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Research article

EFFECT OF CULTURAL CONDITIONS ON LOVASTATIN PRODUCTION BY *ASPERGILLUS NIGER* SAR I USING COMBINATION OF RICE BRAN AND BROWN RICE AS SUBSTRATE

Syarifah Abdul Rashid[1*], Darah Ibrahim[1], and I Nyoman Pugeg Aryantha[2]

[1]Industrial Biotechnology Research Laboratory (IBRL), School of Biological Sciences, Universiti Sains Malaysia, 11800 Minden, Penang Malaysia.

[2]School of Life Sciences and Technology, Institut Teknologi Bandung, Jl. Ganesa No. 10 Bandung, Indonesia 40132

ABSTRACT: A local fungal isolate *Aspergillus niger* SAR I produced high level of lovastatin activity when cultivated under solid substrate fermentation using a combination of rice bran and brown rice as substrate. The highest lovastatin production of 305.08±14.65 ug/g dry solid and 12±0.01 mg glucosamine/g substrate of fungal growth were achieved at the 10th day of cultivation after using all the optimized parameters (5 gram of unground rice bran and brown rice at the ratio of 1:1, water content of 70% (v/w) using sterile distilled water, inoculum size at $1x10^5$ spores/mL and cultivation temperature of room temperature of 30±2 ºC). There was about 90.64% increment in lovastatin production after the optimization of cultural conditions compared with before optimization condition.

Key words: Lovastatin, *Aspergillus niger*, Rice bran

INTRODUCTION

World Health Organization (WHO) reported that about 17.3 million of mortality cases are caused by cardiovascular diseases and by 2030, the estimated number will be increased to 23.6 million. Unhealthy diet, physical inactivity, tobacco use and harmful use of alcohol are the usual causes that welcome hypercholesterolemia, an abnormal range of cholesterol in blood. Cholesterol ($C_{27}H_{45}OH$) is the basic problem of common cardiovascular diseases and generally, it cannot be dissolved in blood. Even though cholesterol consists a water soluble region, it is not sufficient to suspend in the blood as it also carries a fat soluble region. A special combination of lipid and protein known as lipoprotein is used to transport cholesterol to and from the cells in human blood. Regarding the carrier, high density lipoprotein (HDL) and low density lipoprotein (LDL) are the ones people must be most concerned about.

A potential secondary metabolite, statin, a vital hypercholesterolemia inhibitor grabbed attention of medicinal practitioners and scientists. Statin including lovastatin is tagged under natural fermentation derivation and appointed to inhibit an essential enzyme during cholesterol production namely 3-hydroxy 3-methyl glutary coenzyme A reductase or also called HMG-CoA reductase in cholesterol biosynthesis pathway (during the conversion of HMG-CoA to mevalonate). Lovastatin exists in two major forms: lactone (closed form ring) and acid form (open form ring). However, acid form is the only actionable and effective lovastatin in blood. After oral ingestion, the native form of lovastatin (lactone), need to be hydrolyzed to corresponding β-hydroxyacid form, which allowed the inhibition process of HMG-CoA reductase. As a primary site of action, liver became one of a crucial selection by lovastatin for the conversion purpose.

A solid report by Tobert (1987) of announcing lovastatin was the first specific inhibitor for hypercholesterolemia (Reddy, et al., 2011), has induced scientists to explore its production by all means. Again, we are back to our trusted classic system, namely solid substrate fermentation (SSF). Basically, SSF is referred to a process that involves solid in absence and near absence of free water; however, substrates must consist enough moisture to support growth and metabolism of microorganism. The tremendous advantages displayed by SSF in minimizing power and effluent waste, less processing and down streaming stages, and the use of economical substrate, were well reported in recent decades. Thus, the selection of substrates and microorganism should be in equivalent level with the superior SSF.

The selected substrates must be recognized to produce the specific product in SSF. Other substrate characteristics are that they are non soluble, they can act as nutrients and physical support, and they are naturally occurring agricultural crops or agroindustrial waste or inert support, which are required for SSF system (Pandey, 2003). SSF is also acknowledged for supporting various fungi to grow with high density of mycelial, which directly contribute in high final product in production. The exclusive mycelia of fungi permit them to colonize and penetrate the solid substrate, to absorb the needed nutrients. Lovastatin is an intracellular product and mostly accumulated in the mycelia (Reddy, et al., 2011), hence, fungi are suitable for lovastatin production under SSF system.

The present research deals with time coarse production of lovastatin and effect of some critical parameters reported in SSF, namely substrate size, moisture content, temperature and inoculum size. A shake flask system was implemented to produce lovastatin production by filamentous fungus, *A. niger* SAR I.

MATERIALS AND METHODS

Microorganism, culture maintenance and preparation of inoculum

Aspergillus niger SAR I provided by Industrial Biotechnology Research Laboratory, School of bIological Sciences, Universiti Sains Malaysia was used in the present study. It was maintained on potato dextrose agar slants, stored at 4 ^{0}C and subcultured every 4 weeks to ensure its viability.

Spore suspension was prepared using a well sporulated slant. The slant was added with 10 mL of sterilized distilled water. The surface of the fungal colony was gently scratched with an inoculation needle. The concentration of spore suspension was measured using a haemocytometer and adjusted to 1 x 10^7 spores/mL by diluting it suitably. A spore suspension of this concentration was used as inoculums throughout the study.

Production profile of lovastatin

A combination of 5 g rice bran and brown rice in the ratio of 1:1 was used and placed in a 250 mL Erlenmeyer flask. Moisture content was adjusted to 70% (v/w) by adding sterile distilled water and adjusted to pH 6.0 prior to autoclave. The flasks were sealed with cotton plug and aluminum foil and autoclaved at 121 ºC for 15 min. After cooling, media was inoculated with 1.0 mL (1 x 10^7 spores/mL) and mixed thoroughly to have a uniform spore distribution in the substrate. The inoculated flasks were incubated at room temperature (30±2 ºC) for 16 days aerobically. Each day 3 flasks were taken out and assayed for lovastatin and fungal growth.

Improvement of fermentation process for enzyme production

Various process parameters influencing lovastatin production during SSF were optimized. The strategy followed was to optimize each parameter (lovastatin activity and fungal growth), independent of the others and subsequently optimal conditions were employed in all experiments.

Physical parameters

Improvement of cultural conditions in shake flask system for a maximal lovastatin production involves various physical parameters. They were substrate particle sizes (0.1, 1.0, 3.0, 6.0 mm and unground), water content (50, 60, 70, 80 and 90%, v/w), cultivation temperature (25, 30±2, 35 and 40 ºC) and inoculum size of (10^4, 10^5, 10^6, 10^7 and 10^8 spores/mL). The cultivations were carried out for 10 days before determining the lovastatin activity and cell growth determination. All the experiments were performed in triplicate and the values were reported as standard deviations.

Time course of lovastatin production under SSF condition

A 16-day profile was conducted in a shake flask system and the basic conditions of SSF was applied. For every 24 hr, the samples were harvested, dried out and proceeded to HPLC analysis. All experiments were carried out in triplicates and the results were presented as the mean of the triplicates.

Extraction of lovastatin

Fresh fermented substrate samples were dried at 60 ºC for 48 hr in an oven. The dried samples were then suspended in 30.0 mL acetonitrile and shook for 1 hr at 220 rpm. Afterwards, samples were centrifuged at 3000 g for 10 min.

Two milliliter portions of aliquot were mixed up with 2.0 ml distilled water and 50.0 µL of concentrated phosphoric acid before filtered through nylon syringe filter (pore size of 0.45 µm). The filtered samples were subjected onto Thin Layer Chromatograpgy (TLC) plate and High Performance Liquid Chromatography (HPLC) using modified method of Szakacs et al. (1998).

Fungal growth determination

An essential chitin presented in cell wall of most fungi which can be converted to glucosamine, shall indicate fungal growth indirectly. Method by Tsuji et al. (1969) directed on how to convert chitin to glucosamine while Swift (1973) guided to prepare assay with Erhlick's reagent and acetyl acetone reagent in distinguishing glucosamine. Glucosamine was detected spectrophotometrically at 530 nm and the growth was expressed as mg glucosamine per g of substrate. Glucosamine powder was used as a standard with concentration range of 0.1 to 1.0 mg/mL.

Reversed phase analytical procedure, HPLC

Analysis of lovastatin was carried out using HPLC system (Waters Corporation) at UV range wavelength (238 nm) in reverse phase Symmetry column which comprised acetonitrile, dimethyloctadecylsilyl bonded amorphous silica (C18), 4.6 mm x 250 mm and 5.0 µm particle diameter. Under Breeze software system (Waters Breeze System), all elutions were employed at 1.0 mL/min flow rate. Acetonitrile and ortho phosphoric acid (mixed in distilled water and adjusted to pH 3.0 using concentrated ortho phosphoric acid) were labelled as mobile phase eluents. Breeze system was equilibrated isocratically with the mobile phase ratio of 77:23 (v/v), respectively. For each run, 20 µl of samples were injected by needle sample loop into the column. To prepare standard solution, 25 mg of lovastatin (99.7% purity, Merck) was dissolved in acetonitrile and then concentrated ortho phosphoric was added (Szakacs, et al., 1998). The retention time (R_t) between standard and samples were compared to identify lovastatin via HPLC. Results were expressed as µg lovastatin per g dry solid.

RESULTS AND DISCUSSION

Lovastatin time course production

There are many reports on the advantages of using SSF in lovastation production which produces higher yield of product. The fermentation profile of *A. niger* SAR I is shown in Figure 1. The lovastatin production increased slowly from the inoculation day until day 6 and shot up drastically to achieve maximum yield of about 160.03±3.79 µg/g dry solid was obtained at day 10^{th} of cultivation and after that, it slightly decreased. A fluctuating fungal growth was noticed which symbolised a not growth depending characteristic of lovastatin. The indicated fungal growth at day 10^{th} was 1.31±0.03 mg glucosamine/g substrate.

The lovastatin yield increased, which explained that lovastatin is a kind of secondary metabolite and its accumulation in the fungal mycelia. The decrease in lovastatin production after achieving its maximal production could be due to the onset of death phase of fungus and nutrient depletion. A tight interaction between fungal mycelia and solid substrates that did not permit a full revival of biomass which finally delayed the estimation of growth rates and yields could also be the reason.

Influence of substrate size towards lovastatin production

Microorganism penetration depends on the physical condition of the substrate such as surface, assessment of the area and porosity. The undefined size of rice bran provided a preferable attacking surface area for *A. niger* SAR I, while the existence of unground brown rice in between rice bran particle allowed a better aeration including mass transfer of various nutrients, which was good for fungal growth. This combination provided good substrate porosity for fungus to grow, besides serving as good anchorage medium for fungal growth.

As reported by Prakasham et al. (2006), the size alteration contributed in intra-particulate associated aeration, available surface area for microbial attachment and substrate mass transfer and subsequent growth and product production. In the present study, a combination of unground brown rice and undefined rice bran indicated the highest production of lovastatin of 177.80±1.35 µg/g dry solid with 1.27±0.14 mg glucosamine/g substrate of fungal growth compared with other sizes (Figure 2). The decrement of substrate sizes (brown rice) parallels with the lovastatin production. The figure also shows the substrate size of 0.1 mm revealed the lowest lovastatin production (172.05±2.66 µg/g dry solid). Based on the results, it is clear that substrate sizes did not influence lovastatin production significantly. Therefore, the original size for both substrates was selected for the next parameter as it demanded no costly process such as grinding.

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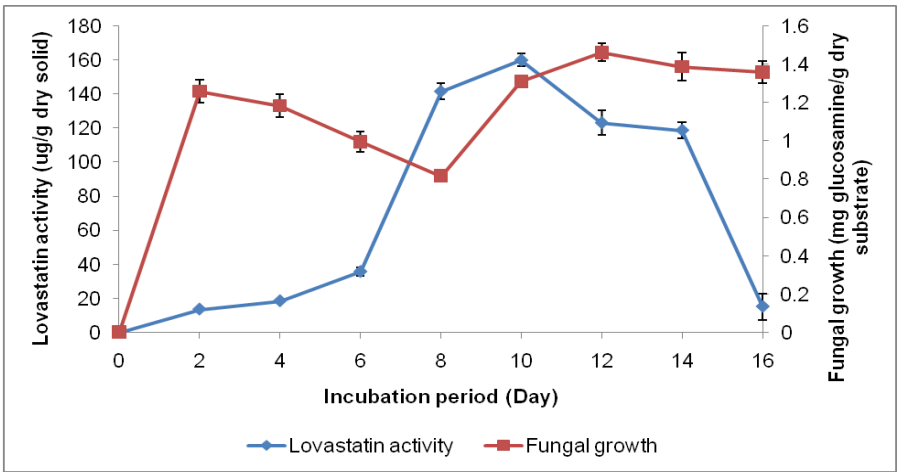

Figure 1: Time course profile of lovastain production and fungal growth under SSF using combination of rice bran and brown rice before the optimization of physical parameters

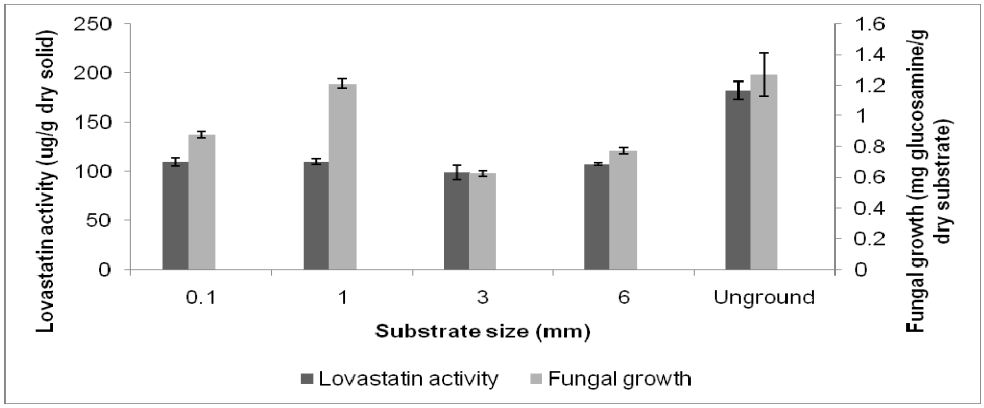

Figure 2: Effect of substrate size on lovastatin production and fungal growth by *A. niger* SAR 1

Influence of water content

Water activity is one of the critical factors in SSF because it is related to the definition of SSF itself. Lenz et al. (2004) declared that water activity or moisture level as a key factor in SSF system as the major process including hypha elongation, spore growth, and production of metabolite matter, are very sensitive to water tension. However, SSF only requested a limited and optimum level of water. Figure 3 indicates that 70% (v/w) of water content gave the best lovastatin production which was the optimum level of water required in this system (204.28±2.82 µg/g dry solid and 0.87±0.03 mg glucosamine/ g substrate of fungal growth). Lower water content was identified to induce sporulation process of microorganism. At the same time water reduces the absorbance of substrate nutrient, problematic during mixing, and also increase the water tension (Lonsane *et al*., 1985). Those symptoms might occur all through 50% (v/w) of water content condition which depicted the lowest production (168.34±0.09 µg/g dry solid and 0.89±0.02 mg glucosamine/g substrate of fungal growth). Shrinking phenomenon of rice bran can be also a factor for the productivity dwindle. Rice bran swells once it absorbs water and later becomes shrivel when it loss the moisture. Lower level of water can not afford to cover this loss throughout 10 days of cultivation period. Thus, it directly affects lovastatin production and fungal growth.

As the moisture content in the fermentation medium increases, the air present in the void volume decreases, resulting in less oxygen available for the process. Without forced aeration and with low moisture content, the available oxygen is sufficient but the water content is not enough to support good metabolic activity and dissipation of heat generated and may account for lower lovastatin production. The optimal water content promotes the hyphal growth and their good tolerance to low water activity (Aw) and high osmotic pressure conditions make it efficient and competitive in natural microflora for bioconversion of solid substrates.

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Influence of temperature

In SSF, the net temperature in the system is influenced not only by the environmental temperature, but also by the increase in temperature generated from the metabolic activities of the fungi growing on the solid substrates (Pang *et al*., 2006). *A. niger* SAR I is a mesophilic fungus and the optimum temperatures for growth is in between 20 and 40 ^{0}C (Manpreet, et al., 2005). As observed in Figure 4, there is no significant different between temperature of 25 ^{0}C and 30±2 ^{0}C, but, the best production was obtained during 30±2 ^{0}C (209.31±3.08 µg/g dry solid and 0.64±0.03 mg/g substrate of fungal growth). As for 25 ^{0}C, the production was about 205.38±6.44 µg/g dry solid and fungal growth was 1.25±0.02 mg/g substrate. Both of temperatures are likely to be the natural habitat of *A. niger* SAR I, thus, those surrounding heat competent in obtaining lovastatin. Temperature of 40 ^{0}C still denoted a good production even though not as competitive as other temperatures (173.67±1.25 µg/g dry solid and 0.89±0.04 mg/g substrate of fungal growth). However, lovastatin production reduced gradually above the optimal incubation of 30±2 ^{0}C. With further increase in temperature, more heat is accumulated in the medium during mesophilic aerobic SSF, which leads to poor heat dissipation, thus reducing the oxygen level and thereby the growth of microorganism, as lovastatin is growth related product. These results are coinciding with those previously reported for lovastatin production. Moreover, when culture was cultivated at 40 ºC the medium dried quite fast and the spores did not developed well. Therefore, the optimum temperature was considered as 30±2^{0}C.

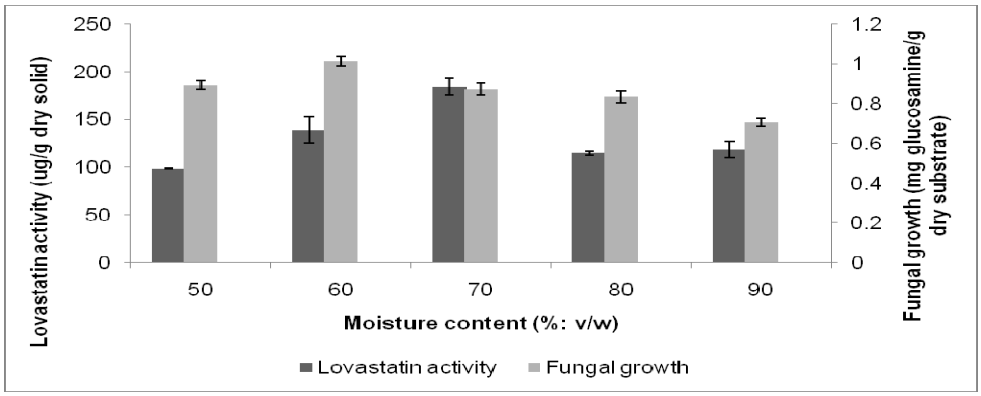

Figure 3: Effect of moisture content on lovastatin production and fungal growth by *A. niger* SAR 1

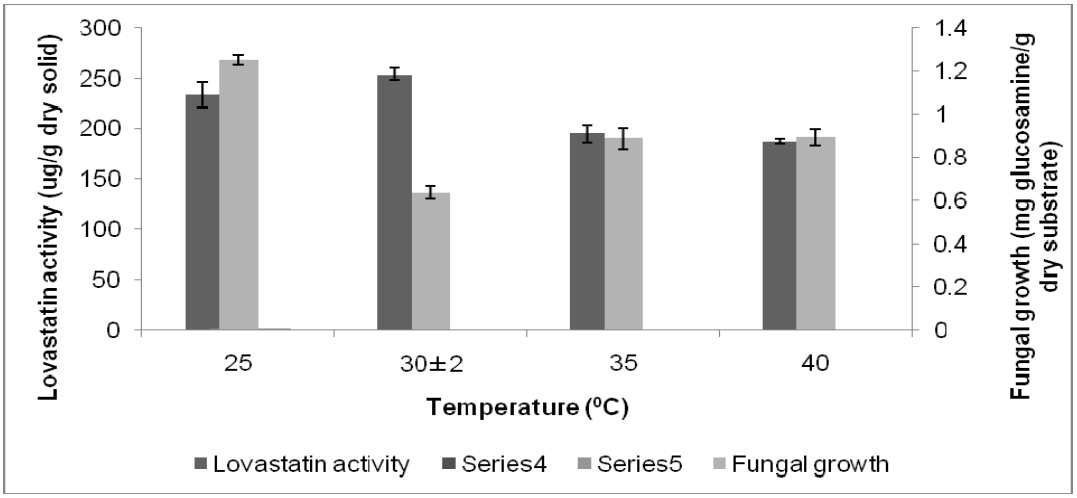

Figure 4: Effect of cultivation temperature on lovastatin production and fungal growth by *A. niger* SAR 1

Influence of inoculum size

A range of inoculum sizes (1 x 10^4 to 1 x 10^8 spores/mL) allowed an overall observation of cell competition in a limited nutrient condition. A lower inoculum size dedicated a long lag phase which indirectly requested longer fermentation process to produce the product.

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Meantime, a higher inoculum size competed among the cells in shorter time, which will then rise an issue of whether the secondary metabolite can be generated at a maximum level. As observed in Figure 5, lovastatin was produced less during the application of 1 x 10^8 spore/mL (208.73±19.84 µg/g dry solid, 1.78±0.05 mg/g of fungal growth) into SSF system, compared to others. Later, the activity of 1 x 10^4 spore/mL (214.22±6.34 µg/g dry solid, 0.90±0.08 mg/g of fungal growth) appeared to be the comrade for the 1 x 10^8 spore/mL inoculum size. During the addition of 1 x 10^6 and 1 x 10^7 spore/mL into SSF, an inductive activity was produced; 249.49±2.82 ug/g dry solid (1.56±0.09 mg glucosamine/g substrate of fungal growth) and 223.95±16.82 ug/g dry solid (1.47±0.02 mg glucosamine/g substrate of fungal growth), respectively. After 10 days of cultivation time, 1 x 10^5 spore/mL of inoculum size was the best concentration to generate maximal lovastatin. The obtained activity was 297.64±0.56 ug/g dry solid and 0.93±0.05 mg glucosamine/g substrate of fungal growth.

Optimization of inoculum size (spores/mL) is necessary in SSF because too little spores lead to insufficient biomass to form mycelia and produce lovastatin, whereas too many spores will lead to over production of biomass leading to quick depletion of available nutrients in the cultivation medium, yielding poor mycelial growth and thus promoting less product formation.

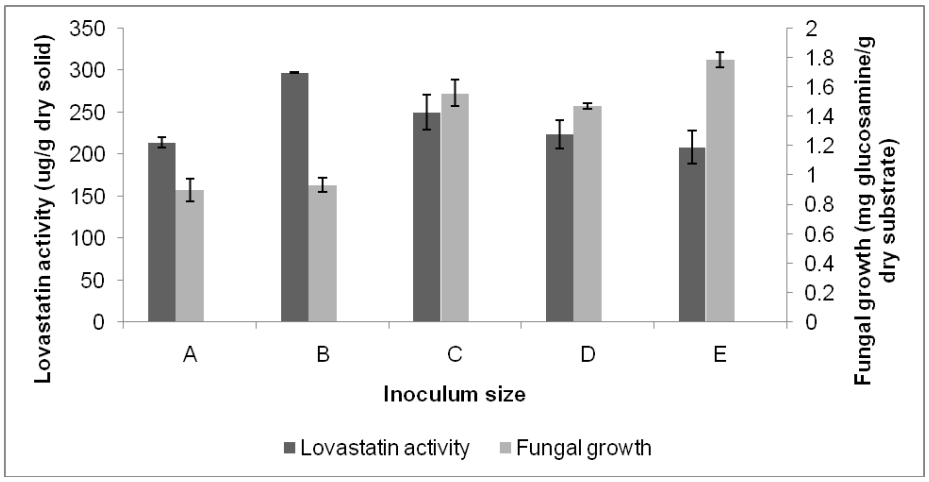

Figure 5: Effect of inoculum size on lovastatin production and fungal growth by *A. niger* SAR 1 (Indicator: A= 1x10^4, B= 1x10^5, C= 1x10^6, D= 1x10^7, E= 1x10^8 spore/ml)

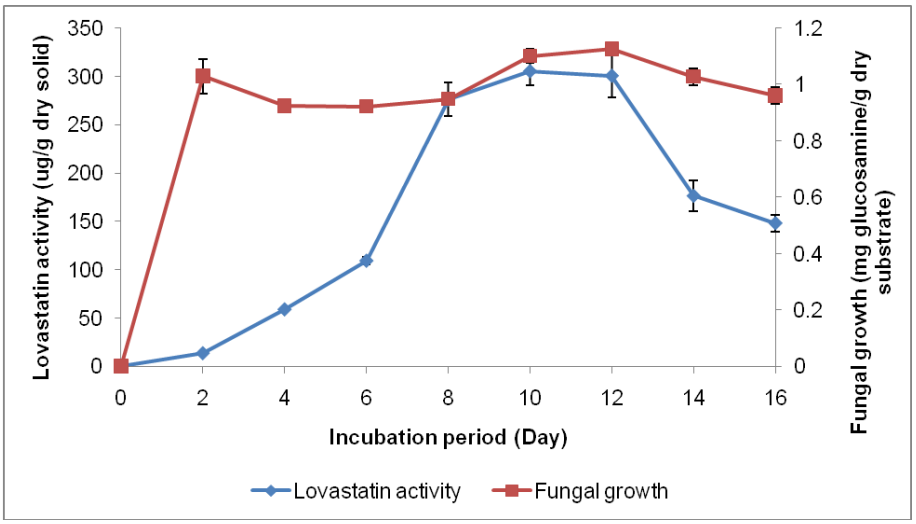

Figure 6: Time course profile of lovastain production and fungal growth under SSF using combination of rice bran and brown rice after the optimization of physical parameters

Time course production after optimization of cultural conditions

Figure 6 shows the time course profile of lovastation production by *A. niger* SAR 1 using all the optimized cultural conditions (5 g of unground rice bran and brown rice at the ratio of 1:1, water content of 60% (v/w) using sterile distilled water, inoculums size at $1x10^5$ spores/mL and cultivation temperature of room temperature which was 30±2 ºC). The cultivation was carried out for 16 days and it was observed that lovastatin production increased from day two until it reached its maximal production of 305.08±14.65 ug/g dry solid at day 10^{th} of cultivation (optimum day). Later, it decreased until the end of cultivation period. Again, the fungal growth curve had a fluctuating pattern and the highest growth was depicted at day 12^{th} of cultivation with about 1.12±0.01 mg glucosamine/g substrate. There was an increase of 90.64% in lovastatin production after the optimization of cultural conditions compared with before optimization.

CONCLUSION

In the present investigation, *A. niger* SAR I has an excellent potential as a lovastatin producer under SSF. The present study has shown a precise detection of lovastatin after 10 days of cultivation period. Analysis of lovastation in HPLC confirmed its identity with that of authentic lovastatin. Furthermore, *A. niger* SAR I cooperates very well with substrate size, water content, temperature and inoculum size.

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