Lovastatin Production by *Pleurotus ostreatus*: Effects of the C:N Ratio

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The types of carbon source and nitrogen source used as well as the C:N ratio in the medium influenced lovastatin production by *Pleurotus ostreatus.* The maximum value of the lovastatin yield was obtained in a medium that contained organic nitrogen.

Key words: Pleurotus ostreatus, Statins, Lovastatin

Introduction

In fungi, the biosynthesis of a secondary metabolite with a complex chemical structure is performed through the polyketide route (Pfeifer and Khosla, 2001). Lovastatin is produced as a secondary metabolite by the fungi *Aspergillus terreus*, *Monascus ruber*, *Penicillium brevicompactum* and *Pleurotus ostreatus.* Lovastatin ($C_{24}H_{36}O_5$, mevicolin, monacolin K) is a potent drug for lowering blood cholesterol. It acts by competitively inhibiting the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA) (Endo, 1992; Bobek *et al.*, 1997).

As with any fermentation product, the culture medium has a significant influence on the lovastatin yield and its rate of production. Therefore, selection and composition optimization of a suitable medium is important when establishing a process for lovastatin production. Of the principal culture nutrients, carbon and nitrogen sources generally play a dominant role in fermentation productivity because these nutrients are directly linked with biomass and metabolite formation.

In previous studies (Alarcón *et al.*, 2003), lovastatin was obtained from cultures in the middle liquid using Hagen medium from a native and commercial strain of *P. ostreatu*s. In the present study, the effect of the C:N ratio in the lovastatin biosynthesis was studied using the native strain PLUBB-127 of *P. ostreatus.*

Materials and Methods

Organism collection

Fruiting bodies of *P. ostreatus* were collected from forests in the VIII Región of Chile, growing on *Nothofagus* sp. during autumn and spring 2001. Mycelial cultures of the strain were derived from the spore print of the fruiting body. A voucher specimen of the mushroom was deposited in the herbarium of the Departamento de Ciencias Básicas de la Universidad del Bío Bío, Chillán, Chile.

Fungal strain and culture conditions

The strains of *P. ostreatus* (PLUBB-127) were kept on potato dextrose agar (PDA), and incubated for 7–10 d, and then stored at room temperature. Fermentation was carried out in Hagen medium containing the following (per liter of distilled water): 0.05 g $CaCl_2 \cdot 2H_2O$ (Merck), 0.025 g KH_2PO_4 (Merck), 0.25 g $(NH_4)_2HPO_4$ (Merck), 0.15 g $MgSO_4 \cdot 7H_2O$ (Merck), 1.3 ml $FeCl_3$ 1% (Merck), 3.0 g malt extract (Merck) and 10 g glucose (Merck). In a 500 ml Erlenmeyer flask containing 250 ml of medium with aeration and agitation (150 rpm), the fermentation was performed; 125 ml of well-grown culture (7 d) in the same medium were used as inoculum. The fermentation was stopped after 10 and 30 d. The pH value of the medium was adjusted to 6.5 with HCl (2 M) or KOH (2 M). Eleven culture media were used. Table I indicates the composition of the chemically defined fermentation media developed in this study.

Statins isolation

Culture filtrate (250 ml) obtained by filtration was acidified to pH 3 with 0.01 M HCl and extracted with ethylacetate (3 × 100 ml). The combined extracts were dried (Na_2SO_4) and concentrated to a final volume of 5 ml.

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Table I. Medium composition for screening of C- and N-sources.

	Experimental media											
Component	Hagen	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11
$CaCl_2 \cdot 2H_2O$	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06
KH_2PO_4	0.025	0.025	0.025	0	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025
$(NH_4)_2HPO_4$	0.25	0.25	0.25	0	0.25	0.25	0.25	0.25	0.25	4.12	0	0
NH_4Cl	0	0	0	0	0	0	0	0	0	0	3.21	0
$NaNO_3$	0	0	0	0	0	0	0	0	0	0	0	5.41
$MgSO_4 \cdot 7H_2O$	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
$FeCl_3$ (1%)	1.2	1.2	1.2	0	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2
Malt extract	3	13	3	10	13	13	13	13	13	0	0	0
Glucose	10	10	10	4	10	10	10	10	10	10	10	10
Yeast extract	0	0	4	4	4	4	0	4	0	4	4	4
Peptone water	0	0	0	0	0	4	4	8	8	0	0	0

The mycelial mass was washed with 0.05 M HCl and stirred at room temperature for 1 h, then filtered, and after acidification extracted with methylene chloride (3 × 100 ml) and ethylacetate (2 × 200 ml) for 1 h at 40 °C under stirring. The extract was dried (Na_2SO_4) and concentrated to a final volume of 5 ml.

The lovastatin structure was established based on the spectroscopic and GC-MS studies, and the spectral data was compared to data from the literature or from authentic samples.

Analytical determinations

Lovastatin identification and quantification were performed on the filtrated culture and extract by HPLC, using a Merck LiChrospher 100 RP18 reverse phase column with a diode array detector eluted at the flow rate 2 ml/min. Solvent A was 0.05% H_3PO_4 in water, and solvent B was acetonitrile. The separation gradient was linear, starting with 95% solvent A and 5% solvent B, reaching 50% solvent A and 50% solvent B in 45 min, 30% solvent A and 70% solvent B in 46 min, 10% solvent A and 90% solvent B in 48 min, 0% solvent A and 100% solvent B in 60 min, and finally continuing with an isocratic run for 5 min. Initial conditions were maintained for 6 min to re-equilibrate the column.

Results and Discussion

In mushrooms, the biosynthesis of secondary metabolites is subject to complex regulation. This

Table II. Media compositions for screening of C- and N-sources.

Experimental run	C-source concentration [g/l]	Medium [g C/l]	N-source concentration [g/l]	Medium [g N/l]	C:N ratio	Lovastatin content* (mg%)
Hagen	13.00	4.96	0.25	0.24	20.67	0.415 ± 0.062
M1	23.00	8.16	0.25	0.87	9.43	4.817 ± 0.356
M2	17.00	5.12	4.25	2.84	1.80	9.591 ± 0.815
M3	18.00	3.36	4.00	3.23	1.04	13.433 ± 2.01
M4	27.00	8.32	4.00	3.47	2.40	14.975 ± 1.24
M5	27.00	8.32	8.00	3.95	2.11	7.517 ± 0.761
M6	23.00	8.16	4.00	1.35	6.07	25.497 ± 1.842
M7	35.00	8.32	12.00	4.43	1.88	21.809 ± 1.912
M8	23.00	8.32	8.25	4.43	1.88	11.893 ± 1.643
M9	14.00	4.16	4.12	3.47	1.20	3.305 ± 0.382
M10	14.00	4.16	3.21	3.47	1.20	3.569 ± 0.216
M11	14.00	4.16	5.41	3.47	1.20	2.001 ± 0.165

* Means ± standard error.

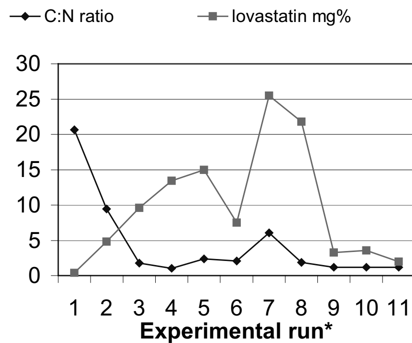

Fig. 1. Relationship between lovastatin content and C:N ratio.
* Experimental run, corresponding to the type medium culture used in this study.

study on the potential use of Chilean strains of *P. ostreatus* for lovastatin production in a liquid medium specifically analyzed the influence of N and C sources on the regulation of lovastatin biosynthesis in *P. ostreatus.* Under each cultivation condition, *P. ostreatus* produced lovastatin in a different content.

The results show that the wild strain PLUBB-127 presents a smaller capacity to produce lovastatin (4.15 mg/l) in comparison with the commercial strain PL-136, which produces 43 mg/l of lovastatin (Table II).

In all the performed experiments, with the exception of experiment M3, glucose was used as the carbon source in a constant amount (10 g/l). Malt extract, which contains 1% of nitrogen and 80% carbohydrates, was used as an additional carbon source. Two nitrogen sources, an organic and an inorganic one, were used. The organic source used were yeast extract (Merck) and peptone water (Merck). The inorganic sources used were $(NH_4)_3PO_4$ (Merck), NH_4Cl (Merck) and $NaNO_3$ (Merck). It can be observed that the lovastatin content increased in all the measured media as the contribution of carbon and nitrogen increased.

The results (Fig. 1) show an important increase in the lovastatin content in relation to increased nitrogen concentration in the media. This increase is significant in those media that use an organic nitrogen source (peptone water or yeast extract). Furthermore, it can be observed that inorganic nitrogen did not significantly influence lovastatin production. Similar results have been reported in studies performed with *Aspergillus terreus* (Hajjaj *et al.*, 2001).

The incorporation of yeast extract and/or peptone water contributes glutamic acid, histidine and glycine among other amino acids to culture medium. Previous studies have demonstrated that glutamic acid, histidine and to a lesser extent glycine are necessary for the biosynthesis of lovastatin. Additionally, histidine and glutamic acid play a key role in generation of ideophase conditions through the formation of α-ketoglutarate, which stimulates aflatoxin synthesis through tricarboxylic acid cycle inhibition (Bhatnagar *et al.*, 1986). Different works have demonstrated the influence of nitrogen in the biosynthesis of secondary metabolites in fungi (Shim and Woloshuk, 1999; Luchese and Harrigan, 1993; Pfeifer and Khosla, 2001).

Our results coincide with the results from studies performed with *Aspergillus terreus* or other filamentous fungi.

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