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Effect of amino acids on pigments, citrinin, and lovastatin production by *Monascus purpureus* under static conditions

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Monascus spp. is known to produce many secondary metabolites including pigments, statin, and undesired mycotoxin.

M. purpureus MTCC 410 strain was grown statically for ten days in potato dextrose broth supplemented with 1% UV sterilized amino acids. Whatmann filter No. 1 was used to separate the developed mycelia from the broth to get the intracellular pigment as the extracellular one is left in the filtrate. The pigment concentration was estimated by the calorimetric method for different wavelengths and expressed in colour value units (CVU). The presence of citrinin in the growth medium was checked under UV light at 350 nm and quantification was done with high-performance liquid chromatography column along with loop injector of 20 µl, and Shimadzu CLASS-VP version 5.032 software.

The maximum biomass (143.6 g/l) was observed with supplementation of 1% D-serine to the medium, whereas the maximum intracellular pigment yield was observed with supplementation of L-histidine monohydrochoride (yellow – 4.48, orange – 3.97 and red pigment – 2.0 CVU/ml). The maximum extracellular pigment yield was observed with supplementation of glycine (yellow – 2.18, orange – 1.65 and red pigment – 1.38 CVU/ml to the growth medium). The maximum lovastatin yield was observed with supplementation of L-cysteine mono hydrochloride and concentration of 2064 mg/l. Maximum citrinin (1.29 mg/l) was observed with supplementation of DL-norleucine to the growht medium.

M. purpureus requires suitable concentration of organic nitrogen in the form of amino acids for a higher yield of secondary metabolites such as supplementation of 1% L-cysteine monohydrochloride or L-tyrosine in the growth medium under submerged cultivation. None of the tested amino acids produced citrinin under experimental conditions making the outcome beneficial for industrial purposes.

Keywords: extracellular pigment, citrinin, amino acids, *Monascus purpureus*, biomass

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INTRODUCTION

Monascus purpureus is a mould species, purplish-red in colour, in Ascomycota division, Monascaceae family. For a long time it has been traditionally important in China, Thailand, and Japan for production of red rice wine, red soybeans, cheese, and ang-kak rice. It was found capable of producing safe natural pigments along with other therapeutically important secondary metabolites, including citrinin and statins (Dufosse et al., 2005).

It contributes to consumers' favourite food colours, from orange-red to violet-red, by producing at least six major related pigments preferred over other synthetic pigments nowadays used in food industry, and are categorized into three main groups: yellow pigments (monascin and ankaflavin), orange pigments (rubropunctain and monascorubin), and red pigments (rubropunctamine and monoscorubramine) (Wang et al., 2007). They are synthesized as water insoluble, aminophiles and unstable in extreme pH in the cytosol from acetyl-coA through multi-enzymatic polyketide synthase complex (Balakrishnan et al., 2014) becoming water soluble after being in contact with present amino acids. Their aminophile characteristic makes them to be associated with cellular proteins or with the cell wall, and this makes them difficult to be extracted. Therefore their extraction requires cell breakage and dissolution in an organic solvent like methanol (Patricia et al., 2005; Liu et al., 2014).

However, as an undesired product, hepatonephrotoxin citrinin is known to be produced along with pigments and it limits the use of *Monascus* as a producer of natural food colourants (Jongrungruangchok et al., 2004). Its production is the main concern worldwide and it has to be prevented using any possible techniques like the use of mutagenic agents inducing mutation in a specific gene of an organism or inhibition of a specific responsible polyketide enzymes (PKSs) (Kang et al., 2014). Supplementation of specific amino acid and cultivation under controlled conditions can enhance the pigment production and inhibit citrinin production (Sameer et al., 2005; Liu et al., 2005).

Lovastatin works as another cholesterol-lowering agent by suppressing cholesterol synthesis via inhibition of HMG-CoA reductase enzyme, which mediates the biosynthesis of cholesterol in liver and is known as a potent inhibitor simvastatin (Ahmad et al., 2009). Thus the presence of lovastatin in bioproducts of an organism is an additional advantage making *Monascus* spp. important in medical and pharmaceutical industries by cooperatively working with other compounds or individually to stimulate body cells in the treatment of Alzheimer's disease or cancer, induction of apoptotic cell death, reduction of inflammation, inhibition of tumorigenesis and viral replication (Nicoleta et al., 2012).

The present study was conducted to screen amino acids for biomass, pigment, and citrinin and lovastatin production under static conditions.

MATERIALS AND METHODS

Culture

Monascus purpureus MTCC 410, obtained from IMTECH, Chandigarh India, was maintained on Potato Dextrose Agar (PDA) medium at 30°C for seven days and preserved at 4°C, with subculturing once every four weeks; its purity was checked regularly (Dikshit, Tallapragada, 2013).

Submerged fermentation

Potato dextrose broth was sterilized by autoclaving, supplemented with UV sterilized 1% amino acids respectively, inoculated with a loopful of *M. purpureus*, and incubated for ten days under static conditions (Dikshit, Tallapragada, 2013).

Biomass estimation

After incubation, the mycelia was separated from the broth using Whatmann filter No. 1, obtained biomass was then weighed to determine the fresh biomass in grams per litre (Dikshit, Tallapragada, 2013).

Pigment estimation

Ten millilitres of both extracts were taken in two separate conical flasks and extra 10 ml of methanol was added and incubated in the shaker

incubatorfor 45 minutes, the filtrate was centrifuged at 10,000 rpm for 10 minutes. The pigment concentration was then determined by the colorimetric method where absorbance was measured at 450, 470, and 540 nm for yellow, orange, and red pigments respectively, and the colour value units were calculated. Colour value = O.D. x dilution x volume of extract/amount of sample (ml) (Ratana, Toshima, 1987).

Citrinin analysis

The extract was transferred into vials and saturated until the volume reduced to half of the original, 10 microliters of it was applied to Silica gel 60 F254 Aluminium sheets (Merck, Germany) using Ethyl acetate: acetone: water (4: 4: 1 v/v) as the mobile phase, and dried plates were examined under UV light at 350 nm to observe fluorescent yellow bands indicating the presence of citrinin. For its confirmation, High Performance Liquid Chromatography (HPLC) analysis was carried out, column with C18 with 250 × 4.6 mm width ID Lichrosper 100 and 5 µm particle size, along with loop injector of 20 µl, and Shimadzu CLASS-VP version 5.032 software, with a mobile phase made of Acetonitrile/water (65:35 v/v and 3.5 pH) acidified with ortho-phosphoric acid (Panda et al., 2010). Citrinin concentration was then estimated using the formula: Area of the sample/Area of the std × Amount of std/Dilution of std × Dilution of sample/amount of sample.

Estimation of lovastatin

Stock solution of lovastatin (6 mg/ml) was prepared in ethanol using pure and standard lovastatin gifted by Biocon, Bangalore, India. Hydroxylamine hydrochloride (12.5%) and sodium hydroxide (12.5%) both in methanol were prepared by refluxing 12.5 g of the solid material with 100 ml of methanol for few minutes, then their equal volumes were mixed and the precipitated sodium chloride was filtered off to obtain alkaline hydroxylamine reagent being used in 4 hours.

Ferric perchlorate (stock solution) was prepared by adding 0.8 g of iron powder into a 50 ml beaker, then in a closed fuming hood, 10 ml of 70% perchloric acid was added and heated until the iron dissolved. As iron dissolves very rapidly when the acid is hot, the reaction may become vigorous and the content was cooled in the fuming hood. Then 40 ml of this stock was mixed with 12 ml of 70% perchloric acid and the volume was made up to 100 ml with ethanol to make Ferric perchlorate reagent (Mohie et al., 2010).

Lovastatin was estimated by different aliquots of standard lovastatin (6 mg/ml) ranging from 20 µl to 100 µl transferred into a series of test tubes: 1 ml of alkaline hydroxylamine reagent was added to each test tube, then pH was adjusted to 1.2 ± 0.2 by adding an appropriate volume of 2 M HCl, followed by addition of 2 ml of ferric perchlorate reagent. After 25 minutes of incubation at room temperature, the absorbance values of the red colour product formed were read at 510 nm (Mohie et al., 2010). The same procedure was followed for the test samples.

RESULTS AND DISCUSSION

Identification of *Monascus purpureus* MTCC 410

The morphological characteristics of *Monascus purpureus* were identified by the pattern of its growing on potato dextrose agar. The mycelium was white in the early stages; however, it rapidly changed to rich pink and subsequently to distinctive yellow-orange colour (Fig. 1a, b). Deep crimson colour was formed as the culture aged (Patcharee et al., 2007).

Effect of amino acids on biomass production

Among all 24 different amino acids tested for biomass production, the results showed that *M. purpureus* produced maximum fresh biomass of 143.66 g/l with supplementation of 1% D-serine (Table 1). Amino acids promoted biomass production.

The growth and sporulation pattern of filamentous fungi are showing variable nature with supplementation of different amino acids and nitrogen sources. Growth and biomass production will also vary with the organisms used and provided growing factors, including

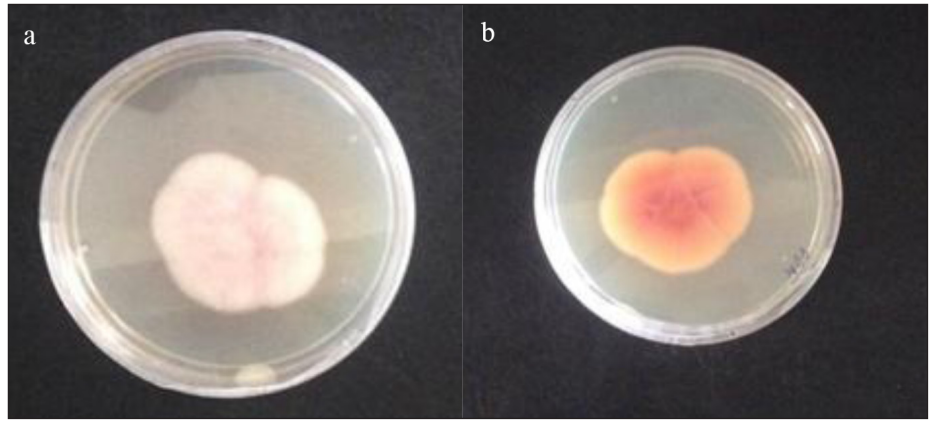

Fig. 1a. Dorsal view of *M. purpureus* 1b: Ventral view of *M. purpureus*

Table 1. **Screening of amino acids for biomass and pigment production under static conditions**

Sl. No.	Amino acid	Fresh biomass (g/L)	Intracellular (cvu/ml)			Extracellular (cvu/ml)		
			yellow	orange	red	yellow	orange	red
1.	DL-alanine	2.6	0.25	0.12	0.24	0.20	0.08	0.19
2.	DL-2-amino-n butyric acid	43.3	0.84	0.70	0.77	0.23	0.09	0.15
3.	L-a mono-hydrochloride	66.6	0.32	0.19	0.27	0.36	0.22	0.17
4.	DL-aspartic acid	30	0.31	0.17	0.18	0.07	0.14	0.05
5.	L-cysteine mono-hydrochloride	33.3	0.09	0.04	0.03	0.07	0.16	0.04
6.	L-cysteine	66.6	3.04	2.58	2.26	0.73	0.61	0.43
7.	3-(3,4-dihydroxy phenyl) DL-alanine	–	–	–	–	–	–	–
8.	L-glutamic acid	120	2.37	1.86	2.0	0.57	0.46	0.34
9.	Glycine	136.6	1.02	0.89	0.61	**2.18**	**1.65**	**1.38**
10.	L-histidine mono hydrochloride	136.6	**4.48**	**3.97**	**2.0**	0.68	1.21	0.3
11.	L-hydroxyl proline	60	2.24	1.81	1.92	0.35	0.21	0.14
12.	L-leucine	90	0.9	0.8	0.61	0.63	0.53	0.38
13.	L-isoleucine	120	0.34	0.23	0.28	0.56	0.46	0.36
14.	DL-norleucine	70	0.57	0.46	0.44	0.62	0.51	0.38
15.	L-lysine mono hydrochloride	66.6	1.92	1.44	1.68	0.26	0.12	0.10
16.	L-methionine	113.3	1.78	1.33	1.49	0.24	0.11	0.16
17.	DL-ornithine mono hydrochloride	77.6	1.10	1.00	0.82	0.84	0.73	0.59
18.	L-proline	8.3	0.1	0.02	0.12	0.34	0.22	0.28
19.	DL-β-phenyl alanine	128.3	0.84	0.74	0.7	1.00	0.92	0.72
20.	D-serine	**143.6**	3.22	2.8	3.41	0.14	0.01	0.10
21.	L-tyrosine	8	0.32	0.08	0.34	0.68	0.44	0.58
22.	DL-valine	28	0.16	0.18	0.20	0.32	0.08	0.30
23.	DL-threonine	24	0.32	0.04	0.38	1.44	1.14	1.34
24.	DL-tryptophan	30	1.2	0.74	0.84	2.06	1.50	0.68

the conditions like static fermentation condition. On the other hand, the nitrogen source plays a major role in enhancing sporulation, so that the spore germination results in increased mycelia (biomass). Amino acids have been essential nutrients; they participated in the metabolic transformations during spore germination leading from polar to non-polar phases of growth (Rajendra, 2006).

Effect of amino acids on pigment production

Supplementation of 1% L-histidine monohydrochloride yielded maximum yellow, orange, and red intracellular pigments of 4.48, 3.97 and 2.0 CVU/ml, respectively, and 1% glycine yielded maximum yellow, orange and red extracellular pigments of 2.18, 1.65 and 1.38 CVU/ml, respectively.

As the *Monascus* culture ages, the pigments are extracellular, safe, nontoxic, and with high stability against pH, light, and temperature (Patcharee, 2007). Submerged fermentation medium with amino acids promotes better production of extracellular red pigments, most of them being a complex of pigments and amino acids (Blanc et al., 1994). Histidine monohydrochloride mainly increased the intracellular red pigment, while the extracellular one was kept very low, almost equal to that of the control. Presumably, the amino acid might have entered the *Monascus* cell where the nitrogen in its amino group might have replaced the oxygen of the orange pigment resulting in the red pigment, so, the intracellular red pigment was higher than extracellular.

Glycine resulted in a large quantity of extracellular pigments. Very likely it changed the permeability of the membrane allowing the intracellular orange pigment to be secreted out of the cell and to react with corresponding amino acid in medium resulting in red pigments.

Conversely, supplementation of 3-(3, 4-dihydroxy phenyl)-DL-alanine completely inhibited pigment production. Similarly, L-cysteine monohydrochloride mainly reduced the intracellular but not extracellular pigment production. The results revealed a close relationship between the supplementation of amino acids and the production and secretion of the pigment. This was significantly meaningful for changing the components of the pigment and increasing the intracellular and extracellular pigment production (Xiao et al., 2013).

Citrinin analysis of *Monascus purpureus* by TLC and HPLC

The presence of citrinin was identified by means of TLC (Fig. 2) and further confirmed by HPLC; its production was found completely

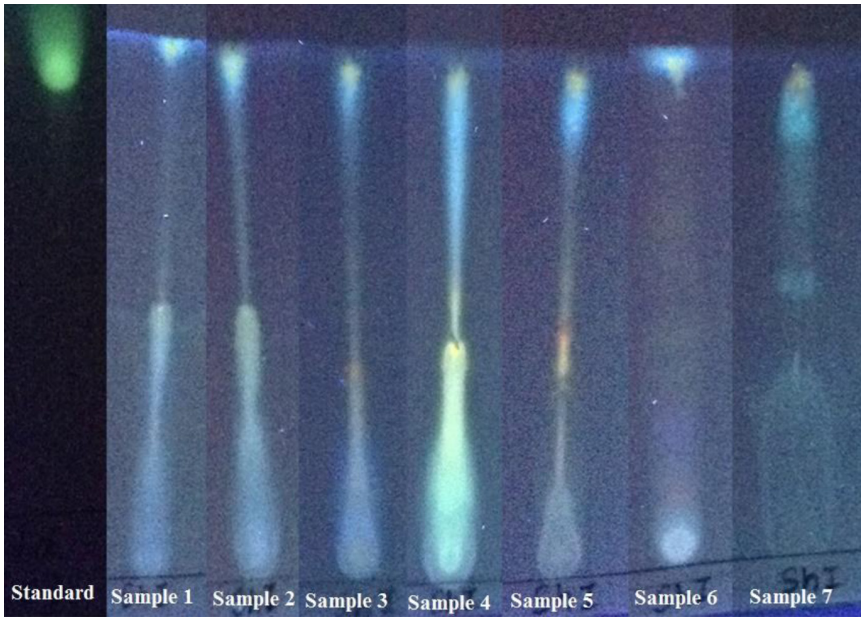

Fig. 2. Citrinin determination by TLC

inhibited under static conditions. Hajjaj et al. (2012) suggest that the precursor for both pigments and citrinin is tetraketide formed by condensation of one acetyl-CoA molecule and three malonyl-CoA molecules.

To obtain *M. purpureus* bio-products with low or zero citrinin, 24 different amino acids were screened under static condition and only few amino acid-supplemented medium was able to produce citrinin. Maximum citrinin (1.29 mg/l) was observed with supplementation of DL-norleucine (Table 2).

This might have inhibited the production of citrinin. Citrinin production also showed dependency on static and shaking culture conditions. Under static conditions a significantly lesser quantity of citrinin was produced. Hence, supplementation of appropriate amino acids under static conditions can be recommended for the production of larger quantities of the pigment and lovastatin and for a lower concentration of citrinin.

Production and quantification of lovastatin

Production and quantification of lovastatin was observed by the colorimetric method (Mohie et al., 2010) where estimation was performed using pure standard lovastatin. The lactone ring of these drugs reacts with hydroxylamine in alkaline medium to form the corresponding hydroxamic acid derivatives which, treated with ferric ions in acid medium, yield highly coloured ferric chelate complex read at 510 nm. Among 24 different amino acids tested, 3-(3,4-dihydroxy phenyl) DL-alanine and L-isoleucine did not produce lovastatin, L-cysteine monohydrochloride produced maximum extracellular lovastatin (2064 mg/L) and obtained the concentration of lovastatin compatible to the one reported by Nirogi et al. (2007). The amount of intracellular and extracellular lovastatin produced statically was higher than the one produced under shaking conditions, and the observations revealed a possible reason for this increase: it is possible that the rotational speed fragments the mycelium of *M. purpureus* thereby reducing the lovastatin production. Under static conditions, the culture was undisturbed thus enhancing the production of lovastatin. Conversely, 1% L-cysteine monohydrochloride-supplemented medium biomass was comparatively low, so our results revealed that the biomass production had no relation with lovastatin production. However, production depends on the organic nitrogen source such as L-cysteine monohydrochloride in the medium. Our results were in agreement with Tsuyoshi et al. (2006).

The glucose present in the medium strongly repressed lovastatin production. The appropriate release is achieved by optimizing the medium composition to produce high amount of lovastatin. This production is via polyketide pathway, which is responsible for synthesizing many secondary metabolites with complex chemical structures. The genes and enzymes involved have been identified and characterized (Kennedy et al., 1999). It is becoming apparent that both carbon and nitrogen sources regulate lovastatin biosynthesis at the level of glucose repression.

Table 2. **Citrinin concentration produced by *Monascus purpureus* MTCC 410**

Sl. No.	Amino acid	Retention time (min)	Area (mV.s)	Concentration (mg/L)
1.	Standard	2.043	81.103	
2.	DL-alanine	2.113	3.746	0.92
3.	L-cysteine	2.067	0.784	0.19
4.	DL-norleucine	2.113	5.257	**1.29**
5.	L-proline	2.057	3.679	0.90
6.	D-serine	2.020	0.670	0.16

Table 3. **Estimation of lovastatin produced intracellularly and extracellularly by *Monascus purpureus* under static conditions**

Sl. No.	Amino acid	Amount of lovastatin produced (concentration in mg/L)	
		Intracellular	Extracellular
1.	DL-alanine	24	960
2.	DL-2-amino-n butyric acid	96	78
3.	L-arginine mono-hydrochloride	120	–
4.	DL-aspartic acid	174	792
5.	L-cysteine mono hydrochloride	–	**2064**
6.	L-cysteine	–	96
7.	3-(3,4-dihydroxy phenyl) DL-alanine	–	–
8.	L-glutamic acid	174	288
9.	Glycine	288	156
10.	L-histidine mono-hydrochloride	330	270
11.	L-hydroxyl proline	210	306
12.	L-leucine	–	–
13.	L-isoleucine	138	138
14.	DL-norleucine	288	192
15.	L-lysine mono hydrochloride	24	60
16.	L-methionine	24	24
17.	DL-ornithine mono hydrochloride	210	234
18.	L-proline	156	768
19.	DL-β-phenyl alanine	252	192
20.	D-serine	252	192
21.	L-tyrosine	210	**1944**
22.	DL-valine	234	1392
23.	DL-threonine	156	1128
24.	DL-tryptophan	96	960

CONCLUSIONS

The study revealed that specific amino acids at 1% influence the type and amount of extracellular or intracellular bio-products and fresh biomass produced *M. purpureus* under submerged static conditions. As it is produced by industrially important pigments, *M. purpureus* can be manageable in the way of setting adequate conditions to produce the desired pure products (pigments, lovastatin, biomass) in large amounts and to inhibit undesired ones like citrinin. At 1%, D-serine must be used as nitrogen source to produce large amounts of fresh biomass under static conditions. For the purpose of pigments, either L-histidine monohydrochloride or glycine can be used to produce a large amount intra- or extracellularly, respectively. This is a new tool for substitution or replacement of industrial detrimental synthetic pigments. Now Monascus citrinin is no longer a problem as amino acids like L-histidine monohydrochloride and glycine, which are pigment enhancers under static condition, completely inhibit the citrinin production. *M. purpureus* requires a suitable concentration of organic nitrogen in the form of amino acids for high lovastatin production, therefore supplementation of 1% L-cysteine monohydrochloride

or L-tyrosine to the potato dextrose medium with submerged cultivation is beneficial in seeking maximum amount of lovastatin production. Supplementation of amino acids under static conditions is a novel methodology to enhance biomass, pigments, lovastatin production, and to reduce citrinin production, and can be adapted by industries for maximum production of pigments and lovastatin by *Monascus* spp.

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AMINORŪGŠČIŲ POVEIKIS PIGMENTŲ, CITRININO IR LOVASTATINO GAMYBAI IŠ *MONASCUS PURPUREUS* STATINĖMIS SĄLYGOMIS

Santrauka

Monascus rūšys gamina daug antrinių metabolitų, įskaitant pigmentus, statinus ir nepageidaujamus mikotoksinus. *Monascus purpureus* yra rausvai raudonos spalvos pelėsių rūšis, pajėgi gaminti saugius natūralius pigmentus ir kitus svarbius antrinius metabolitus, įskaitant citrininą ir statinus. Šio tyrimo metu *M. purpureus* MTCC 410 padermė 10 dienų buvo statiškai auginta bulvių dekstrozės terpėje su 1 % UV sterilizuotų aminorūgščių. Gauto pigmento koncentracija įvertinta kolorimetriniu metodu, esant skirtingam bangos ilgiui, ir išreikšta spalvinės vertės vienetu (CVU). Citrininas nustatytas UV šviesoje (bangos ilgis 350 nm) ir kiekybiškai įvertintas su skysčių chromatografija. Didžiausia biomasė (143,6 g/l) nustatyta terpėje su 1 % D-serino papildu, o didžiausia viduląstelinio pigmento išeiga gauta terpėje su L-histidino monohidrochloridu (4,48 geltono, 3,97 oranžinio ir 2,0 CVU/ml raudono pigmento). Didžiausia ekstraląstelinio pigmento išeiga gauta terpėje su glicinu (2,18 geltono, 1,65 oranžinio ir 1,38 CVU/ml raudono pigmento), didžiausia lovastatino išeiga (2 064 mg/l) gauta terpėje su L-cisteino monohidrochloridu, o citrinino (1,29 mg/l) – terpėje su DL norleucinu.

Raktažodžiai: ekstraląstelinis pigmentas, citrininas, aminorūgštys, *Monascus purpureus*, biomasė