DOI 10.1007/s11094-020-02195-x
Pharmaceutical Chemistry Journal, Vol. 54, No. 3, June, 2020 (Russian Original Vol. 54, No. 3, March, 2020)

LOVASTATIN PRODUCTION FROM *ASPERGILLUS TERREUS* ATCC 20542 UNDER VARIOUS VEGETABLE OILS USED AS SOLE AND SUPPLEMENTARY CARBON SOURCES

Pattana Sripalakit[1,2,*] and Aurasorn Saraphanchotiwitthaya[1,3]

Original article submitted December 16, 2019.

The effect of vegetable oils used as sole and supplementary carbon sources on the production of lovastatin by *Aspergillus terreus* ATCC 20542 and their biomass in submerged fermentation has been examined. Eleven different types of edible vegetable oils were tested including camellia tea oil, canola oil, coconut oil, corn oil, olive oil, palm olein oil, rice bean oil, safflower oil, sesame oil, soybean oil, and sunflower oil. All selected oils can improve the yield of target product at least 2 times. The maximum yield was 87.18 g/L with supplementary 1% w/v coconut oil, which was about 11 times higher than that obtained from the oil-free control. Fungal biomass was proportional to vegetable oil concentration, but an excessive concentration of oil resulted in a lower yield. Substitutions of coconut oil and soybean oil at any quantities for lactose used as sole carbon source showed very low concentrations of lovastatin. These findings indicate that vegetable oils can be used for supporting fungal growth more than the secondary metabolite production. It can be concluded that easily available vegetable oil is a very promising adjuvant for lovastatin production.

Keywords: lovastatin; *Aspergillus terreus*; vegetable oils; fermentation; carbon source.

1. INTRODUCTION

Lovastatin is a cholesterol-lowering agent that belongs to the class of medications called statins. It was the second agent of this class discovered and the first statin approved for clinical use [1]. Lovastatin has been isolated from *Aspergillus terreus* by Merck [2] and subsequently developed to simvastatin [3], which is more widely used since it is more effective than the precursor for inhibiting the conversion of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) to mevalonate in cholesterol biosynthesis [4]. Many researchers extensively attempted for increasing lovastatin production. Various researchers concentrated on the optimization of fermentation medium [5 – 13], effects of culturing environments [14, 15] and the selection of fermentation modes [16 – 18]. Almost all reports were focused on *A. terreus* ATCC 20542, while the other have studied *A. terreus* ATCC 20541 [19], *A. terreus* ATCC 74135 [20, 21], the wild strains of *A. terreus* [22 – 28], the mutant strains of *A. terreus* [29, 30], and different fungal species and genus other than *A. terreus* [3 – 33].

In fermentation process, the composition of a nutrient medium is directly involved in the formation of biomass and secondary metabolites. Optimization of carbon sources led to increase in the lovastatin production. In submerged fermentation of *A. terreus*, previous investigations used sugars as sole carbon sources, e.g., lactose [5, 7 – 9, 14, 16, 18, 28, 29], glucose [10, 19, 20, 32], fructose [6] and other sugars [13]. Moreover, the carbon source supplements like glycerol [5, 15, 30], vegetable oils [11], and linoleic acid [28], can be added together with sugars to the medium. Lactose proved to be the best carbon source, but its price highly influenced the overall production cost [26]. Some other sole carbon sources such as cellulose [22], maltodextrin [23], glycerol [6, 30], soluble starch [25], and whey powder [26] were also applied alternatively.

Vegetable oils are the important component of fermentation media used to produce various antibiotics. They have been used as sole carbon source for cephamycin C [34], clavulanic aid [35] and gentamycin [36]. Furthermore, the positive effects of vegetable oils as a low-level supplement on the fermentation yield were reported for tetracycline [37], erythromycin [38], cephalosporin C [39], and clavulanic aid

[1] Pharmaceutical Biotechnology Research Unit, Faculty of Pharmaceutical Sciences, Naresuan University, Phitsanulok 65000, Thailand.
[2] Department of Pharmaceutical Chemistry and Pharmacognosy, Faculty of Pharmaceutical Sciences, Naresuan University, Phitsanulok 65000, Thailand.
[3] Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Naresuan University, Phitsanulok 65000, Thailand.
[*] e-mail: pattanas@nu.ac.th

[40]. In this context, the effect of vegetable oils present in the composition of carbon source on the metabolite production and biomass yield has been studied. The influence of vegetable oils used as sole and supplementary carbon sources in the fermentation media has also been compared.

2. MATERIALS AND METHODS

2.1. Chemicals

Standard lovastatin (in β-hydroxy acid form, ≥ 98% purity) was obtained from Sigma Chemical Co. (St. Louis, MO). Yeast extract (Fluka Chemie GmbH, Buchs, Switzerland) and lactose (Ajax Finechem, NSW, Australia) were used in the culture medium. The types and sources of vegetable oils used in this work are shown in Table 1. All salts, trace elements, solvents and other chemicals were of reagent grade and obtained from standard sources.

2.2. Microorganisms and Culture Medium

A standard fungus used was obtained from the American Type Culture Collection (Manassas, VA), as *A. terreus* ATCC 20542. The fungal cells kept in the form of revival freeze-dried culture were reactivated in nutrient broth (Merck KGaA, Darmstadt, Germany) for 2 days and subsequently grown on malt extract agar (Merck KGaA) for 5 days in incubator (Memmert GmbH & Co. KG, Schwabach, Germany) at 30°C. In each experiment, refreshed fungal strain was maintained on agar and subcultured into the primary seed culture medium. A basal medium, which followed the suggestion of Casas Lopez, et al. [6], contained of (per liter): 10 g lactose, 8 g yeast extract, 1.51 g KH_2PO_4, 0.52 g $MgSO_4 \cdot 7H_2O$, 0.40 g NaCl, 1 mg $ZnSO_4 \cdot H_2O$, 2 mg $Fe(NO_3)_3 \cdot 9H_2O$, 0.04 mg biotin and 1 mL trace element solution. One liter of the trace element solution contained 100 mg $NaB_4O_7 \cdot 10H_2O$, 50 mg $MnCl_2 \cdot 4H_2O$, 50 mg $Na_2MoO_4 \cdot 2H_2O$ and 250 mg $CuSO_4 \cdot 5H_2O$. Before sterilization, pH of the medium was adjusted to 6.5 using 0.1 N NaOH. The seed culture was prepared in a 250-mL Erlenmeyer flask containing 100 mL of medium, which was treated on an orbital shaker (Revco Scientific Inc., Asheville, NC) at 220 rpm for 2 days at room temperature. All aseptic techniques were conducted in a laminar air flow cabinet (Forma Scientific Inc., Marietta, OH).

2.3 Screening of Vegetable Oils as Supplements

Submerged fermentation was conducted in 250-mL Erlenmeyer flask containing 1.0 mL of dispersed spores from the seed medium and 100 mL of fresh basal medium. Experiments were performed by screening the supplementation of Submerged fermentation was conducted in 250-mL Erlenmeyer flask containing 1.0 mL of dispersed spores from the seed medium and 100 mL of fresh basal medium. Experiments were performed by screening the supplementation of 11 different kinds of vegetable oil at a concentration of 1% w/v for lovastatin production. The flasks were incubated at room temperature, on orbital shaker at 220 rpm for 7 days.

Effect of various amounts of vegetable oils as supplement. Submerged fermentation was conducted as described above. For studying the effect of different amounts of vegetable oil on lovastatin production, 1 – 5% w/v of vegetable oils was added to the culture medium. The flasks were incubated at room temperature, 220 rpm on orbital shaker for 7 days.

Effect of vegetable oils used as a sole carbon source. Submerged fermentation was conducted in 250-mL Erlenmeyer flask containing 1.0 mL of dispersed spores from the seed medium and 100 mL of fresh basal medium without lactose. Lactose used as a sole carbon source was substituted by selected vegetable oils with different concentrations (1 – 5% w/v) in the culture medium. Comparison between 1% w/v vegetable oil used as sole and supplementary carbon

TABLE 1. Selected Edible Cooking Vegetable Oils Used as Sole and Supplementary Carbon Sources for Lovastatin Production from *Aspergillus terreus*

Vegetable oil	Grade	Source
Camellia tea oil	Refined	Lam Soon (Thailand) Pcl., Samutprakarn, Thailand
Canola oil	Pure	Sime Darby Edible Products Ltd., Singapore
Coconut oil	-	Katevanich Industry Co., Ltd., Bangkok, Thailand
Corn oil	Refined	Lam Soon (Thailand) Pcl., Samutprakarn, Thailand
Olive oil	Extra virgin	Rafael Salgado SA, Madrid, Spain
Palm olein oil	Refined	P. S. Pacific Co., Ltd., Phetchaburi, Thailand
Rice bean oil	Extra-cold filtered	Coagro Co., Ltd., Bangkok, Thailand
Safflower oil	Refined	Ouiheng Health Consumer Co., Ltd., Bangkok, Thailand
Sesame oil	-	Chaiseri Co., Ltd., Chiang Mai, Thailand
Soybean oil	Refined	Morakot Industries Pcl., Samutprakarn, Thailand
Sunflower oil	Refined	Thanakorn Vegetable Oil Products Co., Ltd. Samutprakarn, Thailand

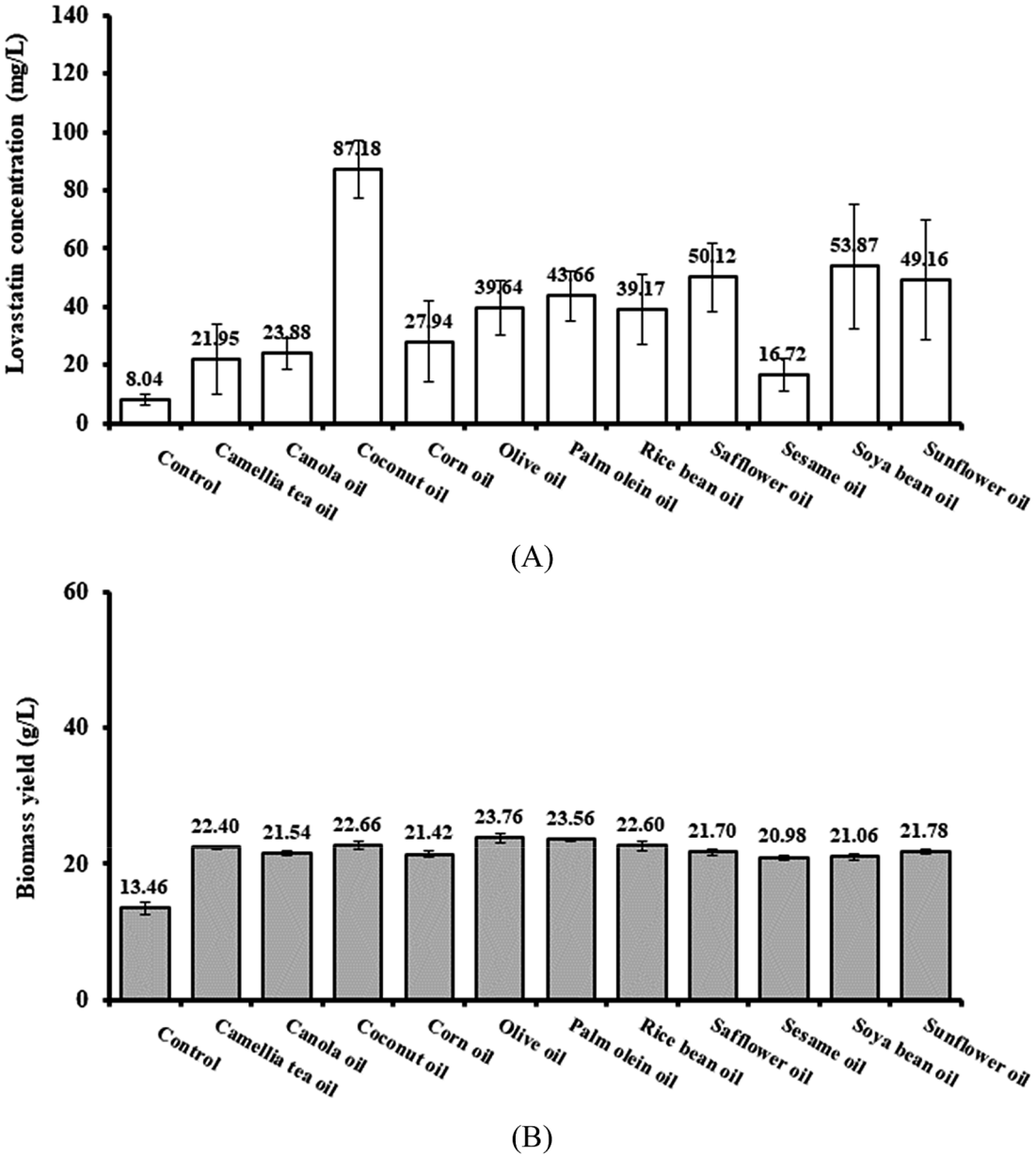

Fig. 1. Lovastatin production (A) and biomass yield (B) from *A. terreus* ATCC 20542 in culture medium supplied with various vegetable oils at 1% w/v. The level of concentrations and weight are expressed as the mean of five replicates ± SD. (Control: fermentation in the absence of vegetable oil).

sources was subsequently studied. All other medium compositions remained fixed and the flasks were incubated as described above.

2.4. Analytical Methods

Assay of lovastatin. At the end of fermentation process, the culture medium was separated from mycelia and adjusted to pH 3 with HCl. The 50-mL clear broth was extracted with ethyl acetate (1:1 v/v) by vigorously mixing for 10 min in a separatory funnel. After separation, 25 mL of organic layer was evaporated and the dried residue was then filled to 2.0 mL with mobile phase. The sample was filtered through 0.45 μm nylon syringe filter. Aliquots (25 μL) were analyzed by high-performance liquid chromatography (HPLC) system comprising Varian 9012 solvent delivery, Varian 9100 autosampler, Varian 9050 variable-wavelength UV-Vis detector (Varian, Palo Alto, CA), and using ODS Hypersil C-18 column (250×4.6 mm i.d.; 5 μm particle diameter; and 250 Å average pore size) (Thermo Electron Corporation, Waltham, MA), using a mobile phase consisting of acetonitrile, methanol and phosphate buffer saline with pH 4.0 (55:12:33 by volume), at a flow rate of 1.0 mL/min, with optical density

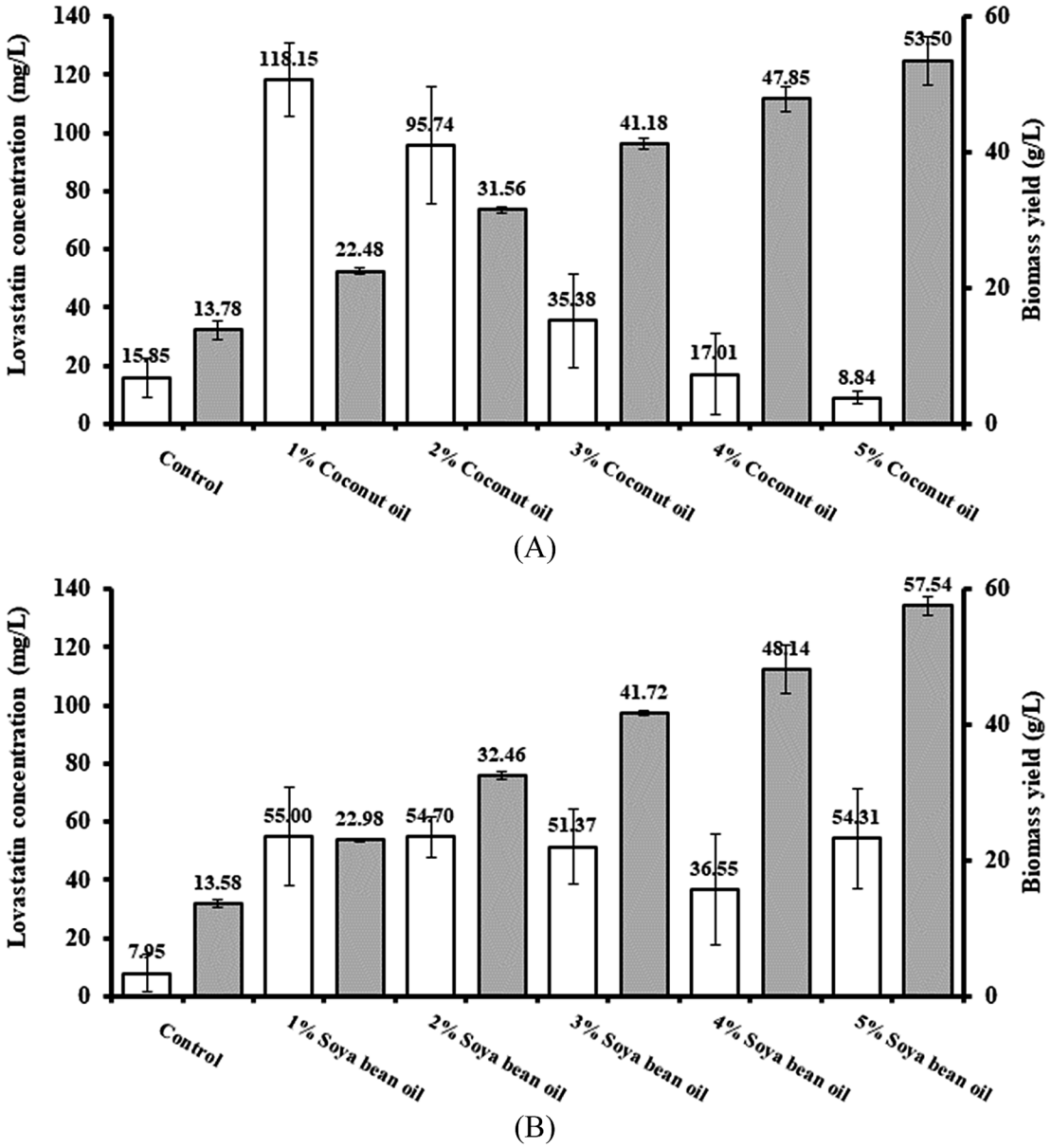

Fig. 2. Effect of different amounts of coconut oil (A) and soybean oil (B) in medium for supplementation on lovastatin production (white bar) and biomass yield (gray bar) in submerged fermentation of *A. terreus* ATCC 20542. The levels of concentrations and weights are expressed as the mean of five replicates ± SD. (Control: fermentation in the absence of vegetable oil).

read at 238 nm. The retention time of the lovastatin peak was observed approximately at 15 min. The lovastatin content was estimated from its concentration in the culture medium, calculated using a calibration curve of authentic sample ranging from 5 to 1000 μg/mL.

Biomass yield. At the end of fermentation, the culture medium was filtered through a filter paper No. 1 (Whatman Plc, Kent, UK), washed the mycelia with sterile distilled water, and then dried in an oven (Contherm Scientific Ltd., Wellington, New Zealand) for 24 h at 70°C. Samples were stabilized at room temperature before the measurement of dry cell weight.

3. RESULTS AND DISCUSSION

3.1 Screening of Vegetable Oils as Supplements

The composition of nutrient medium is one of the key factors that influence the lovastatin production and biomass yield from *A. terreus*. The culture medium usually contained lactose as a main carbon source with the nitrogen source like yeast extract. In the present study, vegetable oils such as camellia tea oil, canola oil, coconut oil, corn oil, olive oil, palm olein oil, rice bean oil, safflower oil, sesame oil, soybean oil, and sunflower oil have been used as a part of the carbon

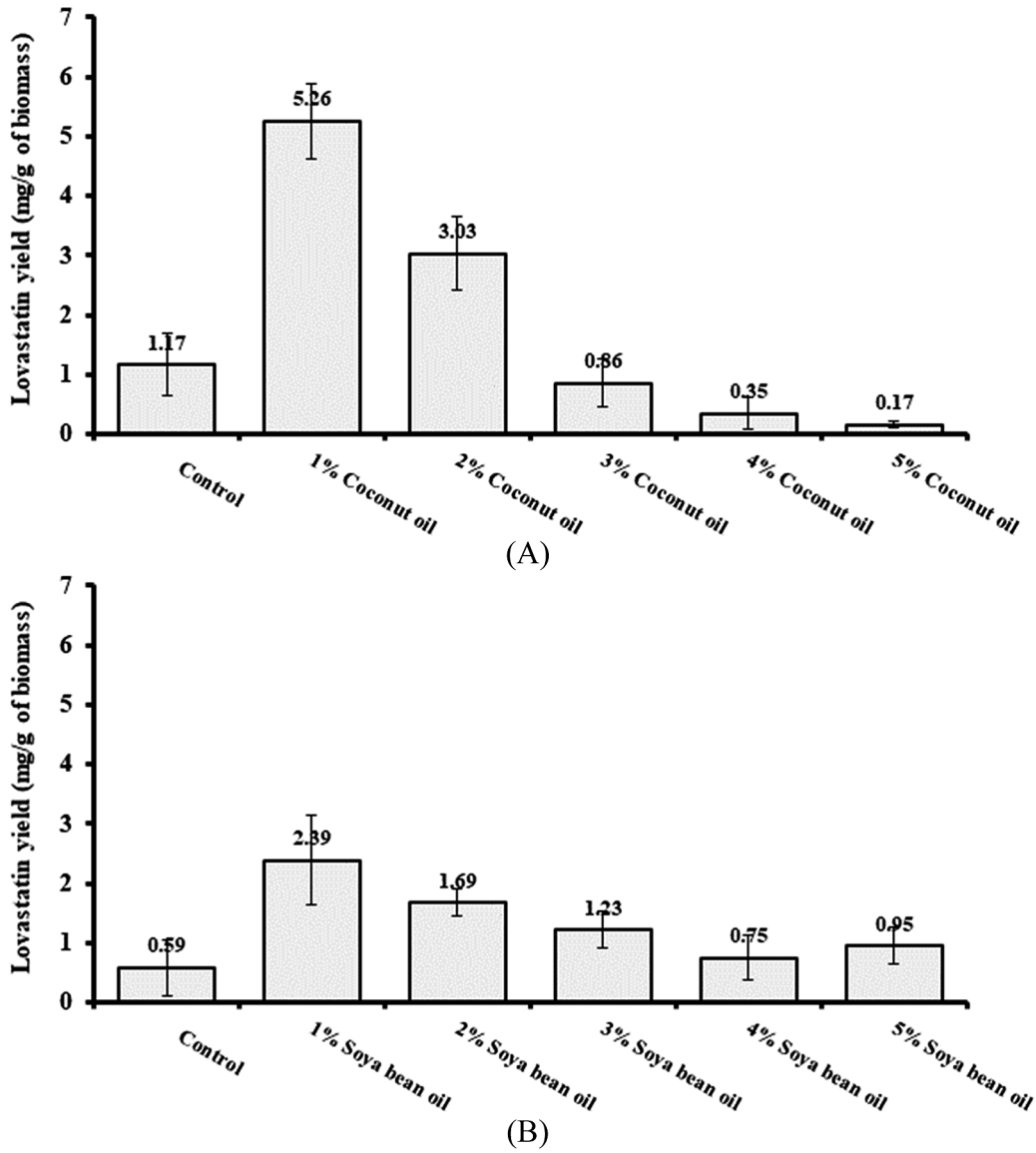

Fig. 3. Comparison of lovastatin yield from medium with various concentrations of coconut oil (A) and soybean oil (B) used as supplementary carbon sources in submerged fermentation of *A. terreus* ATCC 20542. The levels of concentrations and weights are expressed as the mean of five replicates ± SD. (Control: fermentation in the absence of vegetable oil).

source in the concertation of 1% w/v, meanwhile 1% w/v lactose was fixed as a sole carbon source. The production of lovastatin by *A. terreus* and their biomass using different vegetable oils for supplementation are shown in Figs. 1A and 1B, respectively. Best selected vegetable oils increased the product yields and dry weights of fungus compared with the control. Of the vegetable oils screened, the medium containing coconut oil gave the best production results after 7 days with almost 11 times greater yield than that from the oil-free control, while the lowest production was found with sesame oil. The other vegetable oils were roughly grouped according to the levels of lovastatin within 21 – 28 mg/L (camellia tea oil, canola oil and corn oil), 39 – 44 mg/L (olive oil, palm olein oil and rice bean oil) and 49 – 54 mg/L (safflower oil, soybean oil and sunflower oil). There was no difference in dried fungal weight among the vegetable oils used. Soybean oil has beneficial effect on lovastatin level in agreement with Sripalakit, et al. [11] and consistent with the previous reports for their supplements in the production of tetracycline [37], erythromycin [38], cephalosporin C [39] and clavulanic aid [40]. Therefore, coconut oil and soybean oil were both selected for further study in terms of the optimization of their concentrations.

Fatty acids are the major components of any edible vegetable oils [41]. Lovastatin produced by *A. terreus* may depend on the fatty acid composition of the oils used. The addition of linoleic acid can enhance the yield of lovastatin production by regulating the transcription of genes involved in

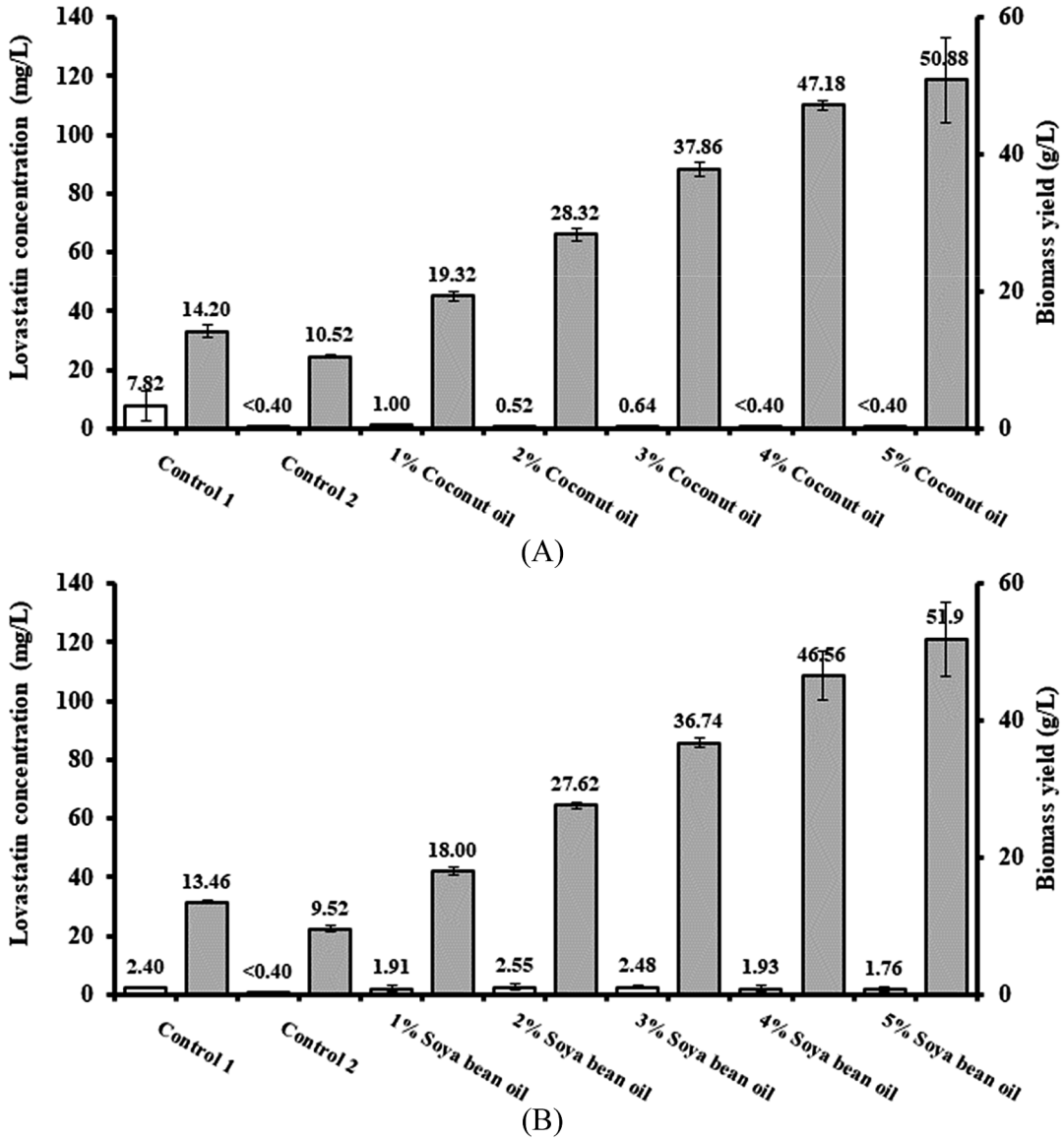

Fig. 4. Effect of different amounts of coconut oil (A) and soybean oil (B) as sole carbon sources on lovastatin production (white bar) and biomass yield (grey bar) in medium for submerged fermentation of *A. terreus* ATCC 20542. The levels of concentrations and weights are expressed as the mean of five replicates ± SD. (Control 1: fermentation in the absence of vegetable oil; Control 2: fermentation in the absence of vegetable oil and lactose).

biosynthesis pathway [42]. In our plant oils studied, linoleic acid and other unsaturated fatty acids such as oleic acid and linolenic acid are frequently found in different profiles. The exception is coconut oil, which mainly contains saturated fatty acids predominantly lauric acid [43]. This may be due to lauric acid that can act as inducer better than linoleic acid by using the same mechanism. Therefore, it seemed that the best oil for lovastatin production must have a high content of saturated fatty acids. This result was opposite to that of Hamedi, et al. [38] and Choi, et al. [36], where the supplementation of unsaturated fatty acids had positive effect on the synthesis of erythromycin and gentamicin, respectively.

3.2. Effect of Different Amounts of Vegetable Oils as Supplements

In general, lovastatin is an intracellular product and mostly accumulated in mycelia, thus its yield is proportional to the amount of biomass [17]. In this study, there was a correlation between the amounts of vegetable oils presented in the medium and the fungal growth, as shown in Fig. 2A and 2B for coconut oil and soybean, respectively. However, lovastatin production in the media containing coconut oil and soybean oil were independent of each other. An initial concentration of 1% w/v of coconut oil as an auxiliary carbon source gave the highest lovastatin concentration at

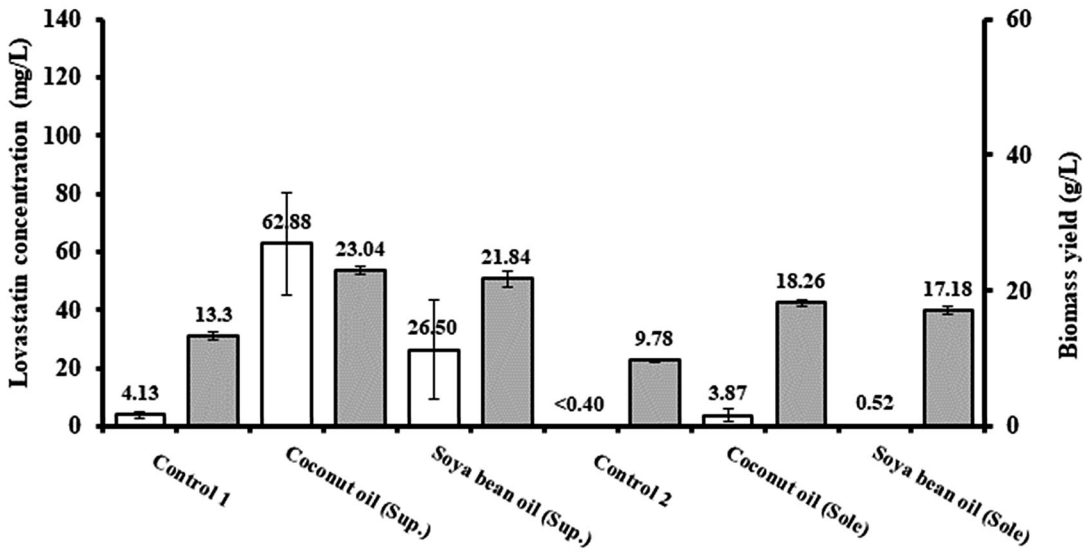

Fig. 5. Comparison of lovastatin production (white bar) and biomass yield (grey bar) between 1% w/v vegetable oil used as supplementary and sole carbon sources in medium for submerged fermentation of *A. terreus* ATCC 20542. The levels of concentrations and weights are expressed as the mean of five replicates ± SD. (Control 1: fermentation in the absence of vegetable oil; Control 2: fermentation in the absence of vegetable oil and lactose).

118.15 mg/L. An increase in coconut oil concentration from 1 to 5% w/v caused a strong (approximately 15-fold) decrease in the lovastatin concentration. On the contrary, increased addition of soybean oil at any concentration within 1 – 5% w/v did not influence the product concentration. In this case, an optimum medium for maximizing the product yield from submerged fermentation should be supplemented with vegetable oil to no more than 1% w/v.

As can be seen from Figs. 3A and 3B, increasing both vegetable oils does not seem to have a positive effect on the lovastatin yield. These results confirm our previous report [11] that adding either soybean oil or palm oil at higher concentration leads to lower yield. If a high concentration of oils is supplied, inhibitory fatty acids may accumulate in the medium, leading to decreased metabolite production. Moreover, unlike the morphology, our findings show no correlation between lovastatin production and microbial biomass. These results are consistent with the utilization of D-galactose in *A. terreus* fermentation, so that only metabolite production is inhibited but not its biomass growth [13]. However, Casas Lopez, et al. [6] suggested that the metabolic pathways for converting carbon to biomass are much faster than the pathways for the synthesis of lovastatin.

3.3. Effect of Vegetable Oils Used as a Sole Carbon Source

The results of lovastatin production in the medium containing vegetable oils as a sole carbon source at different concentrations of coconut oil and soybean oil, compared to that of the medium without any oil (Control 1) and the medium without both oil and lactose (Control 2), are shown in Figs. 4A and 4B, respectively. However, vegetable oils, especially soybean oil, could be used as a sole carbon source for various fungi to produce antibiotics such as cephamycin C [34], clavulanic acid [35] and gentamycin [36]. In this study of an alternative sole carbon source, the oil content and lovastatin production were apparently unrelated. At any quantities of both oils, the process gave metabolite concentrations less than 3% mg/L and lower than the control with lactose, while the amounts of product from the medium without any carbon source were very poor. The addition of vegetable oils can improve the fungal weight in the same way as found in the former experiment, but the fungi are unable to generate more metabolite. Sethi, et al. [44] reported that the mycelial growth of *A. terreus* NCFT 4269.10 was achieved by supplementing the base medium with natural oils. Additionally, *A. terreus* can grow under conditions of carbon starvation under adequate supply of nitrogen source [6]. These observations suggest that increasing concentration of vegetable oils tend to favor mycelial growth instead of metabolite production.

Furthermore, to compare the effect of vegetable oils applied as either sole or supplementary sources of carbon under similar culture conditions, Fig. 5 confirms that lovastatin produced in the medium consisting of either coconut oil or soybean oil used as a single substrate was lower than that in oil-additional medium. More importantly, the lack of lactose in the medium resulted insignificantly lower lovastatin production. Hence, carbon sources especially sugars are directly relevant to the regulation of gene expression and enzyme activity for polyketide pathway [45].

Further investigations are needed using various pure fatty acids, especially lauric acid, in order to find out which component in the vegetable oils is the most effective for lovastatin production.

ACKNOWLEDGEMENTS

The authors wish to acknowledge Miss Janya Riunkesorn for her assistance. This study was financially supported by the National Research Council of Thailand (NRCT) and the Research Funds from Government Budget, Naresuan University, Thailand (grant number. R2554B058).

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