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Full Paper

Utilisation of vegetable oils in the production of lovastatin by *Aspergillus terreus* ATCC 20542 in submerged cultivation

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Abstract: The effect of vegetable oils as a supplementary carbon source during the production of lovastatin by *Aspergillus terreus* ATCC 20542 in submerged culture was investigated. The six vegetable oils tested were sesame oil, sunflower oil, soya bean oil, corn oil, palm oil and olive oil. Lovastatin concentration and biomass were measured. Lovastatin production was higher in several oil-containing media compared to control medium. In particular, palm oil and soya bean oil significantly improved lovastatin production. Yields with palm oil and soya bean oil were 4.5- and 1.4-fold higher respectively, compared with control. Sesame oil and corn oil, however, had a negative effect on lovastatin production. Biomass was proportional to vegetable oil concentration, but an excessive vegetable oil concentration resulted in a lower yield of lovastatin**.** Thus, some vegetable oils appear to be excellent adjuvants for improving efficiency of lovastatin production.

Keywords: lovastatin, *Aspergillus terreus*, vegetable oils, submerged culture

INTRODUCTION

Lovastatin (Figure 1) was the first potent cholesterol-lowering drug to be approved [1]. It acts by competitively inhibiting 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase which catalyses the rate-limiting step of cholesterol biosynthesis [2]. Lovastatin is produced as a

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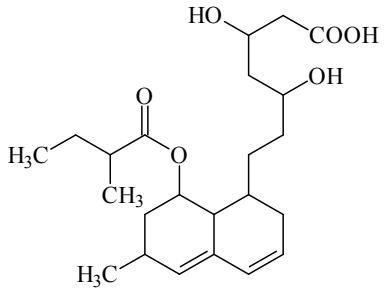

Figure 1. Lovastatin (β-hydroxy acid form)

secondary metabolite of the polyketide pathway by various fungi including *Penicillium* spp. [3], *Monascus* spp. [4], *Trichoderma* spp. [5] and *Aspergillus terreus* [2]. *A. terreus* is known to be the best lovastatin-producing species [6]. Several studies have extensively investigated methods for increasing lovastatin production in *A. terreus*. Many literature reports have focused on culture medium optimisation [7–15]. Further studies have addressed the development of mutant strains [16], the culturing environment [17] and the solid-state fermentation [18–20].

Carbon and nitrogen sources have dominant roles in the fermentation process because these are the major culture nutrients involved in the formation of biomass and metabolites [11]. Vegetable oils are essential components of industrial fermentation media and they are routinely supplemented into media during the production of antibiotics [21]. Vegetable oils are used as sole carbon sources, supplemental carbon sources, anti-foaming agents and precursors during antibiotic synthesis. There are many examples of the successful utilisation of vegetable oils in the production of antibiotics including erythromycin [21], tetracycline [22], cephamycin C [23], cephalosporin C [24], clavulanic acid [25] and gentamicin [26].

However, there seem to be no published reports of the application of vegetable oils in lovastatin production. Thus, we have undertaken to investigate the applicability of a range of vegetable oils to enhancing lovastatin productivity of *A. terreus*. This study also reports an optimised culture medium and culture environment for lovastatin production.

MATERIALS AND METHODS

Chemicals

Standard lovastatin (β-hydroxy acid form) (≥98% purity) was obtained from Sigma Chemical Co. (St. Louis, MO). Yeast extract (Fluka Chemie GmbH, Buchs, Switzerland), corn steep liquor (Sigma Chemical, Co.), lactose (Ajax Finechem, NSW, Australia), D-(+)-glucose monohydrate (Riedel-de Haen Laborchemikalien GmbH, Seelze, Germany) and potato dextrose agar (Merck KGaA, Darmstadt, Germany) were used in the culture medium. Cooking vegetable oils tested were as follows: corn oil (refined, Mass Marketing Co. Ltd, Samutprakarn, Thailand), olive oil (extra virgin, Rafael Salgado SA, Madrid, Spain), palm oil (refined, P.S. Pacific Co. Ltd, Petchburi, Thailand), sesame oil (Chaiseri Co. Ltd, Chiang Mai, Thailand), soya bean oil (refined, Morakot Industries Public Co. Ltd, Samutprakarn, Thailand) and sunflower oil (refined, Thanakorn Vegetable

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Oil Products Co. Ltd, Samutprakarn, Thailand). All other chemicals, trace elements and solvents were of reagent grade and obtained from standard sources.

Microorganisms and Culture Medium

A standard fungal strain of *A. terreus* ATCC 20542 was obtained from the American Type Culture Collection (Manassas, VA). The fungal cells were kept in the form of a revivable freeze-dried culture and reactivated by culturing on potato dextrose agar slants in an incubator (Memmert GmbH & Co. KG, Schwabach, Germany) at 30°C for 3 days. In each experiment, the refreshed fungal strain was maintained on agar and subcultured into the primary seed culture medium. A basal culture medium, previously reported by Casas Lopez et al. [9], was optimised for this study and contained the following in 1 L of distilled water: 10 g lactose, 8 g yeast extract, 1.51 g KH_2PO_4, 0.52 g $MgSO_4.7H_2O$, 0.40 g NaCl, 1 mg $ZnSO_4.H_2O$, 2 mg $Fe(NO_3)_3.9H_2O$, 0.04 mg biotin and 1 mL trace element solution. One litre of the trace element solution contained 100 mg $NaB_4O_7.10H_2O$, 50 mg $MnCl_2.4H_2O$, 50 mg $Na_2MoO_4.2H_2O$ and 250 mg $CuSO_4.5H_2O$. The pH of the medium was adjusted to 6.5 using 0.1 N NaOH before sterilisation. The seed culture was prepared in a 250-mL Erlenmeyer flask containing 100 mL of medium, which was kept on an orbital shaker (Revco Scientific Inc., Asheville, NC) at 220 rpm for 5 days at room temperature. All aseptic techniques were conducted in a laminar air flow cabinet (Forma Scientific Inc., Marietta, OH).

Optimisation of Culture Conditions

A 1.0-mL volume of dispersed spores from the seed medium was added to each flask containing 100 mL of the basal culture medium. The effects of incubation temperature, incubation period and shaking rate on lovastatin production were then examined. Experiments were performed at 25°C and 30°C, orbital shaker speeds of 150 and 220 rpm, and five fermentation periods of 5, 7, 9, 12 and 14 days, while all other conditions remained fixed.

Screening of Carbon and Nitrogen Sources

A 1.0-mL volume of dispersed spores from the seed medium was added to 100 mL of the basal culture medium contained in each flask with different carbon and nitrogen sources. Various medium compositions were tested by varying the carbon and nitrogen sources as follows– lactose and yeast extract; lactose and corn steep liquor; glucose and yeast extract; glucose and corn steep liquor– all with a ratio of 10:8 g L^{-1}. Further, the mass ratios, i.e. 8:10, 10:8, 12:6 and 14:4 g L^{-1}, of the carbon:nitrogen sources were tested, based on the results of preliminary experiments. The total mass of carbon and nitrogen sources was fixed at 18 g L^{-1}.

Effects of Different Vegetable Oils on Lovastatin Production

A 1.0-mL volume of dispersed spores from the seed medium was added to 100 mL of the basal culture medium contained in each flask with different vegetable oils. Experiments were performed by testing six different vegetable oils at a concentration of 1% v/v. A subsequent study tested the effects of using different concentrations (0.5%, 1%, 2% and 3% v/v) of two vegetable oils on lovastatin production.

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HPLC Analysis

Each culture medium was separated from the fungal mass at the end of the incubation period and adjusted to pH 3. The clear broth (50 mL) was extracted with ethyl acetate (50 mL) by vigorously mixing for 10 min in a separating funnel. After separation, the organic layer was evaporated and dried. The residue volume was then adjusted to 2.0 mL with a mobile phase (composition described later in this section), filtered through a 0.45-μm nylon syringe filter and transferred to a sample vial. One hundred microlitres was analysed by an HPLC system that consisted of a solvent delivery system (Varian 9012, Varian, Palo Alto, CA) and a variable wavelength UV-Vis detector (Varian 9050, Varian) equipped with a Rheodyne 7725 sample injector (Rohnert Park, CA) which was fitted with a 100-μl sample loop. Chromatographic separation was conducted using an ODS Hypersil® C-18 column (250 × 4.6 mm i.d.; 5-μm particle diameter; 250Å average pore size) (Thermo Electron Corporation, Waltham, MA) and a mobile phase containing 55% acetonitrile, 12% methanol and 33% phosphate buffer saline (pH 4.0) at a flow rate of 1.0 mL min^{-1}. Detected at 238 nm, lovastatin peak was located at a retention time of 14 min. Produced lovastatin content was obtained from its concentration in the culture medium determined by a calibration curve of the authentic sample between 1–20 μg mL^{-1}.

Measurement of Dry Cell Weight

The culture broth was filtered with a Whatman filter membrane No. 1. The total mycelia obtained were washed with distilled water, dried in an oven for 24 h at 70°C and equilibrated at room temperature before measurement of the dry cell weight.

RESULTS AND DISCUSSION

Optimisation of Culture Conditions

The different culture temperature of 25°C and 30°C had no significant effects on lovastatin production. However, an agitation speed of 150 rpm gave higher lovastatin production compared with 220 rpm. The lovastatin yield increased rapidly between day 3 and day 5, presumably because lovastatin is a secondary metabolite and its accumulation in mycelia appears to be growth-related. The maximum lovastatin yield was achieved on day 7, after which the yield slowly decreased (Figure 2). The decrease in lovastatin concentration after day 7 might be attributable to an insufficient amount of lactose relative to yeast extract and because the nitrogen source could also inhibit lovastatin formation [27]. This production time-course corresponded to others reported by Saminee et al. [6] and Lopez et al. [9]. Lovastatin is an intracellular product, so the product accumulation approximately correlates with cell growth [19]. These results indicate that the cultivation process may be concluded on day 7. Based on these finding, these operating conditions were used in the next experiments.

Screening of Carbon and Nitrogen Sources

The optimum carbon and nitrogen sources for submerged cultures of *A. terreus* have been widely investigated [7–10]. In the present study, different types of carbon and nitrogen sources were

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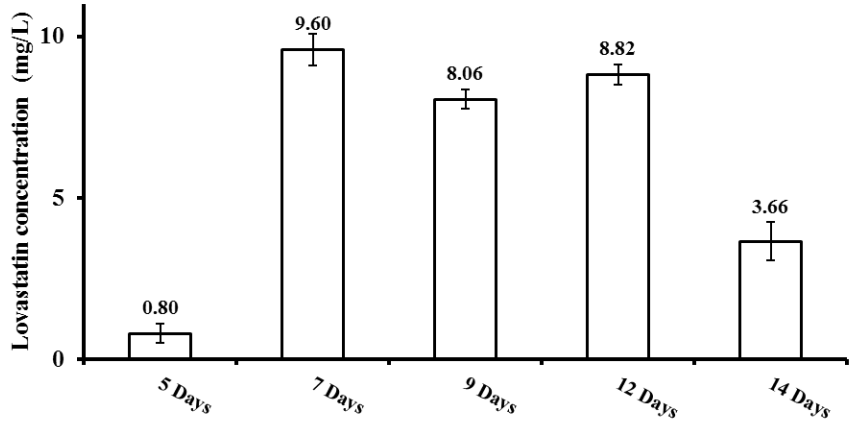

Figure 2. Time-course of lovastatin production by *A. terreus* ATCC 20542 (n = 3)

found to affect lovastatin production. Optimal lovastatin production was found when using 10 g L^{-1} glucose and 8 g L^{-1} corn steep liquor as carbon and nitrogen sources respectively. This medium yielded a lovastatin titre of 33.00 mg L^{-1} within 7 days of culture, a 2.8-fold increase in lovastatin production when compared with that from a basal medium containing 10 g L^{-1} lactose and 8 g L^{-1} yeast extract (Figure 3). These results did not agree with those obtained by Lopez et al. [9] and Lai et al. [10], who preferred lactose and yeast extract as carbon and nitrogen sources rather than glucose and corn steep liquor. The inconsistency might stem from differences in the culture environments as well as the composition and concentration of other culture medium components.

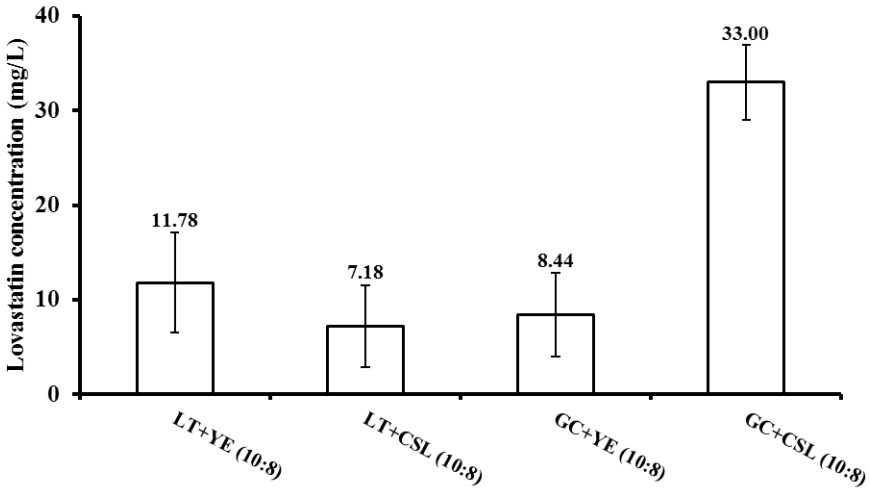

Figure 3. Lovastatin production in cultures containing different carbon and nitrogen sources (g L^{-1}) for *A. terreus* ATCC 20542 (LT = lactose; GC = glucose; YE = yeast extract; CSL = corn steep liquor) (n = 3)

A second set of experiments involved testing the effects of using different glucose:corn steep liquor mass ratios on lovastatin yield. A mass ratio of 12:6 resulted in the highest production (Figure 4). These results agreed with the reports by Hajjaj et al. [8] and Lopez et al. [9]. Lovastatin yield can thus be increased when the carbon source is nonlimiting and growth is only arrested by nitrogen

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source limitation. Based on these findings, glucose and corn steep liquor with a mass ratio of 12:6 was used in the next experiments.

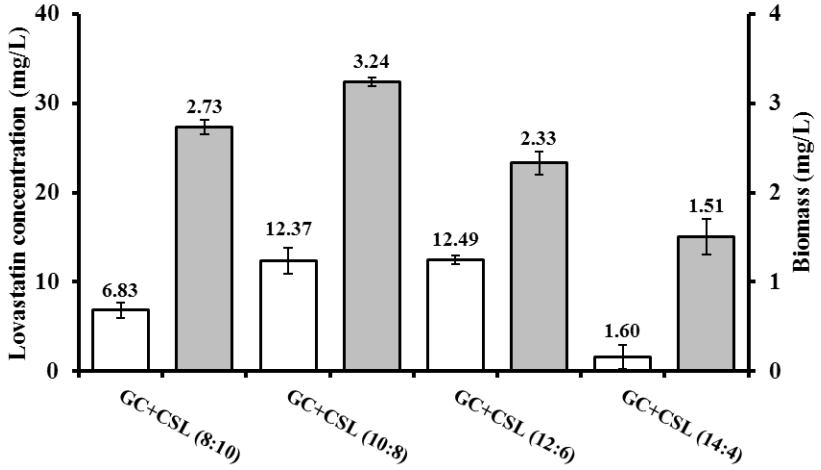

Figure 4. Lovastatin production (light bars) and biomass (dark bars) derived from cultures of *A. terreus* ATCC 20542 with different carbon:nitrogen mass ratios (g L^{-1}) (n = 3)

Effects of Various Vegetable Oils on Lovastatin Production

Separate submerged cultures were grown for 7 days in Erlenmeyer flasks, each containing 100 mL of the production medium and 1% (v/v) of each of the six vegetable oils. Figure 5 shows the effects of various vegetable oils on lovastatin production in two different media containing lactose:yeast extract (10:8) and glucose:corn steep liquor (12:6). Most vegetable oils increased the product yield compared with control, with the exceptions of sesame oil and corn oil. The highest production levels were found with palm oil in the basal medium and soya bean oil in the optimised medium. The maximum production levels were 15.94 mg L^{-1} (palm oil in basal medium) and 41.85 mg L^{-1} (soya bean oil in optimised medium), which were approximately 4.5- and 1.4-fold greater than those obtained from the oil-free controls in basal and optimised media respectively. Soya bean oil was also known to have positive effects on tetracycline [22], cephamycin C [23] and gentamicin [26] production.

In order to find the optimal oil concentration, the experiments were repeated using soya bean oil and palm oil at concentrations of 0.5, 1, 2, and 3% (v/v). The resulting lovastatin and biomass production are shown in Figure 6, which indicates that palm oil and soya bean oil had a similar performance. An oil concentration of 0.5% resulted in the highest lovastatin production for both palm oil and soya bean oil; higher concentrations tended to give lower yields. It is interesting to note that the biomass increased with increasing oil concentration, which suggests that cell growth is related to vegetable oil concentration. These results agreed with a previous report [9], which showed that metabolic pathways governing the synthesis of lovastatin from a carbon source were slower than pathways converting carbon to biomass.

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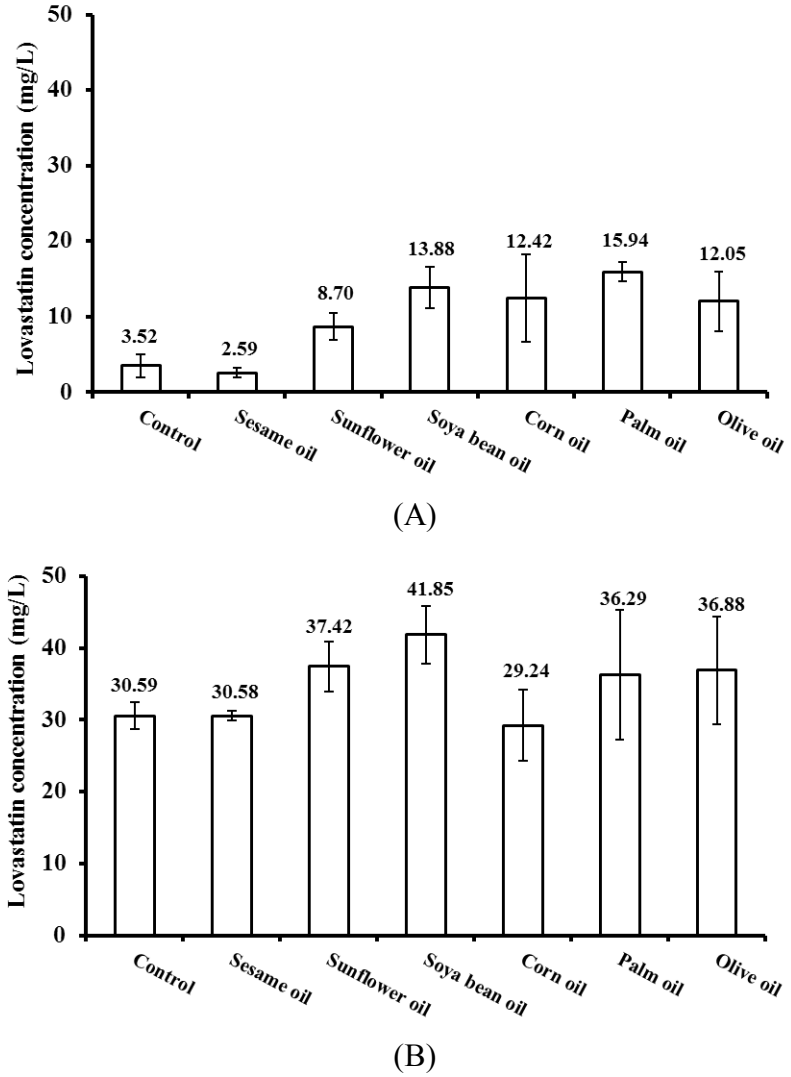

Figure 5. Effects of 1% (v/v) concentration of vegetable oils on lovastatin production by *A. terreus* ATCC 20542 in medium containing (g L^{-1}): (A) lactose:yeast extract (10:8), and (B) glucose:corn steep liquor (12:6) (n = 3)

Although vegetable oils may have a key role as a supplementary carbon source, the data available are still not sufficient to identify any specific components of the vegetable oils used that may be the key factors affecting lovastatin production. Further investigations are required to test different combinations of fatty acids and other components of vegetable oils to identify any major factors affecting lovastatin production.

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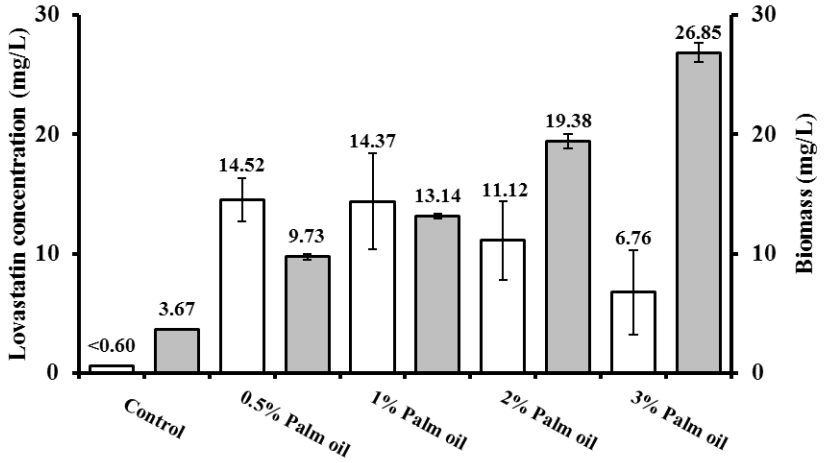
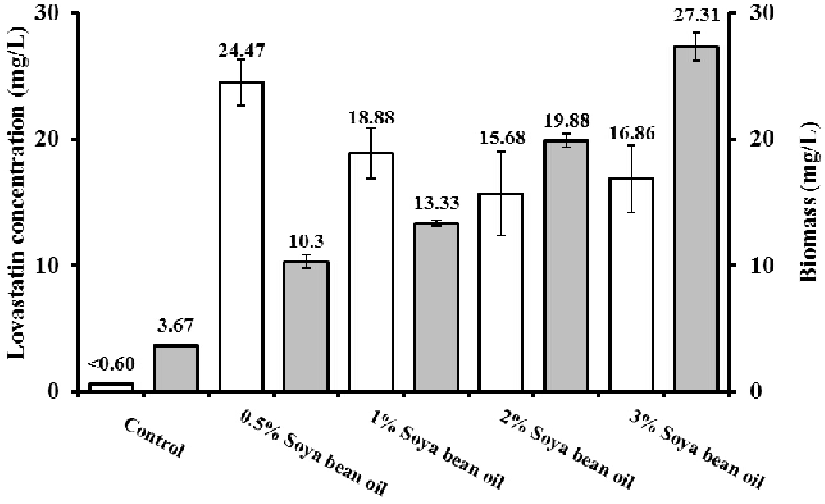


(A)



(B)

Figure 6. Effects of concentration of palm oil (A) and soya bean oil (B) on lovastatin (light bar) and biomass (dark bar) production by *A. terreus* ATCC 20542 in a medium containing (g L^{-1}) lactose:yeast extract (10:8) (n = 3)

CONCLUSIONS

Vegetable oils are a promising substrate as additional carbon and energy source for lovastatin production by *A. terreus* in a submerged culture. Of the six vegetable oils tested, palm oil and soya bean oil significantly improved lovastatin production when present at low levels in the culture medium. The degree of cell growth was also closely associated with the vegetable oil concentration. The type of carbon and nitrogen sources used and their mass ratio also affected lovastatin production.

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