J Ind Microbiol Biotechnol (2003) 30: 589–592
DOI 10.1007/s10295-003-0085-6

ORIGINAL PAPER

Jian-Rong Han · Jing-Ming Yuan

Influence of inocula and grains on sclerotia biomass and carotenoid yield of *Penicillium* sp. PT95 during solid-state fermentation

Received: 24 January 2002 / Accepted: 29 July 2003 / Published online: 3 September 2003

Abstract Various inocula and grains were evaluated for carotenoid production by solid-state fermentation using *Penicillium* sp. PT95. Millet medium was more effective in both sclerotia growth and carotenoid production than other grain media. An inoculum in the form of sclerotia yielded higher sclerotia biomass compared to either a spore inoculum or a mycelial pellet inoculum. Adding wheat bran to grain medium favored the formation of sclerotia. However, neither the inoculum type nor addition of wheat bran resulted in a significant change in the carotenoid content of sclerotia. Among grain media supplemented with wheat bran (wheat bran:grain = 1:4 w/w, dry basis), a medium consisting of rice and wheat bran gave the highest sclerotia biomass (15.10 g/100 g grain), a medium consisting of buckwheat and wheat bran gave the highest content of carotenoid in sclerotia (0.826 mg/g dry sclerotia), and a medium consisting of millet and wheat bran gave the highest carotenoid yield (11.457 mg/100 g grain).

Keywords *Penicillium* sp. · Grains · Sclerotia · Carotenoid · Solid-state fermentation

Introduction

To date, almost all the industrial production of carotenoids using filamentous fungi and yeasts has involved the use of liquid-state fermentation (LSF) [13]. The use of solid-state fermentation (SSF) for carotenoid production is still in the exploratory stage [4]. It is necessary to isolate a strain suitable for use in SSF. Many well-known carotenoid-accumulating microorganisms, such as *Blakeslea trispora*, *Phycomyces blakesleeanus*, and *Phaffia rhodozyma*, are not suitable for SSF due to the difficulty of separating their mycelia or cells from the fermented substrate, making it difficult to collect mycelia or cells for pigment extraction.

We isolated a strain of *Penicillium* sp. PT95 from a soil sample. This strain can form abundant orange, sand-shaped sclerotia (ca. 300 μm in diameter) in which carotenoid is accumulated. Because the sclerotia can be separated easily from their solid medium, strain PT95 is suitable for SSF for carotenoid production. In our previous work [5], we studied the culture conditions influencing sclerotial growth and carotenoid production of strain PT95, demonstrating that the strain could form sclerotia only under SSF conditions and not in submerged culture. Accordingly, SSF is currently the only method available for carotenoid production with strain PT95. In nature, organisms such as species of *Penicillium* and *Aspergillium* typically grow on solid amylaceous substrates such as various grain seeds [7]. We found that corn meal used as a fermentation substrate was more efficient than wheat bran for selective sclerotia growth and carotenoid production of strain PT95 [4]. However, both the sclerotia biomass and pigment yield of PT95 on corn meal medium were too low to be economically practical. The question arose whether the use of grains other than corn, would result in a significant increase in both the sclerotia biomass and carotenoid yield. We also noticed that an old slant culture of the strain consisted of three components: mass conidia, mycelia and a few sclerotia. If one of these were selected as inoculum, what is the result in forming sclerotia? In order to answer these questions, we conducted a series of experiments on the influence of inoculum type, and grain (i.e. corn, rice, wheat, sorghum, buckwheat, millet, broomcorn millet) on the sclerotia biomass and carotenoid yield of strain PT95 during SSF.

J.-R. Han (✉) · J.-M. Yuan
Institute of Biotechnology, Shanxi University,
030006, Taiyuan , People's Republic of China
E-mail: hjr@sxu.edu.cn
Fax: +86-351-7011981

J.-R.Han
Department of Life Science, Shanxi University,
030006, Taiyuan , People's Republic of China

Materials and methods

Microorganisms

Strain PT95 was isolated from soil collected close to Fenyang, Shanxi Province, and identified as a species of the *Penicillium thomii* series described by Raper and Thom [10]. See Han et al. [6] for additional details and a complete bibliography. Strain PT95 was routinely maintained on Czapek's agar slants [9].

Inocula preparation

Strain PT95 was cultured on Czapek's agar slants in the dark at 25°C for 7 days. Spores were washed from slant cultures with sterile water and a suspension of about 10^8 spores/ml was prepared as inoculum. The mycelial pellets obtained by growing mycelium cultures in 250 ml shaken flasks containing 100 ml Czapek's liquid medium (25°C, 125 rpm, 7 days) were homogenized aseptically in a Sorvall Omnimixer for 3 min in an ice bath. The mycelial pellet suspension (5.0 mg/ml) was used for inoculation. The sclerotia as inoculum were obtained from 20-day-old Czapek's agar plate cultures of the strain. To purify sclerotia, the plate cultures were centrifuged (4,000 *g*, 5 min), rinsed aseptically five times with sterile water to wash away spores. The sclerotia were then homogenized aseptically in a Sorvall Omnimixer for 10 min in an ice bath, and the suspension (5.0 mg/ml) was used as inoculum.

Solid-state fermentation

Grains tested in this work (i.e. corn, rice, wheat, sorghum, buckwheat, millet or broomcorn millet) were obtained from the Cereal Quality Laboratory, Shanxi University. The grains were dried at 60°C and then ground to pass through a 2-mm sieve prior to use. The basal medium for SSF consisted of 25 g rice, or other grain, moistened with 20 ml nutrient salt solution that contained the following (per liter of distilled water): $NaNO_3$, 3 g; maltose (Sigma, St. Louis, Mo.), 10 g; soybean oil (Sigma), 2.5 g; K_2HPO_4, 1 g; KCl, 0.5 g; $MgSO_4{\cdot}7H_2O$, 0.5 g; $FeSO_4$, 0.01 g. The initial pH of the salt solution was adjusted to 6.0. The wheat bran added to the basal medium is described below. Grain (25 g) and nutrient salt solution (20 ml) were then each dispensed into 250 ml flasks. After sterilization (121°C, 30 min), 2 ml inoculum was spread aseptically on the surface of the substrate in each flask. Flasks were incubated in the dark at 25°C for 20 days.

Analytical methods

Sclerotia were separated from fermented medium by a procedure described by Han and Xu [5], washed thoroughly with distilled water, and lyophilized. The dried sclerotia were weighed before carotenoid analysis. Pigment extraction and determination were performed as described by Wang and Li [14]. The proportion of β-carotene in the total amount of carotenoid was determined by thin layer chromatography (TLC) and electron absorption spectra [2].

Experimentation and analysis

All experiments were replicated in three flasks and the data are presented as the arithmetic mean ± standard error. Duncan's multiple range test [11] was used to determine significant differences among means at the 1% level of confidence.

Results

Effect of inocula on sclerotia biomass and the content of carotenoid in sclerotia

Regardless of the grain medium used to grow strain PT95, inoculum in the form of sclerotia resulted in a significant increase in sclerotia biomass compared to other two types of inocula ($P < 0.01$), with mycelial pellet inoculum yielding the lowest sclerotia biomass (Table 1). In contrast, no significant differences were found in the carotenoid content of sclerotia among treatments with the three inocula (data not shown). In order to test the effect of inoculum level on sclerotia biomass yield and the amount of carotenoid in sclerotia, the flasks of each grain medium were inoculated respectively with 2 ml of four different suspensions of sclerotium inoculum (i.e. 2.5, 5.0, 7.5 and 10.0 mg/ml), giving culture inoculum levels of 5 mg, 10 mg, 15 mg and 20 mg per flask, respectively. The results (Table 2) showed that, regardless of the medium, sclerotia biomass was significantly affected by the inoculum level. Plotting sclerotia biomass versus amount of inoculum revealed a positive linear relationship: the higher the amount of inoculum, the higher the sclerotia biomass. The sclerotia biomass harvested from the millet medium inoculated with 20 mg sclerotium inoculum per flask reached a maximum of 12.3 mg/100 g grain. However, inoculum level did not significantly affect the content of carotenoid in sclerotia (data not shown).

Inoculum in the form of sclerotia gave the best results of the three inocula tested, and an inoculum level of 20 mg per flask caused a significant increase in sclerotia biomass. Therefore, in subsequent experiments, we used 20 mg pure sclerotia per flask as inoculum.

Table 1 Effect of inocula on sclerotia biomass of strain PT95 grown on various grain media. Values are given as mean ± standard error

Inoculum type	Dry sclerotia weight (g/100 g grain)						
	Corn	Rice	Wheat	Sorghum	Buckwheat	Millet	Broomcorn millet
Sclerotia	$11.52 \pm 0.92^{c*}$	11.30 ± 0.90^{c}	6.05 ± 0.42^{c}	9.43 ± 0.67^{c}	8.50 ± 0.68^{c}	11.24 ± 0.90^{c}	10.30 ± 0.93^{c}
Spores	9.70 ± 0.78^{b}	9.75 ± 0.71^{b}	4.97 ± 0.39^{b}	7.92 ± 0.71^{b}	6.62 ± 0.56^{b}	9.05 ± 0.71^{ab}	8.19 ± 0.65^{ab}
Mycelial pellets	8.20 ± 0.66^{a}	7.95 ± 0.64^{a}	3.54 ± 0.28^{a}	6.50 ± 0.45^{a}	5.20 ± 0.37^{a}	8.00 ± 0.64^{a}	7.05 ± 0.56^{a}

*Superscript letters within a column indicate that values followed by the same letter did not differ significantly ($P < 0.01$) in Duncan's multiple range test

Table 2 Effect of inoculum level for cultures on sclerotia biomass of strain PT95 grown on various grain media

Inoculum level (mg)[c]	Dry sclerotia weight (g/100 g grain)						
	Corn	Rice	Wheat	Sorghum	Buckwheat	Millet	Broomcorn millet
5	11.03 ± 0.88^{a}*	10.60 ± 0.84^{a}	5.30 ± 0.37^{a}	8.85 ± 0.63^{a}	7.90 ± 0.63^{a}	10.70 ± 0.85^{a}	9.60 ± 0.86^{a}
10	11.52 ± 0.92^{a}	11.30 ± 0.90^{a}	6.05 ± 0.42^{a}	9.43 ± 0.67^{a}	8.50 ± 0.68^{a}	11.24 ± 0.90^{a}	10.30 ± 0.93^{a}
15	11.87 ± 0.95^{a}	11.73 ± 0.84^{ab}	6.62 ± 0.52^{ab}	10.05 ± 0.90^{ab}	8.95 ± 0.75^{ab}	11.85 ± 0.93^{ab}	10.95 ± 0.86^{ab}
20	12.27 ± 0.98^{ab}	12.20 ± 0.97^{ab}	6.95 ± 0.55^{ab}	10.76 ± 0.75^{b}	9.40 ± 0.66^{b}	12.30 ± 0.98^{b}	11.45 ± 0.91^{b}

*Superscript letters within a column indicate that values followed by the same letter did not differ significantly ($P < 0.01$) in Duncan's multiple range test

[c]Amount of sclerotium inoculum inoculated in each flask

Table 3 Effect of grains on sclerotia biomass and carotenoid yield. Data in parentheses were obtained from medium consisting of wheat bran plus grain (wheat bran/grain = 5 g/20 g)

Grain	Dry sclerotia weight (g/100 g grain)	Carotenoid content (mg/g dry sclerotia)	β-Carotene fraction of total pigment (%)	Carotenoid yield (mg/100 g grain)[d]	β-Carotene yield (mg/100 g grain)
Corn	12.27^{c}* (15.02)	0.542^{a} (0.518)	71^{a} (71)	6.650^{b} (7.780)	4.722^{b} (5.524)
Rice	12.20^{c} (15.10)	0.540^{a} (0.547)	70^{a} (70)	6.588^{b} (8.260)	4.612^{b} (5.782)
Wheat	6.95^{a} (9.12)	0.709^{b} (0.694)	69^{a} (69)	4.928^{a} (6.329)	3.400^{a} (4.367)
Sorghum	10.76^{b} (13.15)	0.687^{b} (0.644)	69^{a} (69)	7.392^{c} (8.469)	5.100^{b} (5.844)
Buckwheat	9.40^{b} (11.02)	0.818^{c} (0.826)	68^{a} (68)	7.689^{d} (9.103)	5.229^{bc} (6.190)
Millet	12.30^{c} (14.86)	0.803^{c} (0.771)	70^{a} (70)	9.877^{e} (11.457)	6.914^{c} (8.020)
Broomcorn millet	11.45^{bc} (13.32)	0.585^{a} (0.570)	70^{a} (70)	6.698^{b} (7.592)	4.689^{b} (5.314)

*Superscript letters within a column indicate that values followed by the same letter did not differ significantly ($P < 0.01$) in Duncan's multiple range test

[d]Determined as dry sclerotia weight × content of carotenoid in sclerotia

Effect of grains on sclerotia biomass and carotenoid yield

Both the sclerotia biomass and the amount of carotenoid accumulated in sclerotia were strongly dependent on the grain medium used (Table 3). Of the grains tested, millet gave the highest sclerotia biomass, followed by corn, rice, broomcorn millet, sorghum, buckwheat and wheat. Buckwheat was more favorable to carotenogenesis than other grains, and gave the highest carotenoid content, followed by millet, wheat, sorghum, broomcorn millet, corn and rice. Since the total amount of carotenoid in each flask culture was affected by both the sclerotia biomass and the content of carotenoids in sclerotia, we tested the effect of these grains on total carotenoid yields. Wheat gave the lowest pigment yield of 4.928 mg/100 g grain. Millet, which had pronounced effects not only on sclerotia biomass, but also on carotenoid content, gave the highest pigment yield of 9.877 mg/100 g grain, which was twice that achieved on wheat.

In our previous experiments [4], TLC revealed two major pigments. β-Carotene amounted to 71% of the total carotenoids. Although different grains had different effects on the proportion of β-carotene in the total carotenoid, these differences were not significant (Table 3).

Effect of adding wheat bran to grain media

Substituting 5 g wheat bran for 5 g grain in the grain medium (ratio wheat bran:grain 1:4; w/w, dry basis) resulted in a significant increase in the sclerotia biomass compared to the control without wheat bran ($P < 0.01$), but did not result in a significant increase in either the carotenoid content or the proportion of β-carotene (Table 3). After supplementation with wheat bran, rice gave the highest sclerotia biomass (15.10 g/100 g grain), followed by corn, millet, broomcorn millet, sorghum, buckwheat and wheat; buckwheat gave the highest carotenoid content (0.826 mg/g dry sclerotia), followed by millet, wheat, sorghum, broomcorn millet, rice and corn; millet gave the highest carotenoid and β-carotene yields of all media tested (11.457 mg and 8.020 mg/100 g grain, respectively).

Discussion

One conclusion from these results is that the sclerotia inoculum can result in a significant increase in sclerotia biomass compared to either the spore inoculum or the mycelial pellet inoculum. The question arises as to how the sclerotium inoculum can produce such an effect. At this point no satisfactory explanation can be offered. Perhaps the sclerotium inoculum contains substances inducing morphological differentiation of strain PT95, whereas such substances are less abundant in spore inoculum or in mycelial pellet inoculum. Further study may be required to test this hypothesis.

Generally, oxygen penetration into solid medium is one of the main limitations of SSF [1]. Therefore, it is common practice to supply oxygen to the medium during SSF. In this study, oxygen limitation may have been

partially overcome by adding wheat bran to the medium, by increasing the gaps between grain particles and increasing oxygen penetration into the medium, which apparently resulted in the formation of more sclerotia than in the control. No attempt was made to determine the optimal ratio of wheat bran to grain at which the highest sclerotia biomass could be obtained.

SSF, unlike LSF, requires no complex fermentation controls, has the advantages of superior productivity [12], simpler techniques, reduced energy requirements, low wastewater output, and improved product recovery, all of which makes its application of potential interest. For example, SSF has been used for enzyme production and upgrading the values of existing foods, especially oriental foods [3]. Although some strains of *P. blakesleeanus* grown on agar were more carotenogenic than those grown in liquid culture [8], studies on their SSF for carotenoid production in amylaceous substrates, such as cornmeal and other grains, have not been reported. Thus, SSF of strain PT95 for carotenoid production represents an original approach. Due to the inability to form sclerotium and synthesize carotenoids in liquid medium [6], it is impossible to compare pigment yields of strain PT95 under SSF conditions with that under LSF conditions. However, it is certain that the maximal content of β-carotene (0.56 mg/g dry sclerotia) obtained in this work is much lower than the maximal content of β-carotene (33 mg/g dry mycelium) produced by some carotene-overproducing strains of *P. blakesleeanus* under LSF conditions [8]. Therefore, further research needs to be done to increase both the sclerotia biomass and the pigment content of strain PT95 under SSF conditions.

Acknowledgements Support for this research by the Chinese National Natural Science Fund (Grant No. 30070021) and the Shanxi Province Science Foundation (No.20001082) is gratefully acknowledged. We thank Prof. Y.H. Duan for valuable comments on the manuscript.

References

1. Abdullah AL, Tengerdy RP, Murphy VG (1985) Optimization of solid substrate fermentation of wheat straw. Biotechnol Bioeng 27:20–27
2. An GH, Schuman DB, Johnson EA (1989) Isolation of *Phaffia rhodozyma* mutants with increased astaxanthin content. Appl Environ Microbiol 55:116–124
3. Chahal DS (1983) Protein production and growth characteristics of *Chaetomium cellulolyticum* during solid-state fermentation of corn stover. Mycologia 75:597–603
4. Han JR (1998) Sclerotia growth and carotenoid production of *Penicillium* sp. PT95 during solid-state fermentation of corn meal. Biotechnol Lett 20:1063–1065
5. Han JR, Xu J (1999) Solid-state fermentation with *Penicillium* sp. PT95 for carotenoid production. Acta Microbiol Sinica 39:148–153
6. Han JR, Wang XJ, Yuan XE (1998) Studies on the production of carotenoids in sclerotia of PT95 strain of *Penicillium*. Microbiology 25:319–321
7. Hesseltine CW (1977) Solid-state fermentations—part 1. Process Biochem 12:24–27
8. Mehta BJ, Salgado LM, Bejarano ER, Cerdá-Olmedo E (1997) New mutants of *Phycomyces blakesleeanus* for β-carotene production. Appl Environ Microbiol 63:3657–3661
9. Pitt JI (1973) An appraisal of identification methods for *Penicillium* species: novel taxonomic criteria based on temperature and water relations. Mycologia 65:1135–1157
10. Raper KB, Thom C (1949) A manual of the *Penicillia*. Williams and Wilkins, Baltimore
11. Ray AA (1985) SAS users guide: statistics. Cary, N.C.
12. Tengerdy RP (1985) Solid substrate fermentation. Trends Biotechnol 3:96–99
13. Vandamme EJ (1992) Production of vitamins, coenzymes and related biochemicals by biotechnological processes. J Chem Technol Biotechnol 53:313–327
14. Wang YQ, Li QS (1997) Advances in natural carotenoids. Chinese Medicinal Science and Technology Press, Beijing