Effects of Carbon Source on the Production of Lovastatin by *Aspergillus niger* van Tieghem Under Submerge Fermentation

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Abstract

This study aimed to determine the effects of the interaction in the production of lovastatin between carbon sources (lactose, honey and glucose as the control) in four different incubation periods (3, 6, 9 and 12 days) with 1% w/v and 2% w/v concentrations. Results showed that honey was the best carbon source in producing an average concentration of 827.11 ± 382.20 µg mL⁻¹ lovastatin. One percent (w/v) of carbon source produced 1084.00 ± 188.70 µg mL⁻¹ of lovastatin, which is higher than the one produced at 2% (w/v) (396.30 ± 102.44 µg mL⁻¹). Highest yield (815.82 ± 469.07 µg mL⁻¹) of lovastatin was obtained on the sixth day of incubation using honey as the carbon source.

Keywords: Lovastatin production; *A. niger* van Tieghem; Carbon source

Introduction

Lovastatin (C_{24}H_{36}O_{5}) is a member of the drug class of statins and is a secondary metabolite of the fermentation process of various fungi. It is an effective inhibitor of the enzyme that catalyzes the rate-limiting step in cholesterol biosynthesis. This cholesterol-lowering drug acts by competitively inhibiting the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoA) (Alberts *et al.*, 1980; Alberts, 1988). Lovastatin is active not only *in vitro*, in inhibiting cholesterol biosynthesis, but also *in vivo*, in lowering plasma cholesterol levels in humans and animals (Kaneko *et al.*, 1978), thereby making it effective in the therapy of hypercholesterolemia.

Hypercholesterolemia is a primary risk factor for coronary artery disease, the major cause of death in many countries (Goldstein & Brown, 1984). The World Health Organization reported that cardiovascular diseases (CVD) claimed 17.3 million lives in 2008 and predicted that an estimated 23.6 million people will die of CVD by the year 2030. The Philippine National Statistical Coordination Board (PNSCB) also recorded a number of registered deaths that has increased by 16.5% in 2000, 20% in 2008 and 21% in 2009.

Nowadays, lovastatin and its semisynthetic derivatives are very important drugs since the mortality of heart disease is becoming relatively high. Microorganisms, such as fungi, are producers of several pharmaceutical compounds, like lovastatin. Although major improvements in research are generally...
ascribed to the development of superior strains of fungi, nutrient supplies such as carbon source also affect their cellular productivity. In the study that was conducted, *Aspergillus niger* van Tieghem was used for the production of lovastatin, since it is an omnipresent fungus that could produce higher yields of lovastatin, by establishing and optimizing a defined culture condition for the fungus, at a cheaper cost that is commercially produced by submerged fermentation.

As reported by Frisvad *et al.* (2011), *Aspergillus niger* is one of the most important industrial filamentous fungal species used in biotechnology wherein it is used extensively for organic acid production and for the production of extracellular enzymes. Further significant applications include its use as a transformation host of heterologous proteins and secondary metabolites due to the fungus’ high growth rate, low-pH tolerance and high polyketide production rate. Another study by F. Mouafi *et al.* (2016) focused on the screening of various fungal species for lovastatin production using different agro-based wastes. They found out that all different fungal strains in sugar bagasse, olive cake and potato peel yielded good amounts of lovastatin. The yields, specifically by *A. niger*, were 2.41, 2.28 and 2.25 mg/g dry fermented matter, respectively. The factors mentioned are the reasons why *Aspergillus niger* could be one of the good candidates in the production of lovastatin. The effects of the type and concentration of carbon source as well as the incubation period of the fungi on the yield of lovastatin have been investigated.

**Materials and Methods**

**Microorganism**

*Aspergillus niger* van Tieghem was obtained from the Philippine Rootcrops Research and Training Center, Visayas State University, Baybay City, Leyte. The fungus was subcultured on potato dextrose agar (PDA) plates and incubated for 3 days at 28°C. The plates were stored in the refrigerator at 4°C until use.

**Culture Media**

A two-stage culture technique was applied based on the general production of secondary metabolite: seed culture and production culture. The preparation of the seed medium constituents was done and was autoclaved at 121°C, 15 psi for 15 minutes. A mycelial disc (about 0.5 cm) was obtained from the culture grown from a petri dish with PDA at 4°C, and was placed in the broth seed medium. About 600 mL of seed cultures was incubated at 28°C for 24 hours in a rotary shaker at 140 rpm (Siamak *et al.*, 2003).

Six 600-mL production medium, following the procedure of Siamak *et al.* (2003), were prepared separately in six 1000-mL conical flask, in which every three flasks had different concentrations (1% w/v and 2% w/v), and contained different carbon sources (honey, lactose and glucose as the control). The flasks were adjusted to pH 7. Afterwards, 45 mL of production medium was transferred into a 125-mL Erlenmeyer flask and was autoclaved again. Five mL of the seed broth was added as the inoculum and was cultured in the 45 mL production medium, thereby totaling to 50 mL of the production culture contained in the flask. The cultures were incubated at different incubation periods and were swirled ten times every other 2 hours at room temperature.

**Effect of Incubation Period on the Production of Lovastatin**

The effect of incubation period on lovastatin production was investigated using 4 incubation periods (3, 6, 9 and 12 days). The flask was incubated at room temperature (20-25°C). At the end of the incubation period, the lovastatin in the cultured liquid was extracted and analyzed.
Extraction of Lovastatin

The extraction of lovastatin was done following the procedure of Siamak et al. (2003). The culture liquid was adjusted to pH 3 by addition of 6 M HCl. Fifty mL of ethyl acetate was added and shaken at 180 rpm for 2 hours. To separate the organic phase and the aqueous phase, the mixture was then centrifuged at 1500xg for 15 minutes. The organic phase was further treated. The organic phase was completely evaporated in a rotary evaporator at 60°C and the dried residue was dissolved in 10 mL absolute ethanol.

Assay of Lovastatin

Lovastatin was analyzed as described by Rajput & Raj (2009); Jaivel and Marimuthu (2010). About 0.1 mL from each sample was taken into a 10-mL volumetric flask, and the volume of liquid was made up to mark with 20% ethanol. The absorbance of the diluted sample was measured by a UV-Vis spectrophotometer at 238 nm using 20% ethanol as blank. The concentration of lovastatin was calculated from the pure lovastatin standard curve.

Standard Curve

Pure lovastatin was purchased from Merck. A stock solution (40µg mL^{-1}) of pure lovastatin was prepared by dissolving 4 mg of lovastatin in 20 mL pure ethanol, and the volume was adjusted to 100-mL mark with distilled water. Using the dilution technique, fifteen concentrations were prepared in 10-mL volumetric flasks. The solvent used in the dilution up to the 100-mL mark was 20% ethanol. Using the UV-Vis Spectrophotometer (LabMed, Inc.), the absorbances of the different concentrations of standard lovastatin were measured at 238 nm.

Experimental Design

The study was laid out in a 3-Factor Factorial Experiment in a Completely Randomized Design (CRD), with three replications. Twenty-four treatments were prepared as follows:

Results and Discussion

Effect of Carbon Source on the Production of Lovastatin

The composition of a fermentation medium influences the supply of nutrients and metabolism of cells in a medium, therefore the productivity of a fermentation process depends on the culture medium used. Also, the nature and concentration of the carbon source can regulate secondary metabolism through catabolic repression. The effect of the concentrations of several carbon sources on lovastatin production by Aspergillus niger van Tieghem was studied, and a significant interactive effect of the medium constituents on lovastatin titers was observed.

Table 1 shows the concentration of lovastatin produced when employing honey, glucose (control) and lactose as the sources of carbon. The trend of lovastatin production is in the order: honey (827.11 ± 382.20 µg mL^{-1}) > glucose (726.50 ± 415.09 µg mL^{-1}) > lactose (666.61 ± 379.78 µg mL^{-1}). Comparatively, however, the values of 726.50 ± 415.09 µg mL^{-1} and 666.61 ± 379.78 µg mL^{-1} are not significantly different. Honey can be considered as the best carbon source among the sources being studied in the production of lovastatin. Carbohydrates are the main constituents of
Table 1. Concentration (µg mL\(^{-1}\)) of lovastatin as affected by the level of carbon source

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Concentration of lovastatin (µg mL(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Honey</td>
<td>827.11* ± 382.20</td>
</tr>
<tr>
<td>Glucose (control)</td>
<td>726.50* ± 415.09</td>
</tr>
<tr>
<td>Lactose</td>
<td>666.61* ± 379.78</td>
</tr>
</tbody>
</table>

* Values are expressed as means ± SD of triplicate determinations. Means followed by the same letter are not significantly different at α = 0.05, Tukey HSD

Table 2. Concentration (µg mL\(^{-1}\)) of lovastatin as influenced by the level of carbon source

<table>
<thead>
<tr>
<th>Concentration of carbon source (% w/v)</th>
<th>Concentration of lovastatin (µg mL(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1%</td>
<td>1084.00* ± 188.70</td>
</tr>
<tr>
<td>2%</td>
<td>396.30* ± 102.44</td>
</tr>
</tbody>
</table>

* Values are expressed as means ± SD of triplicate determinations. The means followed by the same letter are not significantly different at α = 0.05, Tukey HSD

honey, comprising about 95% of the honey’s dry weight. Oxidation of these carbohydrates by the fungus results in the formation of nine acetyl-CoA molecules, the substrate in the first steps of lovastatin biosynthesis (Buba et al., 2013). Two molecules of acetyl-coA are produced from one glucose molecule.

According to Szczęsna (2006), among the long-chain fatty acid compositions of the honeybee-collected pollen, the predominating acid appeared to be linolenic acid (43% of the fatty acids), followed by palmitic acid (28%) and linoleic acid (14%). Acetyl-coA is produced from the oxidation of fatty acids in the pathway of beta-oxidation. The presence of fatty acid in honey can result to the production of more acetyl-CoA.

Effect of the Concentrations of the Carbon Source on the Production of Lovastatin

One percent (w/v) carbon source leads to the production of higher (1084.00 ± 188.70 µg mL\(^{-1}\)) lovastatin concentration by the fungi than 2% (w/v) (396.30 ± 102.44 µg mL\(^{-1}\)) (Table 2, Figure 1). The lovastatin produced from the two concentrations of carbon source differed significantly.

The results of the study conform with the findings of Sripalaket et al. (2011) about using utilized vegetable oils in the production of lovastatin by Aspergillus terreus ATCC 20542 in submerged cultivation. An oil concentration of 0.5% resulted in the highest lovastatin production for both palm oil and soya bean oil; the higher concentrations (1%, 2% and 3%) tended to give lower yields. Their result indicated that the biomass increased with increasing oil concentration, which suggests that cell growth is related to vegetable oil concentration. These results agreed with their previous report, which showed that the metabolic pathways governing the synthesis of lovastatin from a carbon source were slower than the pathways converting carbon to biomass.

Effect of the Incubation Period

Varying the incubation periods (3, 6, 9, and 12 days) was found to influence the production of lovastatin. The increase in the incubation period resulted in an increase in lovastatin production, with a maximum concentration of 815.80 µg mL\(^{-1}\) in cultures incubated for 6 days (Table 3). All the other incubation periods resulted in lovastatin productivities which were not significantly lower than the concentration obtained on the 6th day of incubation. On the other hand, lovastatin production decreased with further extension of the incubation period to 12 days yielding 791.12 µg mL\(^{-1}\). Similar observations were reported by Siamak et al.
(2003), in the case of *Aspergillus terreus*. They investigated the production of lovastatin at 7 days of incubation, with a level of 55 mg lovastatin per liter of the screened production medium.

During the first phase of growth (trophase) the mycelial growth increased exponentially, and little or no secondary metabolites were produced. This scenario was observed in 3rd day, which was significantly lower than the other higher incubation periods, yielding only 597.07 µg mL⁻¹. When the fungus enters the second phase (idiophase), the biomass becomes stable, and secondary metabolites are synthesized until lysis starts (Burrow et al. 1961; Bu’lock, 1975; Martin and Demain, 1980). Also, the decrease in yield after 6 days may be a manifestation of the onset of the death phase of the organism caused by nutrient depletion. Previous reports indicated that incubation periods of 6-10 days were optimal for lovastatin production by various fungi (Endo, 1979; Moore *et al.* 1985; Gunde-Cimerman *et al.* 1993; Shindia, 1997).

There was a difference between the yields of lovastatin of the two tested concentrations, as influenced by the incubation time. As shown in Figure 2, 1% concentration produces the highest yield of lovastatin at 6th day of incubation and decreases at the 9th day, then increases again at the 12th day. This result agrees with the findings of Burrow et al. (1961), Bu’lock (1975) and Martin and Demain (1980). On the other hand, the 2% carbon source concentration was in its maximum lovastatin production at the 9th day of incubation and decreased at the 12th day, which was different from the trend of the addition of 1% carbon source. By looking at the average yield of lovastatin of the two concentration of carbon source, it was found that the 6th day of incubation is the best incubation period in the production of lovastatin using *Aspergillus niger* van Tieghem.

**Conclusions**

Using submerged fermentation, twenty four treatments with three replications were carried out for the production of lovastatin with carbon source, concentration and incubation periods as variables. The interaction between the two carbon sources (lactose and honey) in four different incubation periods (3 days, 6 days, 9 days and 12 days) were used to optimize the lovastatin production in two carbon concentrations. The concentration of lovastatin was determined using the UV- Vis Spectrophotometer at 238 nm.

Results showed that *Aspergillus niger* van Tieghem produced the highest lovastatin concentration at the 6-day incubation period. Honey, used as carbon source by *Aspergillus niger* van Tieghem produced higher lovastatin concentration than lactose. Also higher lovastatin concentration was produced using 1% (w/v) carbon source concentration than using 2% (w/v) carbon source concentration.
References Cited


