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Strain improvement of *Aspergillus niger* to increase lovastatin production

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Abstract
The aim of the improvements in the present research was to develop strain with improved traits and to enhance lovastatin production. The process of strain improvement of *Aspergillus niger* involved UV irradiation treatment in addition to improvements in the production conditions, that is, the type and concentration of carbon and nitrogen sources in the production medium. The improved strain exhibited lovastatin productivity many folds higher than that of the wild strain. The effect of lovastatin on total blood cholesterol and triglycerides has been conducted using ordinary normal rabbits with high cholesterol feed which showed a significant reduction of both total blood cholesterol and triglycerides levels. Lovastatin was analyzed by UV spectrophotometer and thin layer chromatography (TLC).

Keywords: *Aspergillus niger*, lovastatin, UV irradiation, Cholesterol, Triglycerides.

1. Introduction
Lovastatin ($C_{24}H_{36}O_5$, M.W. 404.55) is the major ingredient of a cholesterol-lowering drugs. It is an important secondary metabolite from fungi through the polyketide pathway.
Lovastatin or mevinolin is a valuable hypocholesterolemic drug, which inhibits biosynthesis of cholesterol by competitively inhibiting 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA), (mevalonate: NADP1 oxidoreductase, EC 1.1.1.34), an enzyme found in liver tissue that plays a key role in production of cholesterol in the body, it catalyzes the rate limiting step (the reduction of HMG-CoA to mevalonate) during synthesis of cholesterol. Its inhibition leads to reduction in the rate of formation of cholesterol in the human body (JIANG *et al.,* 2013) [2]. In recent years, lovastatin has also been reported as a potential therapeutic agent for the treatment of various types of tumors and also play a tremendous role in the regulation of the inflammatory and immune response, coagulation process, bone turnover, neovascularization, vascular tone, and arterial pressure (Kumar *et al.,* 2000) [1].
Lovastatin is commercially produced from *Aspergillus terreus* through fermentation, as the production of lovastatin by fermentation decreases the production cost compared to costs of chemical synthesis (Novak *et al.,* 1997) [4].
In general, the efficiency of lovastatin production is determined by the amount of lovastatin produced by the various fungi strains together with the efficiency of the extraction procedure employed (Kumar *et al.,* 2000) [1].
The major producers of lovastatin is *Aspergillus terreus*, a worldwide distributed fungi but more frequently occurs in tropical and subtropical areas, producing great amounts of lovastatin. It require expensive extraction and multi-step purification procedures to obtain lovastatin. The process of the present research involved improvement of lovastatin production in *Aspergillus niger,* a common contaminant of food, ubiquitous in soil and indoor environments and easily isolated fungi, using simple and inexpensive processes.

Materials and Methods
Microorganism
The soil fungal isolate *A. niger* was used in this strain improvement study.

Methodology

The *A. niger* culture was grown on Potato Dextrose Agar (PDA) medium for 7 days at 30 °C. Spores were then harvested by washing the sporulated mycelia with sterile distilled water and separated by passage through filter paper. Freshly collected spore suspensions were exposed to UV light for 120 min at the distance of 10 cm from the UV source. UV exposure was followed by incubation in dark for overnight to avoid photo- reactivation. The spores then cultured on PDA for 5 days at 30 °C. Spores from the UV exposed strain were again exposed to UV light for 60 min at the distance of 10 cm from the UV source and this was followed by incubation in dark for overnight. The spores were then re-cultured on PDA for 7 days at 30 °C. Spores were harvested and (5ml) spore suspension used to inoculate the seed medium (20ml) and incubated in rotary shaker at 30 °C – 200 rpm for 48hr. Then 5ml from the seed medium used to inoculate 50ml of the production medium. Production medium incubated in rotary shaker at 30ºC - 200rpm for 8 days.

Seed medium composition :(g\l) PH=7

10 lactose, 1 peptone, 1 corn steep liquor, 0.25 KH_2PO_4.

Production medium composition: (g\l) PH=7

(60 lactose, 0.25 peptone, 0.5 corn steep liquor, 0.125 KH2PO4, 0.125 NaNO3) Trace metals 100µl of each ($ZnSO_4$.7H2O4, $MnCL_2$, CuSO4.7H2O, $CaCl_2$.2H2O, $FeSO_4$.7H2O, $MgSO_4$.7H2O).

Extraction of lovastatin from culture medium

At the end of 8 days of fermentation, the fermentation medium was centrifuged at 1500 x g for 20 min and the fermentation broth was acidified to pH 3.0 with concentrated HCl.

The fermentation broth then extracted with equal volume of toluene and the organic phase was separated from the aqueous one and washed with distilled water (half volume of the organic phase) three times to remove water soluble impurities then washed with methanol (half volume of the organic phase) two times to remove some of other impurities.

Analysis of lovastatin

UV spectrophotometer

Lovastatin was detected at 238 nm in UV/Visible spectrophotometer as documented in (Lingappa *et al.* (2004) [4]

Thin layer chromatography (TLC)

TLC was used to detect the presence of lovastatin in the extract. A quantity of 20 µl of the crude extract was spotted to silica gel plate. The plates were developed in a solvent system of dichloromethane: ethyl acetate (70:30 v/v), Ethyl acetate: Hexane: acetic acid (70:30:6 v/v). After the chromatogram was developed in the mobile phase it was stained with iodine vapour and observed under UV light. Individual spots on the TLC plates were marked and R_f values calculated. The plate was developed in the mobile phase for five times. R_f values compared with that documented in (Srinu *et al.,* 2010) [5].

Crystallization of lovastatin

The organic layer was concentrated under vaccum at about 55 °C. The concentrate then cooled to (- 4) °C and stirred at this temperature for 2 hrs, then filtered and the cake washed with pre-cooled toluene (0 °C) then dried at 40 °C and the weight of lovastatin crystals was determined.

Bioassay (Cholesterol lowering effect of lovastatin)

The experimental group consists of 9 ordinary normal rabbits, representing both male and female with body weight of about (1,000-1,500) g. Animals were kept in room temperature conditions. During the experimental period all animals were active, healthy and with normal appetite. The animals were divided into three groups: the first one (control group) consist of (3) rabbits received an unmodified diet. The second group and the third group (Treated groups) each consist of (3) rabbits received food balanced with cholesterol powder. The high cholesterol feed were given over 3 weeks (21 days) to make hypercholesterolemia. At day 22 the high cholesterol feed replaced by normal feed as the control group, group (3) were giving the testing medicine (15mg) orally once a day over 21 days, while group (2) were not. At the end of 21 days blood samples were collected to determine total blood cholesterol and triglycerides levels. Levels of the control group represented the normal level, levels of group (2) represented the hypercholesterolemic level, and levels of group (3) represented the cholesterol lowering effect of lovastatin.

Results and Discussion

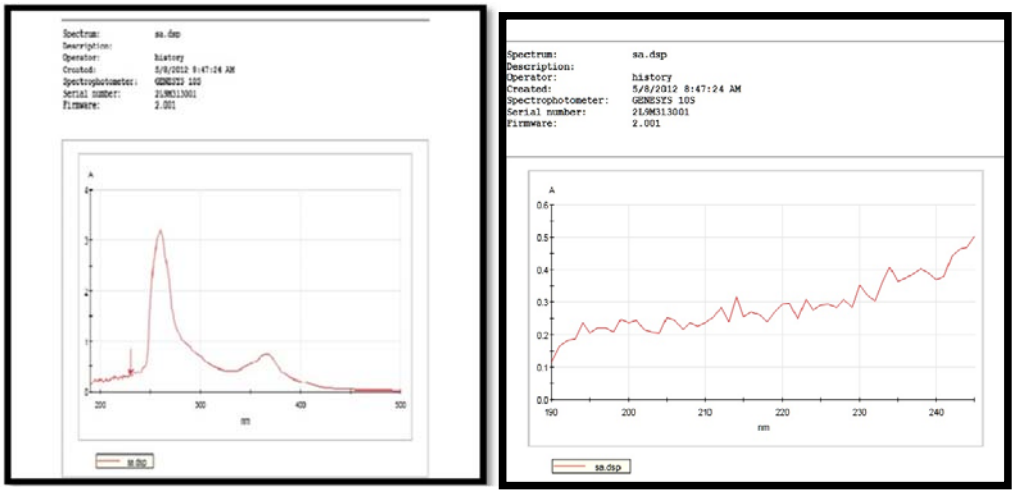


Fig 1: UV Spectrophotometric scanning of the fungal extract.

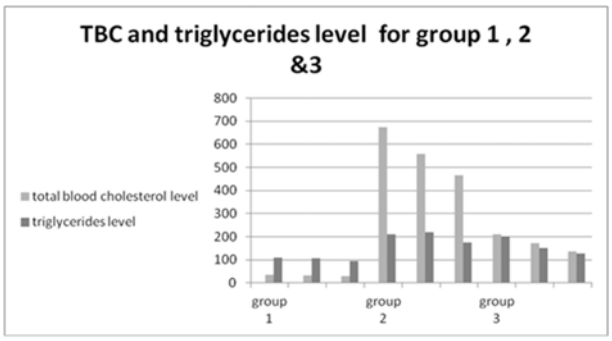
In the figure the fungal extract exhibited a peak at 238 nm in the spectrophotometer scanning.

Table 4.1 R_f values of the fungal extract and the standard lovastatin in different solvents.

Solvent	R_f calculated	R_f of standard lovastatin
Dichloromethane: ethyl acetate (70:30v/v)	0.67	0.67
Ethyl acetate: Hexane: acetic acid (70:30:6)	0.55	0.55

In the table, the fungal extract has the same R_f value of the standard lovastatin in different solvents.

Cholesterol lowering effect of lovastatin



In the figure, lovastatin resulted in a significant reduction of both total blood cholesterol and triglycerides level in group (3) compared with the control group (1) and that of group (2).

Discussion

As with any fermentation product, the culture medium has a significant influence on the yield of lovastatin and its rate of production. Selection of a suitable medium is therefore important for establishing a process for producing lovastatin. Starvation conditions (i.e. no growth because of insufficiency of an essential nutrient) as using slowly metabolized carbon source (lactose) with nitrogen limitation tend to favor the production of lovastatin, in addition UV is considered to be an effective mutagen in the production of lovastatin from *Aspergillus niger*, and thus this high yielding strain on optimized media can further improve the yield of lovastatin.

The optimized fermentation conditions raised the lovastatin titer many folds compared with the wild strain and yield (952.81) mg\l.

The analytical procedures confirm the production of lovastatin in the fermentation broth:

1. The sample exhibited a peak at 238 nm in the spectrophotometer scanning.
2. In the thin layer chromatography it was observed that, the sample spots had the same R_f value as that of commercial lovastatin.

In cholesterol lowering bioassay, lovastatin showed a significant reduction of both total blood cholesterol and triglycerides levels of ordinary normal rabbits which receive high cholesterol feed compared with control group that don't receive high cholesterol feed and this ensure the effectiveness of lovastatin as a potential therapeutic agent for the hyperlipidemia.

Conclusion

This study was investigated that UV irradiation and the culture medium optimization (excess carbon but limiting amounts of nitrogen source) give better productivity of lovastatin and so they give high yielding strain of *Aspergillus niger*.

Lovastatin inhibit HMG-CoA reductase competitively; lower both total blood cholesterol and triglycerides level and so reduce hypercholesterolemia, the major risk factor in cardiovascular diseases.

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