International Journal of Science and Research Archive
eISSN: 2582-8185
Cross Ref DOI: 10.30574/ijsra
Journal homepage: https://ijsra.net/

(RESEARCH ARTICLE)

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Novelty in elicitation of simvastatin production from *Aspergillus terreus* AJ73 and evaluation of immobilized simvastatin on carbon nanotube as anticancer activity

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International Journal of Science and Research Archive, 2026, 18(03), 778-789

Publication history: Received on 04 February 2026; revised on 10 March 2026; accepted on 13 March 2026

Article DOI: https://doi.org/10.30574/ijsra.2026.18.3.0499

Abstract

Simvastatin is a cholesterol-lowering drug widely employed to control cholesterol levels and to prevent stroke and cardiac failure in patients at a high risk of coronary artery disease. Furthermore, Simvastatin has a role as an antimicrobial and anti-cancer agent. The continual increase in productivity that occurs by strain enhancement is crucial for a commercial fermentation process's viability. Elicitation by other microorganisms is a crucial example of these improvements. Three microorganisms strains (live and dead) were utilized for elicitation of local isolate *Aspergillus terreus* AJ73 to enhance the production of simvastatin utilizing solid-state fermentation. These microorganism strains are *Trichoderma viridae, Saccharomyses cerevisiae,* and *Bacillus subtilus*. Three various inoculum sizes of the elicitors (0.25, 0.5, and 1 ml/5gm of $1x10^6$ cells or spores/ml) were employed and incubated at 28 ºC for 6 days (pH 7.0). Elicitors were spent medium with dead cells, spent medium with live cells, live cells, dead cells, and spent medium without live cells or dead cells. The results demonstrated that the maximum production of simvastatin was 260 µg/gm-substrate in the culture elicited with 1 ml/5gm ($1x10^6$ cells/ml) of live *S.cerevisiae* without spent medium compared with the control culture (*Aspergillus terreus* AJ73 culture without any addition) (91.31 µg/gm). While elicitation by some other elicitors showed a low concentration of simvastatin synthesis by *A.terreus* AJ73 utilizing SSF (fluidized bed, consisting wheat bran and oat bran (1:1 w:w), the moisture ratio was (1.2 v:w) by sodium acetate (0.01 M), pH 7.0). The results of cytotoxic activity of free and immobilized simvastatin on carbon nanotubes (MWCNTs) revealed a decrease to a depress in cell viability in a dose-dependent manner on HL-60 cell lines, with a calculating IC_{50} of 133.65 and 73.43 µg/ml, respectively, compare with normal cell line (HdFn cell line) at IC_{50} of 188.34 and 163.17 µg/ml, respectively.

Keywords: Elicitation; Immobilization; Carbon nanotubes (MWCNTs); Anticancer

1. Introduction

Simvastatin is made up of polyketide compounds that certain fungi produce as part of their secondary metabolism. Simvastatin, sometimes referred to as 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA reductase) inhibitor, belongs to class of lipid-lowering drugs that lowers mortality and sickness of patients with a high risk of cardiovascular disease. While the statins block HMG-CoA reductase by competitive inhibition, cholesterol production is slowed down(1). Statins prevent the mevalonate pathway's conversion of HMG-CoA to mevalonic acid (2). Investigations of the production of simvastatin via submerged fermentation (SmF) and solid-state fermentation (SSF) are extensive, and filamentous fungi frequently show significant potential (3). The organisms that production of simvastatin as a secondary metabolism include: *Aspergillus niger, Aspergillus terreus, Aspergillus flavus, Monascus spp, Penicillium purpurogenum,* and *Trichoderma viride*. *Aspergillus terreus* is a significant source for the synthesis of simvastatin and belongs to filamentous Ascomycota (4). Genomic sequence data have revealed the presence of a large fraction of putatively silent biosynthetic gene clusters in the genomes of some microorganisms that encode for secondary metabolites, which are not detected under standard fermentation conditions(5). Microbial studies have

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established that microorganisms in nature exist in complex mixtures of populations in which they normally interact and respond to each other. Much literature mentioned that interspecies interactions importantly affect the bioactive compounds production, particularly antimicrobial agents (6). According to one of the most accepted hypotheses regarding the origin of antimicrobials, antimicrobial products are synthesized to give the producing organism a competitive advantage against surrounding microorganisms that compete for nutrients and space (7). Consequently, using interspecies interactions to produce antibiotics may lead to the stimulation of hitherto unexplored biosynthetic pathways for novel bioactive chemicals or to the improvement needed to produce antibiotic-producing strains(8). The goal of the current work was to enhancement of simvastatin production from local *Aspergillus terreus* AJ73 isolate utilizing solid-state fermentation by various elicitors, and evaluate its anticancer activity.

2. Materials and methods

Sabouraud dextrose agar (SDA), Potato dextrose agar (PDA), Nutrient agar and broth were from Himedia, India. Trifluroacetic acid, Sodium hydroxide (NaOH), Methanol, Ethyl acetate, Ethanol 95%, and other materials were from England, BDH.

2.1 Simvastatin production

Using potato dextrose agar, the local *A.terreus* AJ73 isolate was cultivated. Using a medium made of wheat bran and oat bran (1:1 w:w), the moisture ratio was (1.2 v:w) by sodium acetate (0.01 M), pH (7), and inoculated with 1 ml ($7x10^6$ spores/ml) and incubated for 6 days at 30 °C, simvastatin production from this isolate was then evaluated(9).

2.2 Extraction of simvastatin

Simvastatin concentration was determined following SSF achieved (wheat bran and oat bran (1:1 w:w), the moisture ratio was (1.2 v:w) by sodium acetate (0.01 M), pH 7.0). The culture was extracted using ethyl acetate in a 250 mL Erlenmeyer's flask (pH 3.0). The mixture was incubated for 2 hours at 28 °C on a moving shaker at 140 rpm. After that, the mixture was filtered using Whatman filter paper (No. 1) then employing centrifuge (12000 rpm for 15 min.) to separate the supernatant from the biomass. The supernatant was kept at 4 ^{0}C in glass vials until it was used in further studies (10).

2.3 Simvastatin estimation

Using the technique outlined by (11), simvastatin was calculated as follows: One ml of the supernatant was combined and incubated for 10 minutes with one ml of trifluoroacetic acid (1%). The absorbance of 0.5 ml of this mixture was examined at 238 nm using a UV-Visible Spectrophotometer after being diluted with methanol ten times. By placing the O.D values on a standard chart, the simvastatin concentration present in the sample was determined. The following equation was used to estimate the simvastatin concentration in the SSF system:

Simvastatin concentration (µg/g substrate) = Simvastatin concentration (µg/ml) × Total volume of extraction (ml)/Amount of substrate taken (g) (12).

2.4 Enhancement of simvastatin production utilizing elicitors

2.4.1 Elicitation experiment

Three microorganism's strains (live and dead) were used for the elicitation of local isolate *A. terreus* AJ73 for enhancement of simvastatin production utilizing solid-state fermentation. These microorganisms strains were *Trichoderma viridae, Saccharomyses cerevisiae, and Bacillus subtilus*. Elicitor preparation is explained in the corresponding section (13).

2.4.2 Inoculum preparation

Preparation of fungal elicitors of two fungi including *T.viridae* and *S.cerevisiae*, were cultured on potato dextrose agar and incubated at 28 °C for 5 and 2 days respectively. A one-cm2 area of mycelium (or touch of yeast cells) was cut and transferred to 50 ml of nutrient broth in Erlenmeyer flask (250 ml) and incubated at 28 °C (120 rpm) for 7 days, while the bacterial elicitors of *B.subtilus* were cultured on nutrient agar, then transferred to 50 ml of nutrient broth in Erlenmeyer flask (250 ml) and incubated at 37 °C (120 rpm) for 48 hours. The growth culture of Mold, yeast, and bacteria was equally separated. The first half of the cultures were collected by centrifuge at 6000 rpm and washed twice in sterile distilled water, then homogenized in distilled water utilizing vortex at room temperature. While the other half of the culture remaining for killing by heat (14).

2.4.3 Addition of elicitors

This experiment was carried out in a 250 ml Erlenmeyer flask containing 10 gm of Wheat bran: Oat bran (5:5 gm), 12 ml of sodium acetate buffer 0.2 M, and pH 7.0, inoculated with 3.5 ml of spores suspend (7×10^6 spores/ml) of local isolate *A. terreus* AJ73, incubated at 28 ºC for 2 days. After 48 hours, each flask was inoculated with one of three various inoculums volume of the elicitor (1, 0.5, and 0.25 ml/5gm of 1x106 cells or spores/ml), these elicitors including: spent medium with dead cells, spent medium with live cells, live cells, dead cells, spent medium without live cells, and spent medium without dead cells, then incubated at 28 ºC was completed for 6 days. The simvastatin concentration was estimated.

2.5 Functionalization of carbon nanotube (MWCNTs)

Carbon nanotubes (MWCNTs) can be made functional via an acid oxidation treatment process. One thousand milligrams of carbon nanotubes are combined with one hundred milliliters of a 3:1 concentration of H2SO4 and HNO3 (15). Functionalization will take place while the combination sits in the water bath for 4.5 hours at 40 °C and then in an ultrasonic bath for 15 minutes. After being dried in a vacuum oven for 48 hours at 60 °C, the functionalized carbon nanotube (MWCNT) is ready for collection. The multi-wall carbon nanotube (MWCNT) is utilized after being dried and cooled for 24 hours.

2.6 Immobilization of simvastatin on functionalized carbon nanotube (MWCNT)

Physical means were utilized to immobilize the simvastatin. Dissolving 30 mg of multi- walled carbon nanotubes (MWCNTs) in 10 mL of partial purified simvastatin (the simvastatin was purification utilizing silica gel chromatographic technique to give concentration of 57.8 µg/ml and yield of 73.62 %.). After a thorough mixing, a sample of the produced solution is incubated in an incubator shaker at 30 °C, for 2.5 hours at 140 rpm speed. The functionalized carbon nanotube (MWCNT)- simvastatin conjugate is separated from the composite mixture via centrifugation at 4800 rpm for 15 minutes following incubation. Subsequently, the supernatant is decanted with great care to prevent conjugation loss. After that, the MWCNT- simvastatin composite is washed in a new phosphate buffer solution (pH 7) before being re-dispersed. In order to eliminate the unbound functionalized MWCNT, the procedure of washing and centrifugation must be performed at least three to four times (15).

2.7 Cytotoxic influence of immobilized and free simvastatin

By the following protocol outlined in (16) was used to study the potential cytotoxic effects of simvastatin. Human dermal fibroblasts (HdFn) and leukaemia cells (HL-60) were used to investigate the cytotoxicity of this at various doses. The cells were grown in 96-well microplates at a concentration of 200 µL per well, ranging from $1x10^4$ to $1x10^6$ cells mL-1. After gently swirling the contents of the microplates and sealing them with sterile parafilm, they were placed in an incubator set at 37 °C with 5% CO2 for 24 hours. The medium was withdrawn after incubation, and 200 µL of a 2-fold serial dilution of the free and immobilized simvastatin (25, 50, 100, 200, 400 mg/ml) was added to the wells. Every focus and control was triple-checked. As a control, the microplates were incubated at 37 °C with 5% CO2 for 48 hours. Ten millilitres of MTT solution was added to each well after exposure to simvastatin. So, the microplates were placed in an incubator set at 37 °C with 5% CO2 for duration of 4 hours. With great care, the medium was removed and 100 µl of solution was added to each well. Subsequently, the microplates were placed in an incubator for 5 minutes. A german ELISA reader (Bio-rad) was used to measure optical density at 575 nm. The following equation was used to predict the concentration needed to produce a 50% decline in cell viability for each cell line based on statistical evaluation of optical density data:

$$Y= D+ A-D / 1+10^{(x-logC)B}$$

3. Results and Discussion

3.1 Strategy of elicitation

Since microorganisms usually live in nature with other species, as a result of such inter-species interactions they have evolved complex metabolic and physiological responses. Our strategy for the elicitation of local *A.terreus* AJ73 isolate is based on utilizing some aspects of these interactions by introducing microbial cells to *A.terreus* AJ73 culture. To fully profiteer any eliciting capability, the microbial cells or hypha (live, heat-killed cells (death), spent medium with or without live cells, or spent medium with or without dead cells) were added directly into the local *A.terreus* AJ73 isolate cultures. One of the challenges that may arise in such a situation is the possibility of the introduced live cells of the second microorganisms becoming competitors to the main producers. The motivation of our work is to enhance the production of simvastatin without impacting the growth of local *A.terreus* AJ73 isolate. Therefore, it is important to keep

the elicitor growth under control and this depends on the medium composition and other physicochemical conditions that may support or reduce the elicitor growth. Furthermore, the concentration of the elicitor cells was fixed to be at a level necessary for elicitation but without overtaking the growth of local *A.terreus* AJ73 isolate. Therefore, to prevent this problem, the eliciting capacity of the elicitors' dead cells was compared to live cells. Any impact of the competitor's isolate on the growth of the local *A.terreus* AJ73 isolate can be prevented if the dead cells have an inducing effect.

3.2 Choose of elicitor microorganisms

Microbial natural environments are typically very rich in various kinds of microbial organisms, which can decimate local nutrient resources, resulting in increased competition. The development of antimicrobial compounds is one of the microbial strategies employed to exist in nature. Indeed, elicitation is dependent on mimicking the appearance of another competing species in a culture that is otherwise pure to induce the producer species to turn to their secondary metabolism that produces antimicrobial compounds. Three species of microorganisms; *B.subtilis* and theeukaryo-tic microorganism *T.viridae* and *S.cerevisiae* were chosen as the likely competitors from the natural habitat of local *A.terreus* AJ73 isolate. They were selected for the following reasons: these three microorganisms are common in microbial laboratories and are commonly utilized in bacteriological studies as a guide. Also, they can be found in various environments like air, soil, water, and decomposing plant matter. Furthermore, *Bacillus subtilis* and *S.cerevisiae* are safe and can be cultured easily in the lab. In addition, *Trichoderma viridae* is an isolate previously tested for the production of simvastatin and it cannot produce simvastatin. Lastly, none of these microorganisms are producing simvastatin.

3.3 Elicitation with live and dead hypha or cells with spent medium

The first step in the elicitation experiments was to test the ability of live and dead hypha or cells with spent medium to grow in the production media under conditions optimized for enhancement of simvastatin synthesis from local *A.terreus* AJ73 isolate employing SSF. The preliminary observation showed that live hypha or cells with the spent medium of *B.subtilis*, *T. viridae*, and *S. cerevisiae* could grow well in the defined medium based on the appearance of turbidity in culture after 48 hours of incubation for *B.subtilis* and *S. cerevisiae* and 5 days for *T. viridae*. Therefore, it was necessary to find the concentration of microorganisms that cannot affect the growth of local *A.terreus* AJ73 isolated under the cultural conditions used in this work. Therefore, three different inoculum levels of microorganisms (1, 0.5, 0.25) ml/5gm were added to the local *A.terreus* AJ73 isolate culture, each containing approximately 1x106 cells or spores/ml. These three levels were chosen as being below the inoculation level of local *A.terreus* AJ73 isolate which was fixed to be 3.5 ml that contains approximately 7×106 spores/ml.

3.3.1 Addition of live hypha or cells with spent medium

As can be seen in figure (1), the results of simvastatin production were significantly improved when live cells with spent medium of all microorganisms, were added to fermentation medium of the local *A.terreus* AJ73 isolate . In all elicited cultures, simvastatin production was begin after 48 hours of incubation with 1 ml of 10^6 cells/ ml which is the common time of simvastatin production in the control culture. Maximum production of 246 µg/gm simvastatin was obtained in the culture elicited with 1 ml live cells plus spent medium of *S. cerevisiae* inoculums compared with other elicitors added.

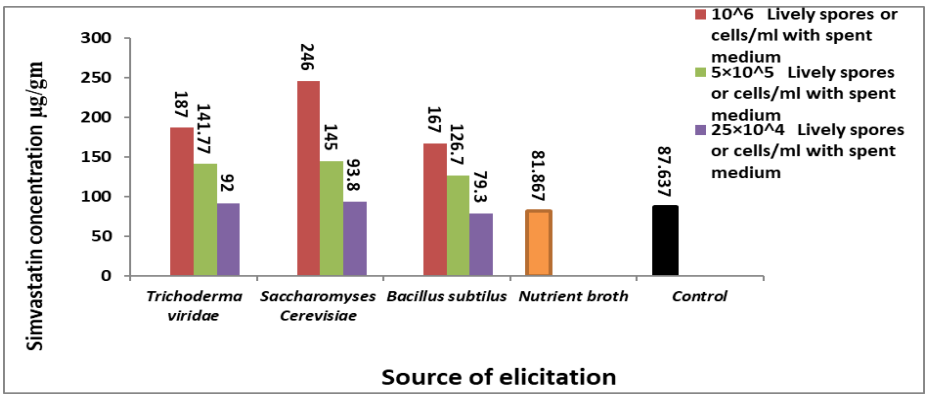

Figure 1 Enhancement of simvastatin production from local *A.terreus* AJ73 isolate by biological elicitation (Live hypha or cells with spent medium) utilizing solid state fermentation (consist wheat bran and oat bran (1:1 w:w), the moisture ratio was (1.2 v:w) by sodium acetate (0.01 M), pH 7.0)

3.3.2 Addition of heat-killed hypha or cells with spent medium

Interestingly, heat-killed hypha or cells of microbial that used had negative impact on simvastatin production by local *A.terreus* AJ73 isolate, whereas the simvastatin yield was decreased with these elicitors. The findings given in figure (2) demonstrated that heat-killed hypha or cells plus spent media had no considerable impact on simvastatin synthesis by local *A.terreus* AJ73 isolate. Compared with production pattern of simvastatin in the hold culture and elicited with dead hypha or cells plus spent media of microbial utilized showed various outcomes. As can be seen in figure (2), simvastatin production of local *A.terreus* AJ73 isolate was decreased when dead hypha or cells with spent medium were added to the fermentative medium after 48 hours of local *A.terreus* AJ73 isolate culture. Also, production of simvastatin was decreased in the cultures elicited with heat killed cells or hypha with spent medium compared with the control culture. These results may due to release of some compounds in intracellular (after death of cells) to the medium, which were affected on simvastatin production.

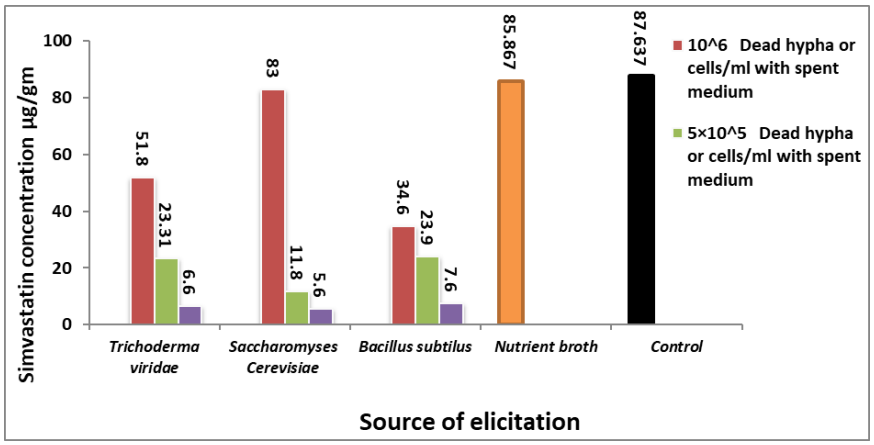

Figure 2 Enhancement of simvastatin production from local *A.terreus* AJ73 isolate via biological elicitation (Dead hypha or cells with spent medium) utilizing solid state fermentation

3.4 Elicitation with live and dead hypha or cells without spent medium

3.4.1 Addition of live hypha or cells without spent medium

The results in figure (3), showed that the utilizing of only living hypha or cells of all microorganisms as elicitors, was significantly enhanced simvastatin production when added to the fermentation medium by local *A.terreus* AJ73 isolate. In all elicited cultures, simvastatin production was started with 48 hours' incubation which is the normal time of production in the control culture. The maximum production of 260 µg/gm simvastatin was obtained in the culture elicited with 1 ml (1×10^6 cells/ml) of live cells without spent medium of *S. cerevisiae* inoculums compared with other elicitors addition.

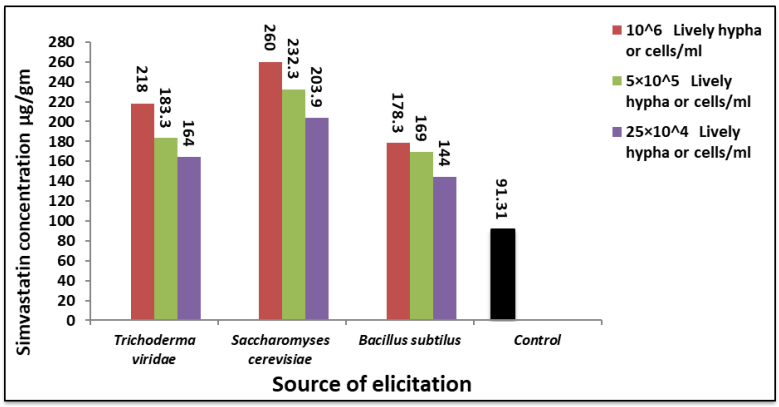

Figure 3 Enhancement of simvastatin production from local *A.terreus* AJ73 isolate by biological elicitation (Live hypha or cells) utilizing solid state fermentation

3.4.2 Addition of dead hypha or cells without spent medium

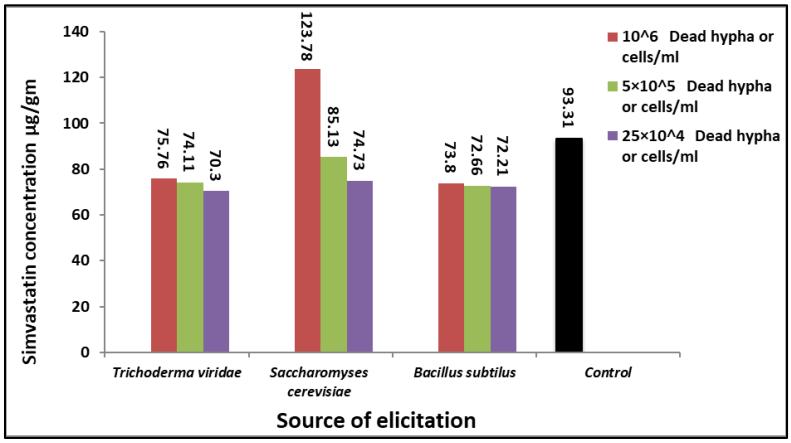

Figure 4 Enhancement of simvastatin production from local *A.terreus* AJ73 isolate by biological elicitation (Dead hypha or cells) utilizing solid state fermentation

Heat-killed hypha or cells without spent medium of *B.subtilis, T. viridae and S. cerevisiae* had the same role as Heat-killed hypha or cells with spent medium of all microorganisms to elicit of local *A.terreus* AJ73 isolate. Figure (4) shows that production of simvastatin was higher in the cultures elicited with Heat-killed hypha or cells without spent medium of *B.subtilis, T.viridae and S.cerevisiae* comparison of the hold culture. Production of simvastatin in the control culture started as normal with approximately 93.31 µg/gm. While the maximum production of simvastatin obtained in the cultures elicited with one ml ($1x10^6$ cells/ml) of *S.cerevisiae* inoculum was 123.78 µg/ml after 6 days of incubation. Elicitation with other hypha and cells of *B.subtilis,* and *T.viridae* were given low yield of simvastatin synthesis via local *A.terreus* AJ73 isolate utilizing SSF.

3.5 Elicitation with spent medium for live and dead hypha or cells

3.5.1 Addition of spent medium for live hypha or cells

Similar to the elicitation with above method for simvastatin production, the outcomes showed that the spent medium for live hypha or cells of *B.subtilis, T.viridae* and *S.cerevisiae* were added to the local *A.terreus* AJ73 isolate culture, production of simvastatin was deterioration (figure 5). Simvastatin in the control culture was produced as normal, after 6 days of incubation and reached its maximum yield of 93.71 µg/gm. In the elicited cultures, maximum production of simvastatin (72.33 µg/gm) was obtained in the culture elicited with 1 ml of spent medium for live *S. cerevisiae* inoculums attained after 6 days of incubation compared with other spent media. Comparing these values with the concentration provided in the control culture, the decrease in the yield of simvastatin may due to there are some of essential nutrient that prolong the period of the log phase, which leads to elongate of the stage of stability in this phase and leads to reduce the production of simvastatin from local *A.terreus* AJ73 isolate by solid state fermentation. Also, the reason may be due to present of compound that effect on the pathway of simvastatin production.

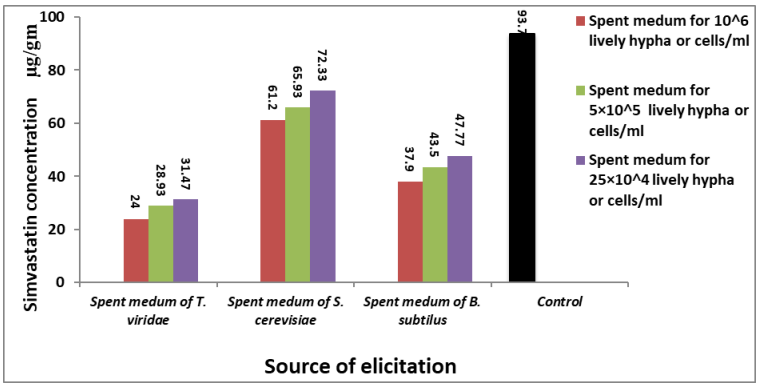

Figure 5 Enhancement of simvastatin production from local *A.terreus* AJ73 isolate by biological elicitation (Spent medium for live hypha or cells) utilizing solid state fermentation(consist wheat bran and oat bran (1:1 w:w), the moisture ratio was (1.2 v:w) by sodium acetate (0.01 M), pH 7.0)

3.5.2 Addition of spent medium for dead hypha or cells

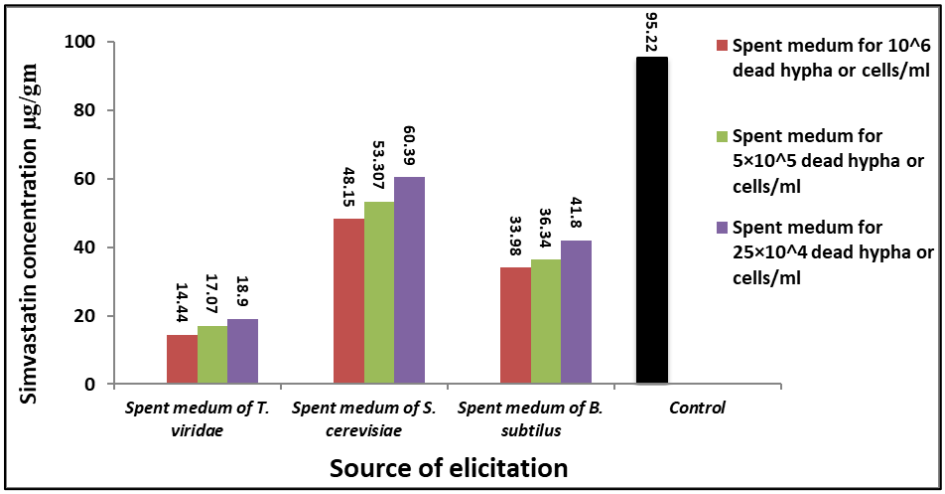

Figure 6 Enhancement of simvastatin synthesis from local *A.terreus* AJ73 isolate by biological elicitation employs (Spent medium for dead hypha or cells) solid state fermentation

The outcomes in figure (6), showed that the simvastatin production from local *A.terreus* AJ73 isolate by SSF was significantly reduced when spent medium for dead hypha or cells were added as elicitors to fermentation medium. In all elicited cultures, simvastatin production was started within 48 hours of incubation which is the normal time of production in the control culture, and the maximum simvastatin production was within 6 days. Generally, comparison to the other elicitors the maximum production of (60.39 µg/gm) simvastatin was obtained in the culture elicited with 1 ml of spent medium for *S. cerevisiae* culture. These results showed that the simvastatin production was decreased and negatively affected with addition of spent media.

From the results obtained in some elicitation experiments it is clear that simvastatin production has been significantly enhanced. Whereas the elicitation with employing spent medium of dead hypha or cells, spent medium for live hypha or cells, and spent medium for dead hypha or cells in which important decreased was observed. The outcomes showed that production of simvastatin from local *A.terreus* AJ73 isolate was significantly higher when using only hypha or living cells as elicitors, especially the living cells of the *S. cerevisiae*. In all the concentrations of *S. cerevisiae* that used as elicitor, the maximum production of simvastatin was 279 µg/gm with 1ml of 1×10^6 cells/ml (table 1). Also, the production of simvastatin was increased when using only dead cells as elicitors but less than the living cells (*S. cerevisiae*), whereas the simvastatin concentration was 137.78 µg/gm compared to control. Simvastatin production was improved when the cells or hypha of *B.subtilis,* and *T. viridae* were utilized as elicitor without spent media, compared with control. But the yield of simvastatin was higher when using live or dead *S. cerevisiae* compared with other elicitors.

3.6 Cytotoxic influence of free and immobilized simvastatin utilizing MTT Assay

The results showed that simvastatin affected the viability of the HL-60 cells in a way that was dependent on the concentration. The cell viability of HL-60 cells and HdFn cell lines was measured for 24 hours using various doses of immobilized and free simvastatin, which ranged from 25 to 400 µg/mL, as shown in table (1, 2) and figure (7, 8). The results in figure (7) demonstrate that free partial purified simvastatin appeared a decrease in viability of cell in a dose-dependent manner on HL-60 cell lines, with a calculating IC_{50} of 133.65 µg/ml comparative with normal cell line (HdFn cell line) at IC_{50} of 188.34 µg/ml. Whereas the findings in figure (8) demonstrated that immobilized simvastatin appeared a decrease in viability of cell in a dose-dependent manner on HL-60 cell lines, with IC_{50} about 73.43 µg/ml comparative with normal cell line (HdFn cell line) at IC_{50} of 163.17 µg/ml.

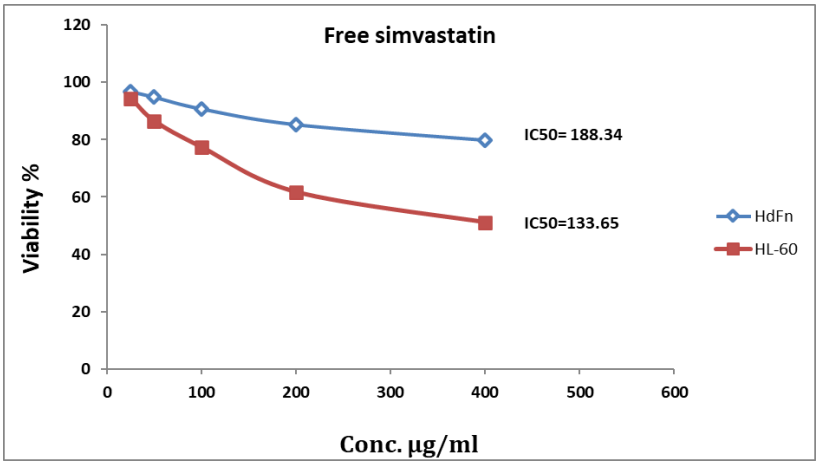

Figure 7 The influence of several concentrations of free simvastatin on the viability of HL-60 and HdFn cell line via assay of MMT. The untreated group (control) is equal to 100%, Data are represented as the mean±SD of five repeat

Table 1 The cytotoxicity of concentrations of free simvastatin on HL-60 and HdFn cell lines.

Free simvastatin	**HdFn**	**HL-60**
Conc.	Mean ± SD	Mean ± SD
400	79.81233 ±0.692338	51.14733 ±2.25378
200	85.252 ±1.399237	61.718 ±1.518635
100	90.73033 ±1.516961	77.343 ±0.536345
50	94.82 ±0.594375	86.448 ±0.682758
25	96.74933 ±0.690224	94.39567 ±0.917138

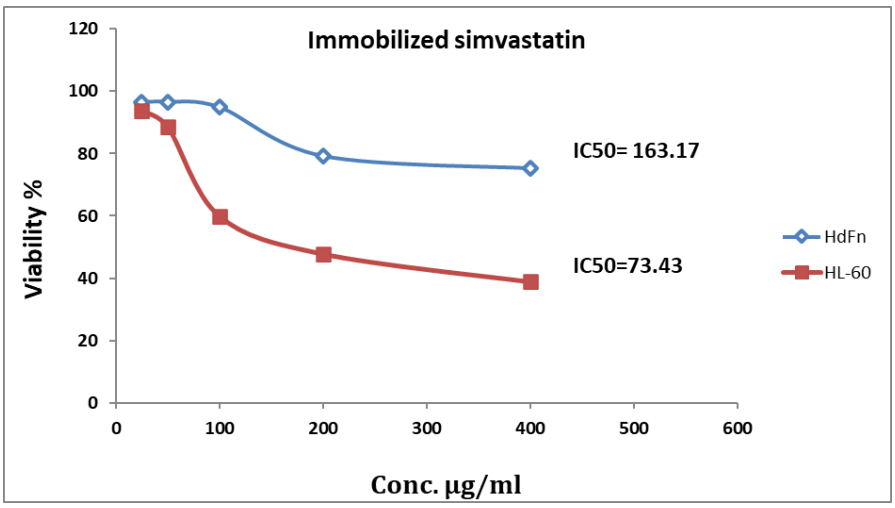

Figure 8 The influence of several concentrations of immobilized simvastatin on the viability of HL-60 and HdFn cell line via assay of MMT. The untreated group (control) is equal to 100%. Data are represented as the mean±SD of five repeat

Table 2 The cytotoxicity of concentrations of immobilize simvastatin on HL-60 and HdFn cell lines.

Imm. simvastatin	**HdFn**	**HL-60**
Conc.	Mean ± SD	Mean± SD
400	75.13133 ±2.200382	38.9062 ±1.762711
200	79.105 ±1.556425	47.8182 ±1.7019
100	94.84567 ±2.200382	59.8942 ±2.454443
50	96.58169 ±1.282882	88.44353 ±2.39069
25	96.466 ±0.921206	93.84487 ±0.64409

4. Discussion

The outcomes showed that the use of living cells with the spent medium as an inducer enhanced the production of simvastatin from the local *A.terreus* AJ73 isolate by SSF, whereas the yield of the simvastatin increased with the with a concentration of 10^6 cells/ml inoculum compared with control. While the use of other elicitors increased the concentration of simvastatin but less than the use of yeast. In the case of stimulation of the production of simvastatin by using dead cells with the spent media, the production is significantly reduced to be less than the control. While utilizing of the spent medium without cells as elicitors, the production of simvastatin is significantly lower than the control. Generally, live cells are more simvastatin productive when utilized as elicitors compared with dead cells, due to that the dead cells were less impact or induce for simvastatin production utilizing local *A.terreus* AJ73 isolate compare with elicited by living cells. This is because the local *A.terreus* AJ73 isolate was prevent the growth of living cells (as elicitors) by producing the largest amount of simvastatin as a defense agent against living elicitors. Also, the outcomes of this work showed that the use of dead and live cells in the production of simvastatin increased compared with the control, but the production of simvastatin by employing dead cells is less than that produced by living cells. Furthermore, the outcomes demonstrated that when utilizing only spent media of dead or living cells as elicitors to induce the local *A.terreus* AJ73 isolate for enhancement of the simvastatin production. Simvastatin yield was very low and these results may be explained by several probabilities: firstly; the spent media may contain the nutrients that may prolong the Log phase and shorten the stationary phase of local *A.terreus* AJ73 isolate, which affects the production of simvastatin.

On the other side, microorganisms used to stimulate the production of simvastatin may be associated with the production of inhibitors leading to the inhibition of simvastatin production. In terms of elicitors, the elicitor's microorganisms may participate in the production of another compound, which works to adjust or change (inhibit) the metabolic pathway for the synthesis of simvastatin from local A.terreus AJ73 isolate, leading to produce another compound in the same metabolic pathway. For example, some studies have developed a strategy for the direct production of monacolin J via fermentation processes that are focused on the disruption of the lovF gene of a fungal strain leading to transformants that can accumulate large amounts of this intermediate more than simvastatin (15). Regarding the production, Simvastatin may be produced in large quantities but may be addition of bread yeast significantly to 263 µg/gm with a concentration of 106 cells/ml inoculum compared with control. While the use of other elicitors increased the concentration of simvastatin but less than the use of yeast. In the case of stimulation of the production of simvastatin by using dead cells with the spent media, the production is significantly reduced to be less than the control. While utilizing of the spent medium without cells as elicitors, the production of simvastatin is significantly lower than the control. Generally, live cells are more simvastatin productive when utilized as elicitors compared with dead cells, due to that the dead cells were less impact or induce for simvastatin production utilizing local *A.terreus* AJ73 isolate compare with elicited by living cells. This is because the local *A.terreus* AJ73 isolate was prevent the growth of living cells (as elicitors) by producing the largest amount of simvastatin as a defense agent against living elicitors. Also, the outcomes of this work showed that the use of dead and live cells in the production of simvastatin increased compared with the control, but the production of simvastatin by employing dead cells is less than that produced by living cells. Furthermore, the outcomes demonstrated that when utilizing only spent media of dead or living cells as elicitors to induce the local *A.terreus* AJ73 isolate for enhancement of the simvastatin production. Simvastatin yield was very low and these results may be explained by several probabilities: firstly; the spent media may contain the nutrients that may prolong the Log phase and shorten the stationary phase of local *A.terreus* AJ73 isolate, which affects the production of simvastatin. On the other side, microorganisms used to stimulate the production of simvastatin may be associated with the production of inhibitors leading to the inhibition of simvastatin production. In terms of elicitors, the elicitor's microorganisms may participate in the production of another compound, which works

to adjust or change (inhibit) the metabolic pathway for the synthesis of simvastatin from local *A. terreus* AJ73 isolate, leading to produce another compound in the same metabolic pathway. For example, some studies have developed a strategy for the direct production of monacolin J via fermentation processes that are focused on the disruption of the lovF gene of a fungal strain leading to transformants that can accumulate large amounts of this intermediate more than simvastatin (17).

Regarding the production, Simvastatin may be produced in large quantities but may be connected (or converted to other compounds) with another compound (released from elicitors microorganisms or/and media) to alter the original structure of simvastatin, which leads to the difficulty for the detection by using the detection methods, that used in this study to detect free simvastatin. In other studies, the simvastatin converted to monacolin J during chemical processes involving very low to high temperatures, organic solvents, long alkali treatments, or developed a strategy for producing monacolin J directly through fermentation processes (18). In the same way, chemical reactions may be occurred between simvastatin and other compounds (like an enzyme), leading to hydrolyzing of the simvastatin for two parts (18) , leading to difficulty in detection (19). Sorrentino, *et al* (19), suggested a novel methodology for improving the production of lovastatin in *Aspergillus terreus* cultures via investing linoleic acid-derived signaling molecules, which are necessary utilized in fungal cell-cell communication. Raina, *et al* (20), demonstrated that butyro-lactone I have a novel role in the enhancement of lovastatin production from filamentous *Aspergillus terreus* as well as they proposed that it has the ability to affect both lovastatin and addition to its own production. Sun *et al* (21), found that adding *Sporobolomyces huaxiensis* isolated from tea leaves into the *Monascus purpureus* culture during growth lead to increasing in monacolin K production (446.92 mg/ml concentration) for 6 times maximal than that of the hold culture compared with three times maximal than the hold culture after the UV light irradiation in the existence of 1.0 % lithium chloride in the medium.

According to Klawitter *et al* (22), lovastatin prevented breast cancer cell lines from proliferating. MDAMB231 breast cancer cells were more susceptible to its effects, and lovastatin acid was generally more effective than lovastatin lactone at modifying protein expression. Bhargavi *et al* (23) discovered that HeLa cells were affected by partial purified lovastatin from *A. terreus* (KM017963), which was purified by adsorption chromatography. This was due to its effects on mitochondrial membrane potential, cell numbers, DNA fracture, and antioxidant properties, including hydroxyl radical scavenging and reduced glutathione levels on the one hand. The final results provide evidence for the anticancer effects of lovastatin and its potential usefulness in the chemotherapy of cervical malignant growth, while its effect on the cell cycle was through cell apoptosis and cell cycle regulation in the G0/G1 phase. According to Janani *et al* (24), lovastatin derived from soil fungi demonstrated strong antitumor activity against the human lung cell line A549. Through an apoptotic experiment, Raghunath *et al* (25) showed that the lovastatin derived from fungal manifest had a strong cytotoxic activity in the in-vitro culture of the human cancer cells (HeLa and HepG2).

5. Conclusion

- The results have revealed the presence of a large fraction of putatively silent biosynthetic gene clusters in the genomes of *A.terreus* AJ73 that encode for secondary metabolites, which are not detected under standard fermentation conditions, or its production can be increased, by using some elicitors.
- From the results obtained in some elicitation experiments it is clear that simvastatin production has been significantly enhanced. Whereas the elicitation with dead hypha or cells with spent medium, spent medium for live hypha or cells, and spent medium for dead hypha or cells in which important decreased was observed.
- The live cells of *Saccharomyces cerevisiae* was best elicitor for enhancement of lovastatin production by local isolate *A.terreus* AJ73.
- The free and immobilized simvastatin on carbon nanotubes (MWCNTs) showed a decrease in cell viability of HL-60 cell lines, and a IC50 of their were 133.65 and 73.43 µg/ml, respectively.

Compliance with ethical standards

Disclosure of conflict of interest

The authors declare no conflict of interest.

Funding

This research received no external funding

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