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Influence of medium design on lovastatin and mevastatin production by *Aspergillus terreus* strains

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Abstract - In order to investigate the influence of medium design on lovastatin and mevastatin production by *Aspergillus terreus* strains, several nitrogen complex sources, such as vegetal flour and peptones of different origin (animal and vegetal) were tested, together with the addition of methionine, an aminoacid that is directly involved in lovastatin biosynthetic pathway. Soybean peptone generally allowed the best lovastatin yields to be achieved (250-280 mg l^{-1}), particularly in the presence of soybean and peanut flours. For mevastatin, the best results (300-320 mg l^{-1}) were obtained at 7 days fermentation with modified base medium (CLD), and at 14 days with standard medium (STD), not being possible in this case to associate the best yield with a defined flour and/or peptone. The results show that lovastatin production is influenced by the presence of soybean peptone and by the addition of methionine; instead, the production of mevastatin appears more strictly strain-associated and not directly dependent on the complex ingredients employed.

Key words: *Aspergillus terreus*, filamentous fungi, lovastatin, mevastatin, statins.

INTRODUCTION

Statins are a class of molecules, obtainable by fungal secondary metabolism and characterized by a polyketide structure, that can inhibit hydroxylmethyl glutaryl-coenzyme A (HMG-CoA) reductase, the first enzyme committed in cholesterol biosynthesis (Alberts, 1988). In human subjects with a regular lipid metabolism, only one-third of the total body cholesterol is diet derived, two-thirds being synthesized directly from intracellular precursor by various organs of the body (Alberts *et al.,* 1980; Furberg, 1999). For this reason the control of cholesterogenesis by inhibiting its biosynthesis is an important means of lowering plasma cholesterol levels.

Different types of statins are currently available for therapeutic use, though lovastatin and mevastatin remain the only natural statins obtained directly by fermentation. They possess a common main portion with different side-linked chains (Manzoni and Rollini, 2002). One of these chains, 6-α-methylic group, derived from methionine, is present in lovastatin, but not in mevastatin (Shiao and Dong, 1987). Investigation on the biogenesis of lovastatin carried out in *Aspergillus terreus* strains employing labelled precursors, indicated that lovastatin biosynthetic pathway starts from acetate units (4- and 8- carbons long) linked to each other in a head-to-tail fashion to form two polyketide chains; the methylic group present in some statins in the side chain or at C6 derives from methionine, as frequently occurs in fungal metabolism, and is inserted in the structure before the closure of the ring (Chan *et al.,* 1983; Shiao and Dong, 1987).

Lovastatin was the first statin to be approved in 1987 by the Food and Drug Administration and became available on the pharmaceutical market as an anticholesterolemic drug (Tobert, 1987). However, mevastatin was actually the first statin to be discovered, being isolated in 1976 from a strain of *Penicillium citrinum* (Endo *et al.,* 1976). Lovastatin was first obtained from *Aspergillus terreus* (Alberts *et al.,* 1980) and subsequently from *Monascus ruber* (Negishi *et al.,* 1986).

In more recent years, statins development became focused on new molecules with improved biological activity, obtainable by the modification of already characterized statins, such as lovastatin and mevastatin. In this area, microbial biotransformation proved a more practical and economic approach, rather than employing chemical hydroxylation processes (Peng and Demain, 1998).

Large scale fermentation processes have been developed for only a few of the statins described in the literature. A Biocon company, namely Biocon India, Bangalore, obtained FDA approval for lovastatin production in January 2001. The company's process is based on a proprietary fermentation technology, the Plafractor, a large-scale solid-matrix bioreactor that combines the advantages of employing a solid substrate with a submerged fermentation, thus allowing a reduction of downstream processing troubles during lovastatin extraction (Suryanarayan and Mazumdar, 2001).

In applied microbiology, the design of the fermentation media is critical. Although major improvements are gener-

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ally ascribed to the development of superior strains, an optimally balanced culture medium is mandatory for maximizing metabolite production.

The present paper evaluates the production of statins, particularly lovastatin and mevastatin, with regard to culture medium composition, by comparatively testing nitrogen complex ingredients, such as vegetal flours and peptones of different origin (animal and vegetal). Moreover, the influence of adding methionine, an aminoacid directly involved in statins biosynthesis, to the culture, was also investigated.

MATERIALS AND METHODS

Microorganisms and culture conditions. Screening for statins production was carried out with four *Aspergillus terreus* strains, MIM (Microbiologia Industriale, Università di Milano, Italy) G+, G-, B+ and B1. These strains were isolated as macromorphologically different in coulor with respect to the parental *A. terreus* ATCC 20542.

The cultures were monthly maintained on Czapek Yeast Extract Agar (CYA), with the following composition (g l^{-1}): saccharose 30, yeast extract (Costantino, Favria TO) 5, K_2HPO_4 1, agar 15; added to this were 10 ml l^{-1} of a stock saline solution of the following composition (g l^{-1}): $NaNO_3$ 300, KCl 50, $MgSO_4 \cdot 7H_2O$ 50, $FeSO_4 \cdot 7H_2O$ 1, $ZnSO_4 \cdot 7H_2O$ 1, $CuSO_4 \cdot 5H_2O$ 0.5. The medium was sterilised at 118 °C for 20 min. The strains, incubated for 7 days at 25 °C, were then stored at room temperature.

Statins production. Two base media were employed, one with our standard formulation (STD, Manzoni *et al*., 1998) and the other as modification of a medium reported from Lai *et al.,* 2003 (CLD), having the following composition (g l^{-1}):

- STD: glycerol 80, peptone 8, $NaNO_3$ 2, $MgSO_4 \cdot 7H_2O$ 1, glucose 30, pH 6.4, sterilised at 118 °C for 20 min. Glucose was sterilised separately at 115 °C for 30 min and successively added to the medium. The medium was supplemented with 30 g l^{-1} of different vegetal flours (defatted peanut, cotton or soybean flours – Mucedola, Settimo Milanese, Italy), for comparative purposes;
- CLD: lactose 60, glycerol 30, glucose 20, corn-steep liquor 10, peptone 8, methionine 0.1, pH 6.4, sterilised at 115 °C for 30 min. Corn-steep liquor was pre-treated at 121 °C for 20 min, filtered under vacuum, added with peptone and sterilised at 118 °C for 20 min before the final addition to the culture medium. The medium was supplemented with 30 g l^{-1} of the above mentioned vegetal flours. Methionine was added at 72 h fermentation, acting as the lovastatin precursor during the idiophase (secondary metabolite production phase) (Crueger and Crueger, 1989).

Bacteriological and soybean peptones (Costantino) were employed in the two base media for comparison purposes.

Statins production was carried out in 1 l Erlenmeyer flasks, each containing 200 ml of the culture medium. Each flask was inoculated with a standard spore suspension in 0.5% Tween 80 from a 14 day-old CYA culture (10^6 spores ml^{-1} medium). Cultures were incubated at 25 °C on a reciprocal shaker (60 strokes min^{-1}) for a fermentation time of 7 and/or 14 days, depending on the base medium employed.

All fermentation trials were conducted in triplicate, and the results were reported as an average of the values ± standard deviation (SD). The error bars in figures indicate the standard deviations for the mean data points.

Analytical determination. At the end of the fermentation, culture broth was filtered through a Buchner's apparatus. Biomass was discharged, and statins identification and quantification were carried out on culture filtrate samples by HPLC, using a LiChrospher 60 RP-Select B column (Merck), eluted with 65% acetonitrile/35% of 0.05% trifluoroacetic acid. Samples were eluted at 0.75 ml min^{-1} and statins detected by absorbance at 238 nm. Concentration of lovastatin (retention time of the hydroxyacid salt form: 31.2 min) and mevastatin (retention time of the hydroxyacid salt form: 23.7 min) was determined by comparison with authentic standards.

RESULTS

Screening for statins production was carried out employing four *Aspergillus terreus* strains belonging to the internal collection of the University of Milan.

Statins are usually produced as a mixture of lactone and free hydroxyacid forms, evidenced in the HPLC chromatogram as two peaks with different retention times. In acid conditions HPLC chromatograms reveal a balance between the two forms. On the contrary, under alkaline conditions, statins detection proved easier, as any statins present in the lactone form are transformed into the corresponding hydroxyacid (Manzoni *et al.,* 1998).

Consequently, lovastatin and mevastatin production was determined in fully alkalised culture filtrate samples.

Lovastatin and mevastatin yields were evaluated on 14 day-old submerged cultures grown on STD or CLD base medium containing bacteriological or soybean peptone and, as complex ingredient, defatted peanut, cotton or soybean flour.

The two peptones employed differ in origin, the bacteriological peptone being obtained through the enzymatic hydrolysis of protein of animal origin: an analysis of its aminoacid content reveals a higher proline and glycine content (11.7 and 13% w/w respectively) than observed in the soybean peptone (2.6% each one). Other differences relate to total nitrogen content (13.9 and 9.4% for bacteriological and soybean, respectively) and ash (13.9 and 8.1%, respectively). Note that both ingredients present a relatively reasonable cost (35-48 ¤ kg^{-1}), and both can be employed in applicative processes.

The vegetal flours are all defatted ingredients of very low cost (0.80-2.60 ¤ kg^{-1}), differing from each other in protein, lipid and ash content, these being 44, 3 and 3% for peanut, 55, 1 and 8% for cotton, and 47, 2 and 6% for soybean, respectively.

Nitrogen regulation is of wide significance in industrial microbiology, since it affects the synthesis of enzymes involved in both primary and secondary metabolism. Many secondary metabolic pathways are negatively affected by nitrogen sources favourable for growth, e.g. ammonium salts. As a result, complex fermentation ingredients often include a protein source containing slowly assimilated amino acid (such as proline) as the nitrogen source to encourage high production of secondary metabolites (Sanchez and Demain, 2002).

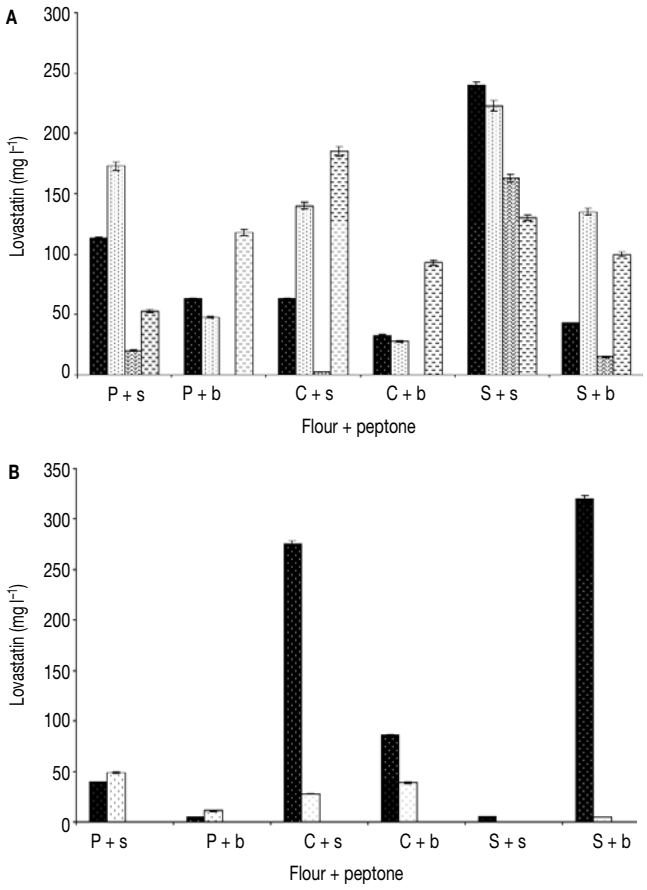

FIG. 1 – Fermentation yields (mg l^{-1}) of lovastatin (A) and mevastatin (B) from *Aspergillus terreus* strains at 14 days, employing the STD base medium supplemented with soybean (s) or bacteriological (b) peptone, and the three vegetal flours: peanut (P), cotton (C) and soybean (S). The error bars indicate the standard deviation for the mean data points. ■ G-, ▨ G+, ▩ B+, ▧ B1.

Statins production in STD base medium

In the first part of the research the STD base medium was employed, and figure 1 shows lovastatin (A) and mevastatin (B) fermentation yields obtained by the four 14 day-old *A. terreus* submerged cultures.

The best lovastatin yields were always obtained employing soybean peptone. From among the tested conditions, the most effective proved to be the use of soybean flour (240 and 223 mg l^{-1} from *A. terreus* G- and G+ respectively). Lower yields were obtained with cotton (185 mg l^{-1} from *A. terreus* B1) and peanut (173 mg l^{-1} from *A. terreus* G+ strain). Bacteriological peptone did not prove to enhance lovastatin production, having obtained 0 to 130 mg l^{-1} independently from the vegetal flour chosen.

As regards mevastatin production, very interesting yields were obtained only from *A. terreus* G- strain, 320 mg l^{-1} with bacteriological peptone and soybean flour, and 276 mg l^{-1} with soybean peptone and cotton flour. No other strain-ingredient association proved to be effective for the production of this metabolite.

So far, from the results obtained employing the STD base medium, lovastatin seems to be a common metabolite in all the four tested strains, its production being enhanced in media containing soybean-derived ingredients. These results are in good agreement with the data reported in previously published research, the lovastatin yields being 235 ml l^{-1} using *A. terreus* strains in media containing defatted soybean flour at 14 days fermentation (Manzoni *et al.,* 1998, 1999).

On the other hand, mevastatin production appears more strictly strain-associated (*A. terreus* G-) and not directly dependent on the complex ingredients employed.

Statins production in CLD base medium

In order to increase statins yields, trials were then carried out employing a base medium reported in the literature to favour lovastatin production in *Aspergillus terreus* strains (Lai *et al.,* 2003). This medium is characterised by carbon and energy sources represented not only by glycerol and by glucose, as in the STD medium, but also by lactose. Moreover, CLD base medium contains a higher concentration of nitrogen sources, represented by both peptone and corn-steep liquor. The most significant difference between the two base media that can be related to statins production lies in the presence, in the CLD medium formulation, of methionine, to be added to the culture at 72 h fermentation. The addition of this aminoacid was proposed by Kimura *et al.* (1990) to serve as an important precursor in the synthesis of monacolin K (lovastatin): considering the methylation step in lovastatin synthesis, D,L-methionine was added to examine its effect on the enhancement of product formation.

Again, comparative fermentation trials were carried out with the three vegetal flours and the two peptones. In this part of the research we wanted to verify the possibility of an earlier biosynthetic phase due to the presence of methionine, so analyses were carried out not only on the 14 day-old samples but also earlier (7 days).

Lovastatin production at 7 days proved very low, with yields ranging from 0 to 50 mg l^{-1} (data not shown). The best results were instead found at 14-days fermentation (Fig. 2). Again, the best lovastatin yields were obtained employing soybean peptone. From among the tested conditions, the most effective proved to be the use of soybean flour (280 mg l^{-1} from *A. terreus* G-) and peanut flour (250 mg l^{-1} from *A. terreus* G+), while a lower yield was obtained with cotton (207 mg l^{-1} from *A. terreus* G+). The addition of bacteriological peptone did not prove to enhance lovastatin pro-

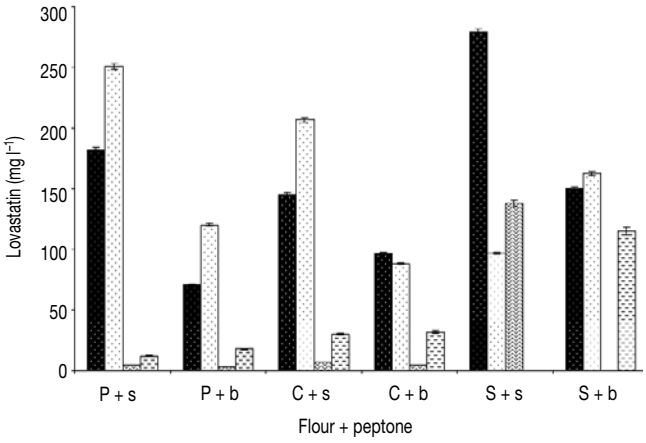

FIG. 2 – Fermentation yields (mg l^{-1}) of lovastatin from *Aspergillus terreus* strains at 14 days, employing the CLD base medium supplemented with soybean (s) or bacteriological (b) peptone and the three vegetal flours: peanut (P), cotton (C) and soybean (S). The error bars indicate the standard deviation for the mean data points. ■ G-, ▨ G+, ▩ B+, ▧ B1.

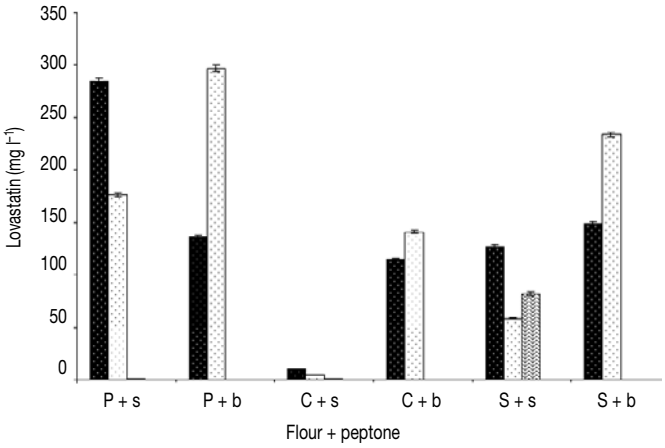

FIG. 3 – Fermentation yields (mg l^{-1}) of mevastatin from *Aspergillus terreus* strains at 7 days, employing the CLD base medium supplemented with soybean (s) or bacteriological (b) peptone and the three vegetal flours: peanut (P), cotton (C) and soybean (S). The error bars indicate the standard deviation for the mean data points ■ G-, ▨ G+, ▨ B+, ▨ B1.

duction unequivocally: only the G+ and B1 strains on soybean flour showed an increase in lovastatin yield when the soybean peptone was replaced by the bacteriological one (from 90 to 163 mg l^{-1} and from 0 to 115 mg l^{-1}, respectively).

As regards mevastatin production, a deep analysis of the results was carried out at 7 days fermentation, when the highest yields were evidenced (Fig. 3). Mevastatin yields proved interesting only in samples from *A. terreus* G+ (bacteriological peptone) and *A. terreus* G- (soybean peptone) strains grown on peanut flour, 300 and 285 mg l^{-1} respectively. Note that the B+ and B1 strains were not good mevastatin producers (0-70 mg l^{-1}).

Again, as evidenced in previous experiments, B+ and B1 strains did not prove to be efficient statins producers. On the contrary, A. *terreus* G+ and G- strains showed good results. Lovastatin best yield (280 mg l^{-1}) was achieved with G- strain and soybean flour at 14 days fermentation, while mevastatin best yield (300 mg l^{-1}) was obtained with the G+ strain employing peanut flour at 7 days. To be noted that lovastatin best results were always obtained at 14 days and those of mevastatin generally at 7.

An overall look at the results employing the CLD base medium showed that the soybean peptone generally resulted in higher lovastatin yields than the bacteriological peptone. As regards mevastatin, the most interesting results were evidenced in the 7-day culture samples: employing peanut flour, 300 mg l^{-1} were obtained with *A. terreus* G+ and bacteriological peptone, and 285 mg l^{-1} with *A. terreus* G- and soybean peptone.

DISCUSSION

The present research, aimed at evidencing any possible influence of culture medium composition on statins production, tested several nitrogen complex sources, such as vegetal flour and peptones of different origin (animal and vegetal). The influence of adding methionine, an aminoacid directly involved in the lovastatin biosynthetic pathway, was also examined.

From an overall view of the results obtained employing the four *A. terreus* strains, it was possible to highlight the followings:

- the best lovastatin yields (280 and 250 mg l^{-1}) were obtained employing the CLD base medium supplemented with soybean peptone at 14 days, with *A. terreus* G- and soybean flour and *A. terreus* G+ and peanut flour, respectively;
- the best mevastatin yield (320 mg l^{-1}) was obtained employing the STD base medium supplemented with bacteriological peptone at 14 days, with *A. terreus* G- and soybean flour; a similar yield (300 mg l^{-1}) was obtained employing the CLD base medium supplemented with bacteriological peptone at 7 days with *A. terreus* G+ and peanut flour.

In conclusion, the experiments revealed that soybean peptone generally led to the best lovastatin yields, particularly in the presence of soybean and peanut flours (*A. terreus* G+ and G- strains). As regards mevastatin, the best results were obtained at 7 days fermentation with CLD base medium, and at 14 days with STD medium. In this case, it was not possible to associate, in a univocal way, the best yields with a defined flour and/or peptone.

The data analysis revealed that lovastatin production was influenced by the presence of soybean peptone, and that mevastatin production did not seem to be influenced by the type of peptone added to the culture. Moreover, a comparison of the results obtained employing the two base media showed that methionine addition (CLD base) seems to favour lovastatin biosynthesis. This behaviour is consistent with the lovastatin biosynthetic steps where the methylic group within the structure derives from methionine, and, like the methylbutyric side chain, it is incorporated inside the structure before ring closure.

Employing the CLD base medium, lovastatin yields were found to be 20% higher than with the STD base medium. This is in accordance with literature data (Lai *et al.,* 2003). With regard to mevastatin, it was not possible to find any univocal relations as yields were not strictly dependent on the type of flour or peptone used.

In conclusion, the present research highlights that some of the *A. terreus* tested strains produce statins in very interesting yields. It was found that the G+ and G- strains led to the highest yields of lovastatin and/or mevastatin, depending on the culture medium formulation.

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