of entomopathogenic fungi.[2] In comparison to organic nitrogen sources, inorganic nitrogen sources gave rise to relatively lower mycelia growth and exo-biopolymer production.[3]

3.3 Effect of mineral source

The influence of various mineral sources on mycelia pellet formation and fungal biomass production was examined at a standard concentration level of 5 mg. Among the various mineral sources examined, K_2HPO_4 yielded good mycelia growth as well as fungal biomass production and for this reason it was recognized as a favourable mineral source (Table 3). Similar observations were made by Xiao *et al.*[2] during experiments involving other mushroom fermentations. K_2HPO_4 could improve productivity through its buffering action, and essential phosphates were favourable for mycelia growth in submerged cultures of mushrooms[4, 5]

Table 2. Effect of nitrogen source on mycelia growth of *C.sinensis* in shake cultures

Nitrogen source (1.0%, w/v)	Biomass (dry wt, g/l)	No. of fungal pellets per flask	Final pH
Rice bran	6.47±0.14	57±0.05	5.5
Malt extract	6.41±0.23	55±0.03	5.3
Peptone	4.45±0.15	41±0.12	4.6
Tryptone	5.23±0.09	28±0.7	5.1
Yeast extract	5.83±0.35	30±0.01	4.3

Cultures were grown for 12 days at 25°C and an initial pH of 5.5. Data are the means ± S.D. of triplicate determinations.

Table 3. Effect of mineral sources on mycelia growth of *C.sinensis* in shake cultures

Mineral source (5 mg)	Biomass (dry wt, g/l)	No. of fungal pellets per flask	Final pH
KH_2PO_4	5.71±0.09	45±0.07	5.5
K_2HPO_4	6.98±0.13	57±0.05	5.1
$MgSO_4 \cdot 5H_2O$	6.18±0.20	55±0.09	56

Cultures were grown for 6 days at 25°C and an initial pH of 5.5. Data are the means± S. D. of triplicate determinations.

3.4 Effects of initial pH and temperature

To investigate the effects of initial pH upon mycelia growth and fungal pellet formation, *C. sinensis,* respectively, *P. hepiali* Cs-4 was cultivated in PDB medium at different initial pH values (4.5-6.0) in shake flasks. The optimal pH levels for mycelia growth and fungal pellets production was 5.5 (Table 4). In order to find the optimal incubation temperature for mycelia growth, the fungus was grown at different temperatures ranging from 20-25°C, and, finally, the optimum of temperature was found at 23°C, being correlated with the appropriate pH level 5.5 (Table 4).

Table 4. Effect of initial pH and temperature on mycelia growth of *C.sinensis* in shake cultures

Initial pH	Initial temp (t°)	Biomass (dry wt, g/l)	No. of fungal pellets per flask
4.5	18	1.90±0.10	12±0.02
5.0	21	2.55±0.05	20±0.14
5.5	23	3.49±0.15	35±0.23
6.0	26	3.37±0.12	30±0.03
6.5	29	2.05±0.23	25±0.15

Cultures were grown for 6 days at 25°C and an initial pH of 5.5. Data are the means± S. D. of triplicate determinations.

3.5 Effect of inoculum age and inoculum size

Amongst several fungal physiological properties, the age and size of the mycelial inoculum may play an important role in fungal pellets development.[6] To examine the effect of inoculum age and inoculum volume, *C. sinensis* Cs-4 was grown on PDA slants for three different time periods (3-12 d) varying the inoculum size (2-10%). As it is shown in Tables 5-6, the inoculum age as well as the inoculum size appeared to have some effects on the mycelial growth and fungal biomass production.

Table 5. Effect of inoculum age on mycelia growth of *C. sinensis* in shake cultures

Inoculum age (h)	Biomass (dry wt, g/l)	No. of fungal pellets per flask
288	2.28±0.15	15±0.01
264	3.79±0.12	25±0.05
240	4.15±0.10	30±0.23
216	4.73±0.12	35±0.90
192	5.35±0.23	41±0.05
168	6.23±0.37	45±0.78
144	7.50±0.20	48±0.03
120	6.20±0.15	43±0.05
96	4.73±0.09	35±0.15
72	2.55±0.05	25±0.23

Cultures were grown for 12 days at 25°C and an initial pH of 5.5. Data are the means ± S.D. of triplicate determinations.

Table 6. Effect of inoculum size on mycelia growth of *C. sinensis* in shake cultures

Inoculum size (%)	Biomass (dry wt, g/l)	No. of fungal pellets per flask
12	3.28±0.15	28±0.23
8	3.49±0.12	35±0.03
4	5.53±0.15	47±0.07
2	2.95±0.23	25±0.15

Cultures were grown for 12 days at 25°C and an initial pH of 5.5. Data are the means ± S.D. of triplicate determinations.

3.6 Morphological changes in mycelia during fungal pellet formation

Morphological features of mycelia were comparatively analyzed using digital microscopic images by which the roughness and compactness of the fungal pellets as well as their total number were used as biomarkers of submerged fermentation (see Figs 1-6).

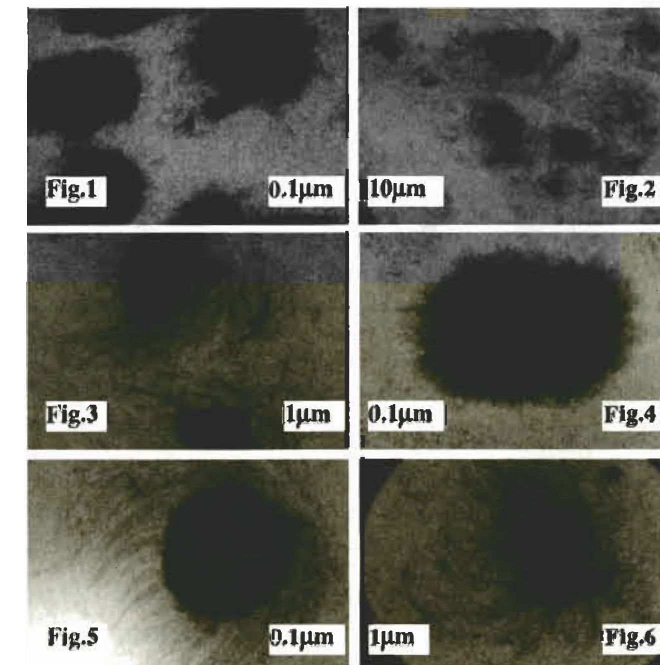

Fig 1-6. Morphological changes in *C. sinensis* Cs-4 mycelia, during growth in submerged culture

Pellet morphology was characterized by the shape and aspect of pellets as well as their dimensions, including circularity parameter, area covered by each pellet, hairiness, roughness and compactness.[7] In each figure, the magnification is shown by a bar marker. The typical morphological changes could be noticed in Figures 1-6, during the whole submerged culture cycle. In this respect, after the second day of fermentation (Figs 1-2) the pellet diameter as well as the pellet surface area increased (Fig. 3), during the specific fungal growth phase and reached a maximum on day 5 and 6 (Fig. 4), when the hairiness declined. After this period of time, the roughness and hairiness of the fungal pellets increased over the five or six days of submerged fermentation (Fig. 5) and between day 8 and day 12 of the whole culture cycle for fungal pellet formation (Fig. 6) the pellet shape as well as the roughness and hairiness of pellet surface showed a maximum. After this period of time, the fungal pellets rapidly broke up into hyphal fragments and flocks, which caused a decrease in broth viscosity as the fungal cells reached the stationary phase and changed their morphology.

4 Conclusions

Taking into consideration that most submerged fermentations of medicinal and entomopathogenic fungi such as *C. sinensis (P. hepiali)* Cs-4 require a specific micro-environment including complex nutrients and relatively long incubation periods, the influence of all physical and chemical factors upon mycelia pellets formation and fungal biomass production has to be optimised by applying the most efficient submerged fermentation. The best applied procedure in order to increase the fungal pellets formation as well as biomass production is that one

to optimise the correlation between the number of fungal pellets and their morphology, especially in the growth phase of each fungal submerged fermentation. The morphological changes of fungal cells are significant biomarkers of the whole submerged fermentation that could be used to increase the efficiency of mycelial pellet formation.

Acknowledgements

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