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The investigation of media components for optimal metabolite production of *Aspergillus terreus* ATCC 20542

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ABSTRACT

Purpose: This study aimed to assess the effect of nitrogen, salt and pre-culture conditions on the production of lovastatin in *A. terreus* ATCC 20542.
Methods: Different combinations of nitrogen sources, salts and pre-culture combinations were applied in the fermentation media and lovastatin yield was analysed chromatographically.
Result: The exclusion of $MnSO_4 \cdot 5H_2O$, $CuSO_4 \cdot 5H_2O$ and $FeCl_3 \cdot 6H_2O$ were shown to significantly improve lovastatin production (282%), while KH_2PO_4, $MgSO_4 \cdot 7H_2O$, and NaCl and $ZnSO_4 \cdot 7H_2O$ were indispensable for good lovastatin production. Simple nitrogen source (ammonia) was unfavourable for morphology, growth and lovastatin production. In contrast, yeast extract (complex nitrogen source) produced the highest lovastatin yield (25.52 mg/L), while powdered soybean favoured the production of co-metabolites ((+)-geodin and sulochrin). Intermediate lactose: yeast extract (5:4) ratio produced the optimal lovastatin yield (12.33 mg/L) during pre-culture, while high (5:2) or low (5:6) lactose to yeast extract ratio produced significantly lower lovastatin yield (7.98 mg/L and 9.12 mg/L, respectively). High spore concentration, up to 10^7 spores/L was shown to be beneficial for lovastatin, but not for co-metabolite production, while higher spore age was shown to be beneficial for all of its metabolites.
Conclusion: The findings from these investigations could be used for future cultivation of *A. terreus* in the production of desired metabolites.

1. Introduction

Carbon and nitrogen are arguably the most important nutrients for fungal growth and their metabolite production. Carbon source is often associated with metabolite production, whereas nitrogen is essential for the growth of the fungus. Normal medium is usually comprised of carbon source (s), nitrogen source (s), water, salts and micronutrients. In industrial fermentation, the use of complex nitrogen sources is unfavourable due to their high cost and low reproducibility. However, a number of studies suggested that complex nitrogen sources are more effective in supporting the growth of *A. terreus* (Kumar et al., 2000; Hajjaj et al., 2001; Casas López et al., 2003, 2005; Bizukojc and Ledakowicz, 2007a), which is a prolific producer of lovastatin, a cholesterol lowering drug. It was suggested that these complex nitrogen sources are only utilised by fungi because of the lack of ammonium ion (Bizukojc and Ledakowicz, 2010), although other studies found that nitrates and ammonium failed to support any lovastatin production (Hajjaj et al., 2001). Additionally, the use of single amino acids such as histidine or sodium glutamate only produced several tens of milligrams per litre (Hajjaj et al., 2001). Most studies using a complex nitrogen source for lovastatin production, such as yeast extract, corn steep liquor and peptonised milk have been met with greater success (Hajjaj et al., 2001; Casas López et al., 2003, 2005).

The presence of macro and micronutrients in the growth medium has also been shown to influence lovastatin production. These nutrients are not essential for microorganism growth, but act as supplements to achieve better fermentation. Certain divalent metal cations (from mineral salts) such as Fe^{2+}, Ca^{2+}, Zn^{2+}, Mg^{2+} and Mn^{2+} were reported to improve the growth of fungi (Jia et al., 2009). The effect of salt can be either ion-specific (Jia et al., 2009) or osmotic-based (Wucherpfennig et al., 2011). In a more specific study conducted by Bizukojc et al. (2007), it was postulated that certain individual vitamin B (B3, B5 and B6) and their combination may enhance lovastatin formation by providing more precursors (for example, NAD(P), FAD,

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coenzyme A and protein biosynthesis) for lovastatin biosynthesis. The addition of butyrolactone I, a self-regulating metabolite that stimulates lovastatin formation, to the culture of *A. terreus* has also been conducted by Schimmel et al. (1998). Their study demonstrated that butyrolactone I increased the branched hyphae, the number of spores and the production of lovastatin (Schimmel et al., 1998).

The pre-culture condition may also influence microbial growth and metabolite production. According to Bizukojc and Ledakowicz (2007b), pre-culture for *A. terreus* is important to speed up the stationary phase into metabolite production phase (Bizukojc and Ledakowicz, 2007b). During pre-culture, an optimal ratio of nitrogen and carbon is required and this may differ from the main culture medium. It is generally accepted that a nitrogen-deficient culture medium favours the growth and metabolite production of *A. terreus* (Casas López et al., 2003), but higher nitrogen is needed during pre-culture conditions to speed up their metabolite-production phase (Bizukojc and Ledakowicz, 2007b). The optimal spore conditions, such as spore age and spore amount, are also essential for the pre-culture to thrive (Bizukojc and Ledakowicz, 2009). Therefore, this study aimed to investigate the effects of different nitrogen sources, carbon/nitrogen ratio, salt types and pre-culture conditions on the production of lovastatin, (+)-geodin and sulochrin. At the same time, the morphology of the fungus was also analysed. The effect of carbon sources on this fungus will not be discussed, as it has already been published elsewhere (Rahim et al., 2017).

2. Materials and methods

2.1. Culture conditions

The culture condition was prepared based on the authors' earlier investigation (Abd Rahim et al., 2015). In short, the fungal spore of *A. terreus* ATCC 20542 was rehydrated based on the manufacturer's recommendation (Cryosite Distribution Pty Ltd., Sydney, Australia), maintained on the potato dextrose agar, and counted using a haemocytometer. The inoculation was done using 10^7 spores/mL in 50 mL culture medium, in 125 mL Erlenmeyer flasks.

The culture was cultivated at 185 ± 5 rpm and at a temperature of 30 ± 1 °C in a shaking incubator. The basal medium used for the experiments were 20 g/L lactose, 4 g/L yeast extract (YE), 0.4 g/L KH_2PO_4, 0.2 g/L MgSO4·$7H_2O$, 0.4 g/L NaCl, and 0.001 g/L $ZnSO_4·7H_2O$, at pH 7, unless stated otherwise. The pre-culture was prepared in a similar basal medium but with lower carbon source (10 g/L) and higher nitrogen source at 8 g/L, unless stated otherwise. The fungus was used for cultivation when the diameter of the fungus pellet reached 1.5 ± 0.5 mm(measured using a digital calliper).

The transfer of fungus pellet from pre-culture media to culture media (in new, sterilised 125 mL Erlenmeyer flask) involved several steps (in a sterile condition). A sterilised strainer was used to recover the fungus from the pre-culture flask and washed twice with sterile deonised water. The collected fungus was then transferred to a new flask with the appropriate culture medium. This process was either done under the biosafety cabinet or next to the Bunsen burner.

2.2. Fungal biomass dry weight determination

The biomass yield was determined gravimetrically. Fungus biomass was recovered by filtration using No. 2 Whatman filter paper and washed twice with distilled water, followed by drying at 80 °C for 24 h or until a constant weight was achieved.

2.3. Analytical analysis

Quantification of lovastatin, (+)-geodin and sulochrin was carried out using High-Performance Liquid Chromatography (HPLC), as described previously (Abd Rahim et al., 2015). The HPLC parameters were: Agilent 1200, UV detector (238 nm), XDB Eclipse Zorbax C-18 column at 30 °C, sample chamber temperature at 4 °C, a flow rate at 1.0 mL/min, an injection volume of 10 µL 95% acetonitrile and 0.1% phosphoric acid solution as mobile phases. The lovastatin standards were prepared from the commercially available lovastatin tablet (Lovastin, YSP Industries, Malaysia) (Casas López et al., 2003), while (+)-geodin and sulochrin standard were purchased from Sapphire Bioscience (Sydney, Australia).

2.4. Microscopy

For the detailed viewing of much smaller fungus spore, Scanning Electron Microscope (SEM) was used (Hitachi S4500 FEG-SEM). Before the SEM was used, the samples were prepared and mounted on the special thermanox coverslip (ProSciTech, QLD, Australia) then treated with a special cell-adhering solution (1% polyethylenimine). The spores were adhered to the surface by adding sufficient liquid spore solution on the coated side of the coverslips. After 30 min of incubation, the spore's solution was discarded and primary (2.5% glutaraldehyde) and secondary fixatives (1% osmium tetroxide in 0.1 M PBS) were added for at least 1 h each, before the procedure of dehydration series was performed. The dehydration steps involved several washing procedures using increasing concentrations of ethanol to remove any traces of water. The coverslips were then treated with a hexamethyldisilazane (HDMS) solution for 2 min and then placed in a desiccator with the lid off to allow the HDMS fumes to evaporate overnight. Finally, the coverslips were mounted on stubs and coated with gold for viewing.

2.5. Statistical analysis

All experiments were conducted with a minimum of triplicates. The data obtained for all metabolites were analysed using one-way ANOVA with Tukey post hoc test, with significance when $p < .05$. All statistical analysis was performed using GraphPad Prism, version 6.01. For the plotting of the graph, 95% confidence interval was used for the error bar.

3. Result and discussion

3.1. The effect of pre-culture and salts interactions on the production of lovastatin

Salt is an essential component of the culture media. Besides providing additional nutrients in the form of metal ions, it is also essential for the regulation of osmotic pressure in a microorganism. The use of salts in this experiment is depicted in Table 1 and the type and quantity was pre-determined by referring to the previous literature (Jia et al., 2009; Chatzifragkou et al., 2010, 2011). $MnSO_4·5H_2O$, $CuSO_4·5H_2O$ and $FeCl_3·6H_2O$ formed crystal precipitates in the solution when all the salts were combined and heated together in an autoclave at 121 °C for 15 min under high pressure. The formation of these solid crystals could damage the fungus, leading to lower lovastatin production. Their exclusion from the media was shown to improve lovastatin production by more than three-fold (Treatment 2, Table 1). The addition of $CaCl_2·2H_2O$ in the media led to no significant change in lovastatin production; hence it was also excluded (Treatment 3). Other salts, such as KH_2PO_4, $MgSO_4·7H_2O$, NaCl and $ZnSO_4·7H_2O$ were shown to be important as their absence significantly reduced lovastatin production (Treatment 4, 5, 6 and 7). No-salt negative control showed a significant reduction of lovastatin production compared to the highest lovastatin produced (282% decrease). In all the experiments performed, the biomass did not differ significantly between different treatments. Based on these initial results, we optimised the amount of KH_2PO_4, $MgSO_4·7H_2O$ and NaCl using a full factorial design (3 levels), as depicted in Table 2. As shown in Table 2, the optimal amount of KH_2PO_4 and NaCl is 0.4 g/L, and $MgSO_4·7H_2O$ is at 0.2 g/L, which produced up to 9.96 mg/L of lovastatin yield (set number 20).

Table 1
Optimisation experiment of salt contents in the media. Black-shaded cells indicate exclusion of salt from the solution. Lovastatin yield was measured at day 5. All treatments showed statistically significant increase in lovastatin production when compared to treatment 1 (positive control). Only treatment 2 and 3 are statistically significant when compared to treatment 8 (negative control).

Salts	Amount (g/L)	Treatments							
		1	2	3	4	5	6	7	8
KH_2PO_4	1.0								
$MgSO_4{\cdot}7H_2O$	0.8								
NaCl	0.8								
$ZnSO_4{\cdot}7H_2O$	0.001								
$CaCl_2{\cdot}2H_2O$	0.1								
$FeCl_3{\cdot}6H_2O$	0.08								
$CuSO_4{\cdot}5H_2O$	0.01								
$MnSO_4 \cdot 5H_2O$	0.001								
Lovastatin (mg/L)		3.10	11.85	10.59	9.58	8.42	8.57	8.18	7.12
Significance			***	***	**	**	**	**	*
Biomass (g/L)		7.86	7.79	8.01	7.86	7.67	7.63	7.85	7.55

Table 2
Optimization of the amount of three chosen salts (KH_2PO_4, $MgSO_4{\cdot}7H_2O$ and NaCl) on lovastatin production, using 3-level factorial design). 95% confidence was used as the standard error. Asterisk (*) indicates level of significance.

Set	KH_2PO_4 (mg/L)	$MgSO_4{\cdot}7H_2O$ (mg/L)	NaCl (mg/L)	Lovastatin yield (mg/L)
1	0.4	0.2	0.1	7.84 ± 1.05**
2	0.1	0.1	0.1	6.15 ± 0.45*
3	0.1	0.4	0.2	6.04 ± 0.87*
4	0.1	0.2	0.2	7.67 ± 0.94**
5	0.1	0.1	0.4	7.55 ± 1.34*
6	0.4	0.1	0.1	7.32 ± 1.15*
7	0.2	0.2	0.2	7.75 ± 0.73**
8	0.2	0.2	0.4	7.91 ± 1.26**
9	0.4	0.2	0.2	7.96 ± 1.01**
10	0.2	0.4	0.1	6.32 ± 0.66*
11	0.2	0.1	0.4	7.18 ± 0.69*
12	0.1	0.1	0.2	6.25 ± 1.12*
13	0.4	0.4	0.1	6.68 ± 0.83*
14	0.4	0.1	0.4	9.05 ± 1.47**
15	0.2	0.4	0.2	6.91 ± 0.79*
16	0.2	0.1	0.1	7.17 ± 1.25*
17	0.1	0.4	0.4	6.67 ± 0.94*
18	0.1	0.2	0.4	7.62 ± 1.38*
19	0.4	0.4	0.2	6.59 ± 0.53*
20	0.4	0.2	0.4	9.96 ± 1.03***
21	0.4	0.1	0.2	7.27 ± 1.14*
22	0.4	0.4	0.4	7.56 ± 0.87*
23	0.1	0.4	0.1	6.05 ± 0.48*
24	0.2	0.4	0.4	7.39 ± 1.04*
25	0.2	0.2	0.1	7.34 ± 1.07*
26	0.1	0.2	0.1	6.13 ± 0.91*
27	0.2	0.1	0.2	7.03 ± 0.79*

Pre-culture for *A. terreus* is essential in providing sufficient nutrients and shortening the time of the main cultivation. In this section, we tested the effect of pre-culture and the optimal concentration of nitrogen sources (YE) needed for the pre-culture. The amount of carbon (lactose) for all the treatments was constant at 10 g/L (with 8 g/L YE), except for the control at 20 g/L (with 4 g/L YE). After 24 h, the fungal pellet was transferred into culture media (20 g/L lactose and 4 g/L YE) for 5 days to measure their effect on lovastatin production. The summary of the experiment and results are shown in Table 3.

In our investigation, the optimal YE (as nitrogen source) for pre-treatment was at 8 g/L as it supported high amount of lovastatin (12.33 mg/L), although it did not produce the highest biomass or diameter of the fungal pellet. In a previous investigation, it was found that biomass growth of *A. terreus* is not directly related to lovastatin production (Bizukojc and Ledakowicz, 2007a). Similarly, the bigger pellet diameter may also reduce the metabolite production (Bizukojc and Ledakowicz, 2010). The usage of 4 g/L YE showed significantly lower growth and produced a smaller pellet, which translated into lower lovastatin production (7.96 mg/L). In contrast, the usage of 12 g/L of YE produced a significantly larger pellet and higher biomass, with low lovastatin production.

3.2. Nitrogen sources

Nitrogen source is one of the major components in the fermentation media, and complex nitrogen sources are usually preferred in the cultivation of *A. terreus* (Hajjaj et al., 2001; Casas López et al., 2003, 2005). In this investigation, this theory was revisited and tested using selected nitrogen sources at 4 g/L each, as recommended by Bizukojc et al. (Bizukojc and Ledakowicz, 2007a). Ammonium sulphate (AS) was used as a simple and an easily reproducible nitrogen source. Powdered soybean (SB), yeast extract (YE) and flour (wheat) were used as complex nitrogen sources. Fig. 1 summarises the production of lovastatin, (+)-geodin and sulochrin by *A. terreus* under the influence of different nitrogen sources over a period of 12 days. From our findings, it was clear that the use of AS is not favourable for metabolite production, as a significant reduction was observed across all three metabolites tested. This reduction was also observed by Hajjaj et al. (2001) who observed

Table 3
The effect of nitrogen source (yeast extract) concentration on growth, metabolite production, and diameter during the pre-culture of *A. terreus*. Control experiment consisted of higher carbon source (lactose) at 20 g/L, which is the concentration used after the pre-culture. The diameter was taken 24 h after cultivation while biomass and lovastatin yield were measured at the end of 5-days cultivation. Different superscript letters showed significance, within the same column.

Lactose (carbon) concentration (g/L)	YE (nitrogen) concentration	Ratio lactose:YE	Biomass (g/L)	Lovastatin yield (mg/L)	Diameter (mm)
10	0 g/L YE	10:0	1.69^a	3.19^a	0.40^a
10	4 g/L YE	5:2	6.18^b	7.96^b	1.11^a
10	8 g/L YE	5:4	7.51^c	12.33^d	2.05^b
10	12 g/L YE	5:6	10.83^d	9.12^c	2.51^c
Control (20)	4 g/L YE	5:2	5.10^b	6.54^b	1.46^b

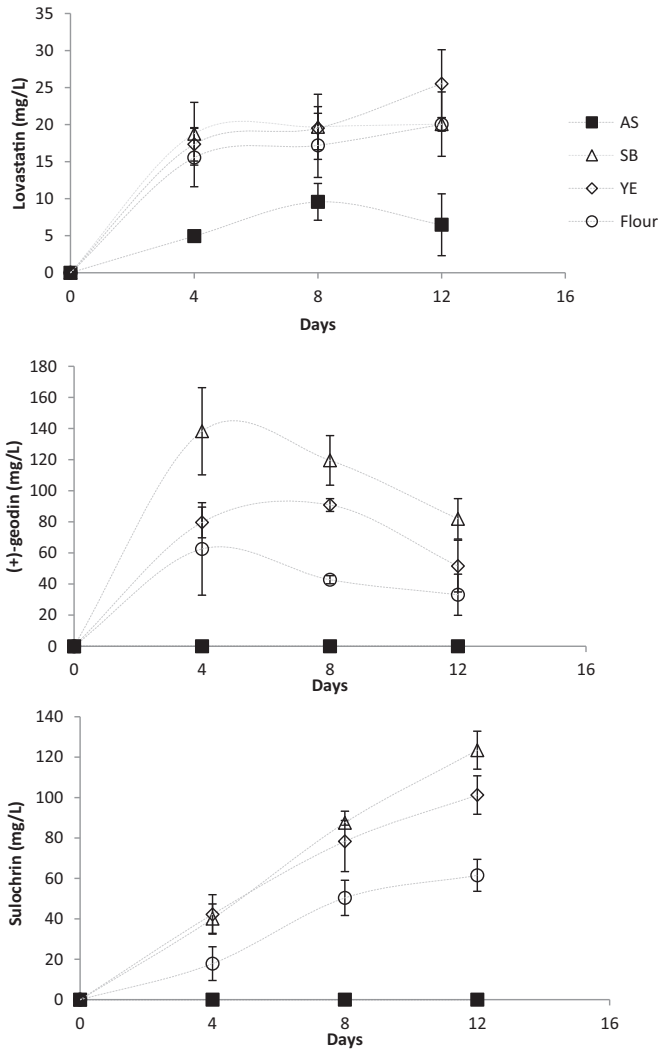

Fig. 1. Lovastatin, (+)-geodin, and sulochrin production over the period of 12 days. Lovastatin, (+)-geodin and sulochrin production of ammonium sulphate (AS) were significantly lower than other nitrogen sources. A) Lovastatin production levelled off after day 4 in all treatments. B) Reduction of (+)-geodin's production was observed only after day 8, while in C) sulochrin's production was still continuing even after day 12. Symbols show experimental values while dashed lines connecting symbols are used as a visual guide only. 95% confidence was used as the standard error.

no lovastatin production when ammonium ion was used as the main nitrogen source. However, they also observed that *A. terreus* primarily utilised ammonium ion for growth, which contrasted with our finding. We found that the cultivation of *A. terreus* in AS produced a significantly smaller and lighter pellet, with a soft, jelly-like appearance as compared to other nitrogen sources. This result indicates that apart from inhibiting metabolite production, the application of AS is also unsuitable for the growth of *A. terreus*. So far, the main reason for this is still unknown, as most microorganisms can readily assimilate ammonium ion for their growth. However, upon further investigation, it was observed that the use of AS acidifies the media (pH 4.1) as was consumed, which might not be conducive for *A. terreus*. Ammonium salts may be a good nitrogen source candidate for fermentation with organic acid production, such as citric acid fermentation (Papagianni, 2007). It is possible that the application of simple nitrogen sources that do not acidify the media would result in better metabolite production.

The use of complex nitrogen sources showed better production of lovastatin, (+)-geodin and sulochrin, without acidification of the media (all produced pH > 6). While the production of lovastatin showed significant improvement, a larger spike in production was observed in (+)-geodin and sulochrin. The pattern of production of (+)-geodin and sulochrin was also highly similar (as they are both produced in the same pathway), which may indicate that their production is influenced by the same factor under normal conditions. SB was shown to be the best nitrogen source for both metabolites, followed by YE and flour. Although the main reason is unclear, Bizukojc M. et al. (2011) proposed that (+)-geodin's production is reduced in the presence of nitrogen (Bizukojc and Pecyna, 2011). It is possible that SB is utilised more rapidly than the other nitrogen sources, leading to the starvation of nitrogen which and consequent increase of (+)-geodin and sulochrin in the broth. Additionally, complex nitrogen sources usually contain a mixture of many other compounds that might be beneficial for fungal growth, such as vitamin B complex. Further analysis of the complete nutritional content of complex nitrogen sources and consumption profile would be beneficial for the confirmation of this theory.

3.3. Optimal spore concentration for the production of metabolites in *A. terreus*

A. terreus can grow either as pelleted or dispersed hyphae in submerged culture. It is generally accepted that increased efficiency of lovastatin production can be achieved with the pellet form of *A. terreus* (Novak et al., 1997; Lai et al., 2003; Casas López et al., 2005). Although the method is still unclear, the formation of *A. terreus* pellet is thought to be either from the single or aggregation of spores (Metz and Kossen, 1977). Fig. 2 (from scanning electron microscope) suggests that the pellet formation likely arises from the aggregate of multiple spores. The main factors studied in this work were the spore amount and age, as shown in Fig. 3. The importance of spore factors has been demonstrated numerous times in other studies (Porcel et al., 2006; Bizukojc and Ledakowicz, 2010). Our observations showed that while spore amount affected the production of all three metabolites, the difference in production was only significant when the spore amount was increased from 10^5 to 10^7 spore/L, but not from 10^7 to 10^9 spores/L. It could be that high spore amounts can reduce the pellet diameter, contributing to higher lovastatin production. This was also observed in another study (Bizukojc and Ledakowicz, 2010). It is currently unknown why (+)-geodin and sulochrin reacted negatively to the increase in spore amount. It is most likely that higher spore levels can reduce resources such as the oxygen content in the media, which is essential for the

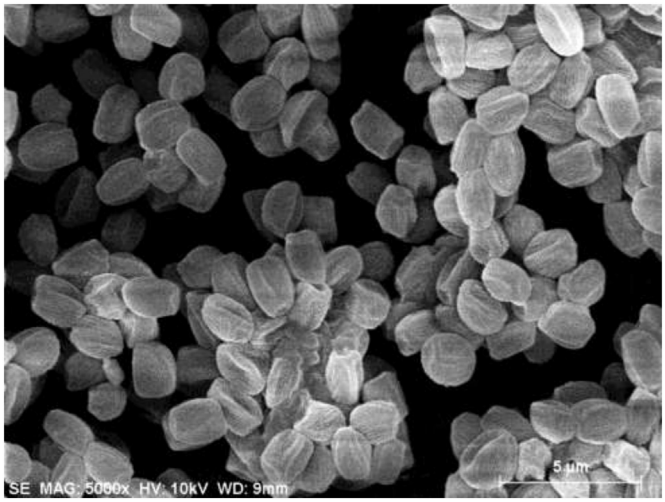

Fig. 2. The scanning electron microscope image of *A. terreus* spores under 5000 times magnification. An aggregate of spores was observed after 4 h post-inoculation, which suggests that the rise of the single pellet resulted from the combination of multiple spores.

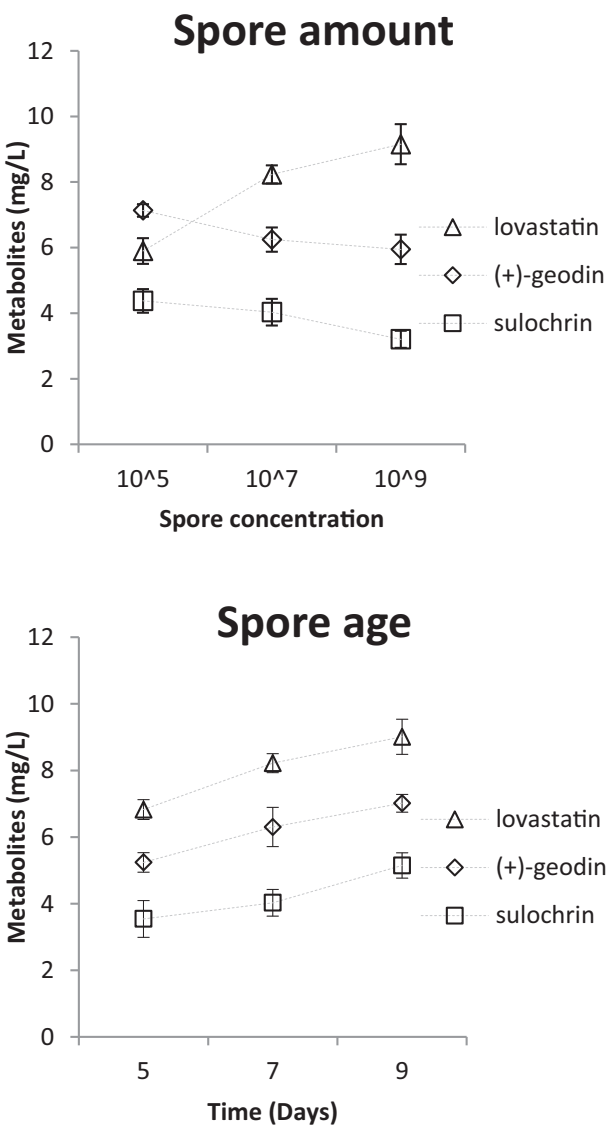

Fig. 3. The investigation of spore factors on the production of metabolites by *A. terreus*. a) Non-significant change of metabolite production was observed when different spore amounts were used. b) Increasing spore age also had minimal improvement on metabolite production. 95% confidence was used as the standard error.

formation of these metabolites (Bizukojc and Ledakowicz, 2009). 10^7 spores/L may be an optimal spore concentration for the cultivation of *A. terreus*, as 10^9 spores/L produce very high biomass, which adds to the complexity of transferring the fungus pellet into new cultivation media, coupled with the low production of (+)-geodin and sulochrin. Spore age is measured based on how long (usually hours or days) the fungus was grown on the solid media before harvested into suspension. Higher spore age produced higher metabolites but was not significant in value from one point to the other.

4. Conclusion

It was found that the different type of salt and nitrogen in the media of *A. terreus* could greatly affect the production of lovastatin. A simple nitrogen source, such as ammonia, is inhibitory to the growth and metabolite production. In contrast, complex nitrogen sources are stimulatory to the metabolite production, and the type of complex nitrogen sources affected (+)-geodin and sulochrin significantly, unlike lovastatin. Spore concentration could be an important determinant in the production of metabolite, unlike spore age which only has minimal effect on metabolite production.

Compliance with ethical standards

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Research involving human participants and/or animals

N/A.

Informed consent

N/A.

Declaration of Competing Interest

N/A.

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