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Original Research Article

Biosynthesis of lovastatin using agro-industrial wastes as carrier substrates

Sadia Javed[1]*, Munazzah Meraj[2], Saqib Mahmood[3], Arruje Hameed[1], Farah Naz[4], Sameera Hassan[4] and Rao Irfan[5]

[1]Department of Applied Chemistry & Biochemistry, Government College University, Faisalabad, [2]Department of Biochemistry, Peoples University of Medical and Health Sciences, Nawabshah, [3]Department of Botany, Government College University, Faisalabad, [4]Centre of Excellence in Molecular Biology, University of Punjab, Lahore, [5]Department of Pharmacy, Peoples University of Medical and Health Sciences, Nawabshah, Pakistan

For correspondence: **Email: drsadiajaved@yahoo.com*

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Abstract

Purpose: *To compare fungal strains including Aspergillus flavipes GCBL-72, Aspergillus flavus GCBL-60, and Aspergillus niger GCBL-45 and determine whether solid- or liquid-state fermentation (SSF or LSF) is more appropriate for lovastatin production using various inexpensive raw materials.*
Methods: *LSF and SSF techniques were used to produce the drug lovastatin. High-performance liquid chromatography was performed out to quantify lovastatin production. A kinetic growth model was applied to estimate product formation at the expense of substrate utilization.*
Results: *Aspergillus flavus GCBL-60 was a superior lovastatin-producing strain consuming wheat bran as the raw material in SSF. The optimum lovastatin production was 28.36 ± 0.76 mg/100mL at 35 °C, pH 5.5, inoculum size 2 mL, 96 h incubation time, and 60 % moisture content. Evaluation of the kinetic growth parameters for lovastatin production confirmed that product formation was improved after fermentation parameter optimization.*
Conclusion: *Our results indicate that Aspergillus flavus GCBL-60 was best lovastatin-producing strain and that SSF was superior to LSF for maximum production. Careful optimization can enhance product formation.*

Keywords: *Hypercholesterolemia, Kinetics, Optimization, Lovastatin, Solid-State Fermentation, Raw materials*

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INTRODUCTION

Lovastatin has an important role in lowering blood cholesterol levels in both human and animals, thus diminishing the risk of hypercholesterolemia. It works by inhibiting the 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, thereby limiting the rate of cholesterol synthesis [1]. This compound is produced as a fungal secondary metabolite. Simvastatin can be derived from fermented lovastatin by enzymatic deacylation of lovastatin. Various *Aspergillus* species such as *Aspergillus niger*, *Aspergillus terreus*, *Aspergillus flavus*, *Aspergillus flavipes*, *Aspergillus parasiticus* and some Monascus species including *Monascus rubber*, *Monascus paxi*, *Monascus anka*, and *Monascus purpureus* can produce this compound [2,3].

Lovastatin was first isolated from *Monascus rubber* for commercial purposes, but *Aspergillus terreus* remains the organism of choice for lovastatin biosynthesis. The lovastatin produced

in fermentation broth medium is the open hydroxy form, which is the biologically active form. The synthetic form of lovastatin is always the lactone form, which is converted to the open hydroxy acid form *in vivo* (Fig. 1) when administered to patients [4]. The solid-state fermentation (SSF) technique is considered to be the best approach for lovastatin production using fungal strains because it offers numerous advantages over liquid-state fermentation (LSF) such as low energy requirements, less waste water, and a large surface area for fungal growth [5-7]. In addition to treating hypercholesterolemia, lovastatin has other clinical applications for coronary heart disease, Alzheimer disease, renal diseases, cancer, bone fracture disease, and multiple sclerosis [8-10].

The present project was designed to identify the best lovastatin-producing fungal strain and optimize fermentation parameters using agro industrial wastes as carrier substrates. This information is valuable for enhancing the bulk production of the cholesterol-lowering drug lovastatin using indigenous sources, which facilitates environmental friendly management of renewable resources.

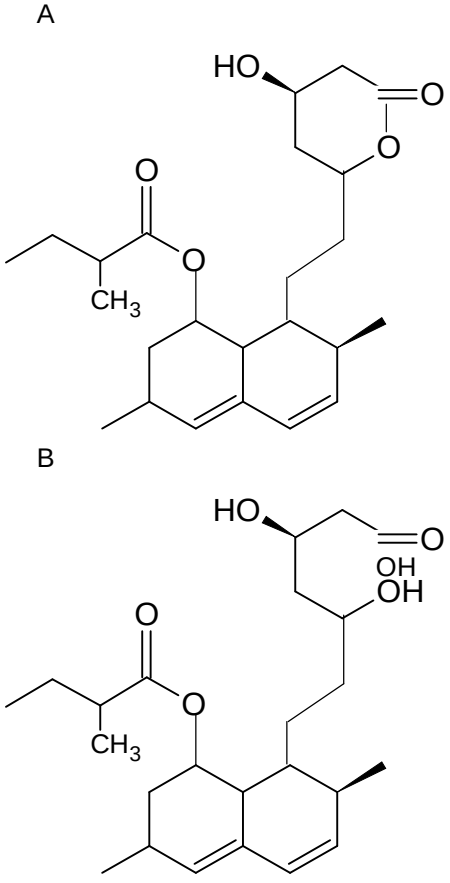

Figure 1: Structural formulae of the (A) lactone and (B) open hydroxy acid forms of lovastatin

EXPERIMENTAL

The pure cultures of fungal strains were isolated by our research group and grown on potato dextrose agar (PDA) slants. They were stored in a refrigerator at 4°C to avoid contamination.

Inoculum preparation

Vogel's medium (KH_2PO_4, 0.5; peptone, 0.1; yeast extract, 0.2; NH_4NO_3, 0.2; $(NH_4)_2SO_4$, 0.4; $MgSO_4$, 0.02; glucose, 50 %; trisodium citrate, 0.5 g/100mL) was prepared in 500-mL flasks. The medium pH was maintained at 5.5 using 0.1M NaOH/HCl solutions. It was sterilized in an autoclave at 121 °C for 15 min. Fungi spores were picked by loop and added into the flask. These flasks were positioned in an orbital shaker at 120 rpm for 48 h (30 °C).

Spore quantification

Spores were counted using a hemocytometer under low-power microscope using the method of Kolmer *et al* [11].

Fermentation medium cultivation

A comparison of two fermentation techniques was made using 250-mL conical flasks kept in a temperature-controlled incubator. For SSF, 5 g wheat bran was placed in triplicate flasks, and inoculum medium was added to keep the substrate at 60 % moisture. After maintaining the pH at 5.5, the flasks were sterilized in an autoclave for 15 min at 121 °C. Next, 5 mL spores of individual fungal strains were inoculated in separate flasks and placed in an incubator for 72 h. For LSF, 100 mL Vogel medium was placed in 250-mL conical flasks. The samples were sterilized at 121 °C for 15 min in an autoclave after verifying the pH. After cooling to room temperature, they were inoculated with 5 mL of fungal spores individually and kept at 30 °C for 72 h on a shaker.

Lovastatin extraction

For SSF, distilled water was added to bring the volume up to 100 mL, and the flasks were placed on a shaker for 1h. These flasks were then acidified with 10 % 1 N HCl to pH 3.0 after 72 h for both SSF and LSF. An equal volume of ethyl acetate was added to the acidified broth, which was kept on a shaker for 2 h at 70 °C. This broth was filtered to separate out the biomass and filtrate, followed by centrifugation at 4,000 g for 8 min before the upper phase was collected. Next, 10 mL 1 % trifluoroacetic acid was added to 1 mL

of the organic phase for the lactonization process, and the extract was vaporized at 80 °C.

Analytical procedures

Lovastatin samples were analyzed using high-performance liquid chromatography (HPLC). The concentrated lovastatin was diluted at a 1:1 ratio with acetonitrile and passed through a filtration assembly (0.45 µm) The samples (20 µL) were injected into the HPLC apparatus (Shimadzu LC-10AT) by syringe and analyzed with a UV detector at 238 nm and Shim pack CLS-ODS (C-18) column at a flow rate of 1mL/min. The mobile phase consisted of 0.1 % phosphoric acid and acetonitrile with a volume ratio of 40:60 [12].

Kinetic growth parameter determination

All kinetic parameters for lovastatin yield and biomass were calculated by the method as described by Okpokwasili and Nweke [13]. The kinetic parameters were studied before and after optimization of culture conditions in the fermentation process.

Statistical analysis

Comparisons of means were done by Duncan's multiple range (DMR) tests, and the statistical significance of differences among means was estimated by analysis of variance (ANOVA) under a complete randomized design [14].

RESULTS

This study was performed to screen three *Aspergilli* strains (*Aspergillus flavipes GCBL-72*, *Aspergillus flavus GCBL-60,* and *Aspergillus niger GCBL-45*) and compare two fermentation techniques for natural lovastatin production using wheat bran, wheat straw, corn stover, and banana stalk as carrier substrates. It was found that the *Aspergillus flavus* GCBL-60 strain had the best capacity to produce lovastatin (23.57± 1.31 mg/100mL) using wheat bran and the SSF technique (Figure 2) as compared with the LSF technique (16.42 ± 2.61 mg/100mL).

Fermentation parameter optimization

An optimum pH is necessary for all biological processes because living things are sensitive to pH change. The greatest amount of lovastatin was produced (23.33 ± 1.57 mg/100mL) with a dry cell mass (5.83 ± 0.051g) at pH 5.5 by *Aspergillus flavus* GCBL-60 (Figure 3A) using SSF. Initially, lovastatin production was enhanced by increasing the pH (3.0-5.5), and it was gradually decreased by further increasing the pH.

Temperature is one of the most useful fermentation factors to optimize because it has a direct influence on lovastatin production. The maximum yield of lovastatin obtained was 25.30 ± 1.75 mg/100mL with a dry cell mass of 6.36 ± 0.93 g by *Aspergillus flavus* GCBL-60 at 35 °C (Figure 3B).

To attain maximize lovastatin yield, media were prepared in different conical flasks with various inoculum sizes (1.0-5.0 mL). The maximum lovastatin yield (26.68 ± 1.34 mg/100mL) and dry cell mass (6.34 ± 1.21 g) were found at 3.0 (Figure 3C). Moisture content optimization is necessary for maximum production of secondary metabolites in SSF. A lower moisture level is very important to avoid fermentation medium contamination and suboptimal product formation due to reduced mass transfer processes, such as diffusion of gas and solutes to the cell. It was observed that the maximum lovastatin yield (27.88 mg/100 mL) was obtained at 60 % moisture content using wheat bran by *Aspergillus flavus* GCBL-60 (Figure 3D).

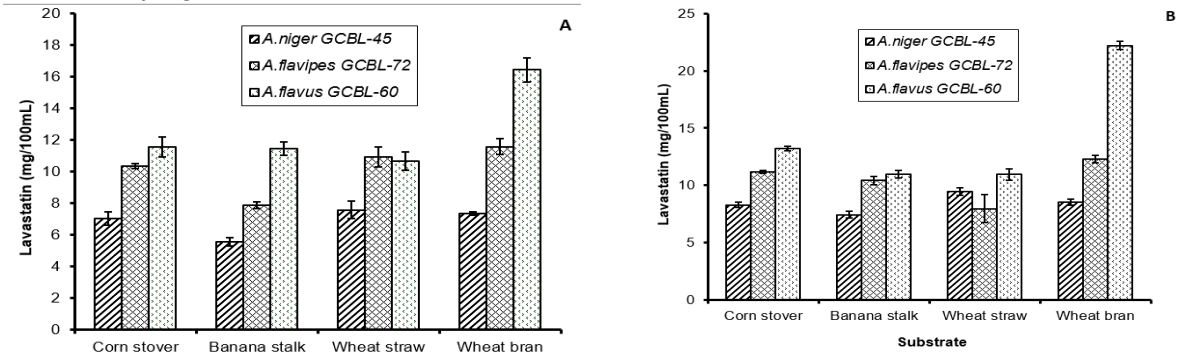

Figure 2: Screening of three fungal strains (*Aspergillus niger* GCBL-45, *Aspergillus flavipes* GCBL-72, and *Aspergillus flavus* GCBL-60) for lovastatin production in (A) LSF and (B) SSF. Values (mean± SD, n= 3, $p \leq 0.05$)

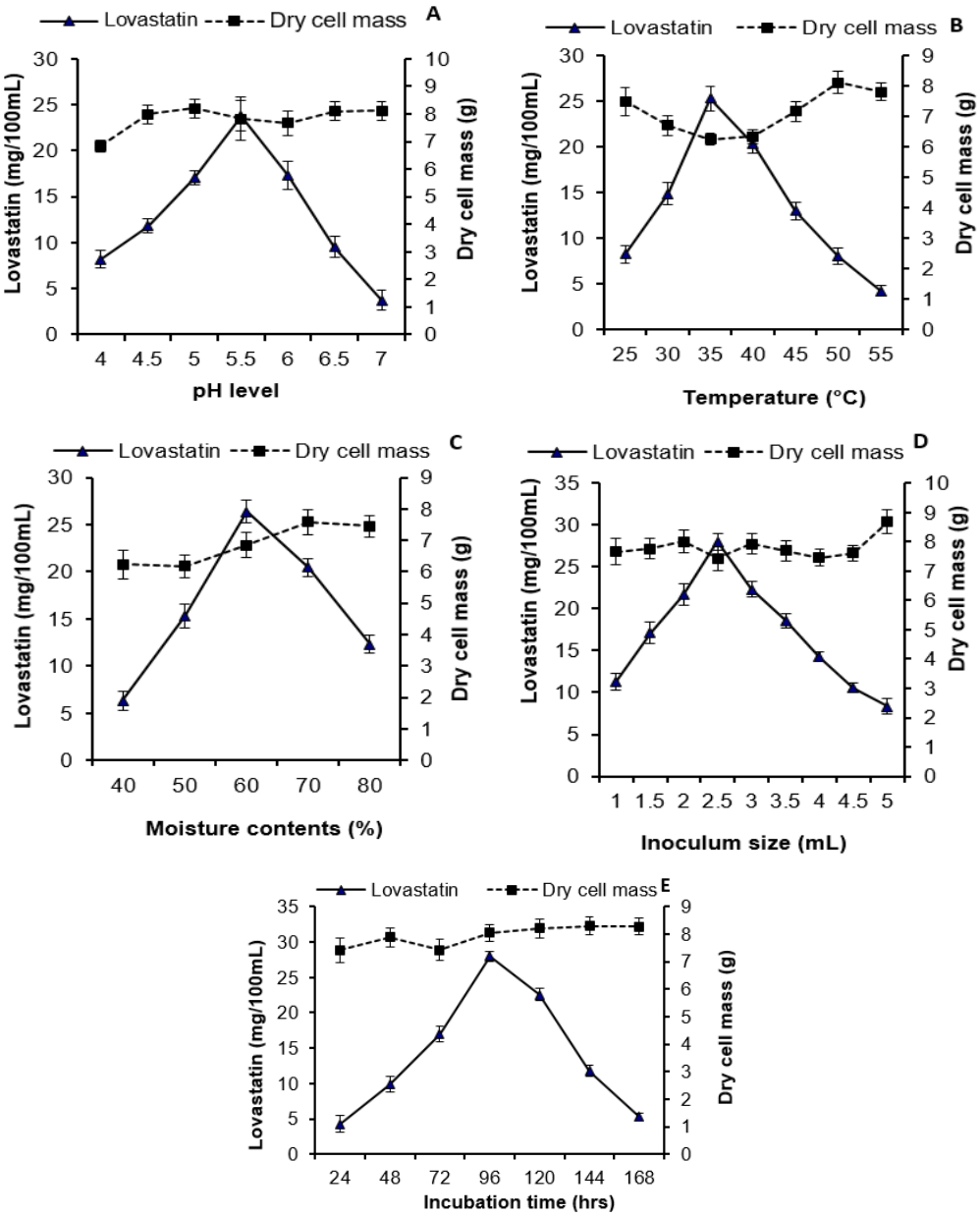

Figure 3: Effects of (A) pH, (B) temperature, (C) inoculum size, (D) moisture content and incubation time on lovastatin production by *Aspergillus flavus* GCBL-60 using wheat bran in SSF. Values (mean± SD, n= 3, $p \leq 0.05$)

Time is one of the most important factors for optimizing any fermentation medium. It was found that the optimum incubation time was 96 h with maximum lovastatin production (28.41 ± 0.33 mg/100 mL) for *Aspergillus flavus* GCBL-60 (Figure 3E). A longer incubation time did not enhance lovastatin production. This might be due to fungal strain age, nutrient depletion, and low nitrogen availability in the medium.

Kinetic parameters

Kinetic parameters of substrate consumption and product formation were estimated before and after optimization of lovastatin production by *Aspergillus flavus* GCBL-60 (Table 1). After optimization, the highest lovastatin quantity (28.41 ± 0.33 mg/100 mL) was noted for *Aspergillus flavus* GCBL-60. Growth parameters were also compared before and after optimization (Table 1). Before optimization, *Aspergillus flavus* GCBL-60 exhibited levels of substrate consumption parameters Yx/s and Yp/x and product formation parameter Yp/s (1.43, 3.18 and 0.44 mg/g, respectively). On optimization, it was observed that these growth parameters were further enhanced to 2.74, 5.60 and 0.489 mg/g, respectively (Table 1). It was also observed that the Q_s, q_s, and Yx/s parameters significantly increased after optimization, indicating that *Aspergillus flavus*

Table 1: Kinetic parameters for lovastatin production by *Aspergillus terreus* GCBL-60 in SSF

Kinetic parameter	*Aspergillus flavus* GCBL-60	Pre-optimization	Post-optimization
Substrate consumption	[A]μ(h^{-1})	0.084	0.190
	[B]Yx/s	1.43	2.74
	[C]Yp/x,	3.18	5.60
	[D]qs(g/g/h)	0.023	0.021
	[E]Qs(g/L/h)	0.16	0.11
Product formation	[F]Qx(g/L/h)	0.072	0.056
	[G]Yp/s	0.44	0.489
	[H]qp (Product/h/g)	0.033	0.058
	[I]Qp (Product/L/h)	0.23	0.314

Lovastatin was produced in Vogel's medium with an initial sugar concentration of 20 g/L (w/v). [A]μ (h^{-1}), Specific growth rate; [B]Yx/s, Drug produced/g glucose consumed; [C]Yp/x, Drug produced/g cell formed; [D]qs (g/g/h), g sugar consumed/g cells/h; [E]Qs (g/L/h), g sugar consumed/L/h; [F]Qx (g/L/h), g cells formed/L/h; [G]Yp/s, Cell formed/g sugar consumed; [H]qp (Product/g/h), Drug produced/g cells/h; [I]Qp (Product/L/h), drug produced/L/h

GCBL-60 grew faster under the new conditions. The maximum product formed (Q_P) was 0.23 g/L/h before optimization, and this improved to 0.314 g/L/h after optimization.

DISCUSSION

Fermentation is a technique that produces secondary metabolites using inexpensive raw materials under SSF. It offers major advantages including cost-effective raw material, simple downstream processing, and less water use [15]. In SSF, fungus directly adsorbs on the substrate and uses it as an energy source to secrete a higher amount of the desired product. Substrate particle size is very important to provide higher porosity for better heat and mass transfer for maximum lovastatin production. Highest lovastatin production (85.88 mg/L) by *Aspergillus flavus* UICC 360 was reported by Mangunwardoyo *et al* [16], and a maximum lovastatin concentration (113 μg/mL) by *P. ostreatus* was observed by Lakshmanan and Radha [17].

Biological fluids are very sensitive to changes in physiological pH. However, maximum lovastatin production was observed at pH 7.0 by Latha *et al* [22]. We found that further increase in pH decreases product formation, while low pH prevents contamination and also denatures or inactivates the fungal strain. The pH range for most of the fungi is reported as 3.5-7.0. The pH affects the transport of different ions and substances across the cell membrane, which supports product formation and cell growth [1].

Fermentation medium temperature is one of the most important factors that directly influences microorganism growth. Temperature affects the production of secondary metabolites, and this effect is variable according to media composition. It was previously reported that the maximum lovastatin production occurs at 28 °C, and further increases in temperature decrease the yield [19]. Optimum lovastatin production was observed at 30 °C. This temperature might be best for the sporulation, proliferation, and growth of mycelial masses. However, Attalla *et al* [20] obtained maximum lovastatin production at 28 - 30 °C.

Appropriate inoculum size is necessary in SSF processes because inappropriate sizes may lead to inaccurate data. Inoculum with too few spores may lead to insufficient biomass, while too many spores results in biomass overproduction and subsequent depletion of nutrients in the medium. These findings are in agreement with those of Latha *et al* [22], who reported the highest amount of lovastatin produced with a 2 % culture of *Aspergillus fischeri*. Some other studies reported varying inoculum size; Hajjaj *et al* [21] and Samiee *et al* [23] found that 3 and 10 % inoculum sizes, respectively, were best for maximum lovastatin production by *Aspergillus terreus*.

Moisture content is one of the most critical factors affecting SSF. An optimum moisture level minimizes the risk of fermentation medium contamination. Low moisture may support the availability of sufficient oxygen, but it also reduces metabolic activity, which may decrease lovastatin production. Conversely, higher moisture level decreases oxygen availability due to insufficient air present in the void volume and poor lovastatin production. It was evident from our study that the 2 % inoculum size yielded the most lovastatin, and further increasing moisture content lowered production. Latha *et al* [22] reported the highest lovastatin yield at 60 % moisture content.

Incubation time is another important factor to optimize for maximum lovastatin production with SSF. It is generally considered that a short fermentation period can effectively contribute to increasing profitability on an industrial scale. Lovastatin is a secondary metabolite produced

during the stationary phase of growth [24]. Latha *et al* [22] reported a maximum yield of lovastatin at 7.43 mg/g of dry substrate with 168 h of fermentation time. Maximum lovastatin production after 96 h may be due to the fact that it was produced as a result of the secondary microorganism growth. The subsequent decrease in lovastatin production was due to feedback inhibition by the existing lovastatin. Mukhtar *et al* [24] found maximum lovastatin production (1,699.87 ± 0.08 µg/mL) by *Aspergillus terreus* after 120 h incubation.

Overall, kinetic parameters exhibited maximum product formation on substrate utilization during the fermentation process. Haq *et al* [25] reported that the cell growth rate of *A. niger* GCB-16 was 0.193/h by GCB-16 for citric acid production. Moreover, Ali *et al* [26] found that the cell growth rate of *Aspergillus niger* was 0.122/h. However, our selected strain *Aspergillus flavus* GCBL-60 showed the maximum cell growth rate following fermentation profile optimization.

CONCLUSION

The findings of current study indicate that the combination of *Aspergillus flavus* with wheat bran and solid state fermentation technique is best for maximum lovastatin production.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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