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ORIGINAL ARTICLE

Comparison of lovastatin, citrinin and pigment production of different *Monascus purpureus* strains grown on rice and millet

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Abstract Six *Monascus purpureus* (red mould) strains were cultivated on brown rice and millet as substrates. They underwent strain selection for high lovastatin and pigment production, and low citrinin mycotoxin production, with particular reference to potential for millet as substrate. For most of these strains, substrate dry matter loss was 54–60% on rice and 46–48% on millet, although the 'MOPU GS1' strain showed 18% and 17% dry matter loss, respectively. 'MOPU GS1' was also the only strain with detectable levels of lovastatin (1.3 and 1.6 mg lovastatin/g substrate dry weight [dw], respectively) and citrinin under the limit of detection. In the other strains, citrinin varied from 0.3 to 18.2 mg/g substrate dw. Among the six strains, 'EBY3' provided high pigment production when grown on rice, although it produced 1.1 mg citrinin/g substrate dw. Millet showed good potential as an alternative substrate to rice, due to higher lovastatin and lower citrinin production; however, rice was the better substrate for production of *M. purpureus* pigments.

Keywords *Monascus purpureus* · Lovastatin · Citrinin · Pigment · Rice · Millet

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Introduction

Monascus purpureus is a species of red mould cultivated according to a long tradition in East Asia, where rice products fermented by this fungus (i.e., red yeast rice) are consumed as food and food additives (Turgay and Azirak 2004). This mould produces a plethora of pharmacologically active ingredients, the most well-known and investigated of which is lovastatin (also known as monacolin K) (Ma et al. 2000). It also produces other similar bioactive polyketides that are involved in the regulation of cholesterol levels in humans, as well as different pigments that are used as food colorants; e.g. 'red rice' (ang-kak, or anka) (Pfohl-Leszkowicz et al. 2002; Pohleven et al. 2015). As the most important of the *M. purpureus* secondary metabolites, the potent cholesterol-lowering actions of lovastatin arise from its inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase, the key enzyme in cholesterol synthesis (Wang et al. 2004). Lovastatin is also biosynthesised by several other *Monascus* species, such as *M. ruber*, *M. pilosus*, *M. vitresus*, *M. anka*, *M. serorubescens* and *M. pubigerus* (Panda et al. 2009).

The *Monascus* pigments can be divided into three major groups, as yellow (e.g., ankaflavin, monascin C, monascidin A), orange (e.g., rubropunctatin, monascorubin) and red (rubropunctamine, monascorubramine) pigments (Erdogrul and Azirak 2004; Patakova et al. 2015). Red *Monascus* pigments are recognized as the most important, as these can be used as substitutes for nitrites in meat products as well as for synthetic colourants (Carvalho et al. 2005). Consumption of *Monascus* pigments is rapidly increasing (Mostafa and Abbady 2014), and the yearly market value for these biopigments in the USA is estimated to be several hundreds of millions of US dollars (Carvalho et al. 2014).

The end-products of *M. purpureus* fermentation also include: dimerumic acid and γ-aminobutyric acid, which along with lovastatin can lower plasma cholesterol levels (Patakova et al. 2015); various sterols (e.g., β-sitosterol, campesterol), which facilitate the absorption of cholesterol into the small intestine (Erdogrul and Azirak 2004); isoflavones; unsaturated fatty acids; dietary fibre; magnesium; and vitamin B complex (i.e., niacin) (Pohleven et al. 2015). Some *Monascus* species also produce the hepatotoxic polyketide citrinin (also known as monascidin A) (Pfohl-Leszkowicz et al. 2002; Wang et al. 2004), which has been implicated as a cause of the endemic nephropathy in the Balkans (Pfohl-Leszkowicz et al. 2002). Citrinin has a bacteriostatic effect towards the genera *Bacillus*, *Streptococcus* and *Pseudomonas* (Erdogrul and Azirak 2004), which has indicated its potential use as a partial substitute for nitrate/nitrite salts in the preservation of meat and poultry products in Europe (Chen et al. 2015). However, due to the toxic effects of citrinin, it is still an undesirable *Monascus*-derived product, and European Commission Regulation (EC) No 212/2014 defines a temporary maximum limit of 2 µg citrinin/g food product following supplementation based on rice fermented with *M. purpureus* (Sánchez et al. 2017). Some Asian countries have also introduced their own limits, such as 0.05 µg and 0.2 µg citrinin/g food product in South Korea and Japan, respectively (Patakova et al. 2015).

In nature, *M. purpureus* grows on solid substrates (Bibhu et al. 2008) as a spoilage organism (Barbosa et al. 2017). In most commercial situations, rice is used for solid-substrate cultivation of *M. purpureus*, although it can also be grown on other starch-containing substrates (Pohleven et al. 2015), such as wheat, corn, soya, cassava, potatoes, corn flour, peanut flour (Ogbonna 2016), buckwheat flour, tapioca flour (Dikshit and Tallapragada 2012), barley, sorghum, buckwheat grain (Ivaništova et al. 2017), millet (Zhang et al. 2018) and germinated finger millet (Venkateswaran and Vijayalakshmi 2010). The qualities and quantities of the secondary metabolites biosynthesised by *Monascus* species during the stationary phase depend on type of cultivation medium, *Monascus* strain used, availability of oxygen, water activity, temperature (Patakova et al. 2015), duration of cultivation, volume of inoculum (i.e., number of spores), substrate pH (Panda et al. 2009) and humidity (Yii-Lih et al. 2008). Furthermore, fermentation is generally carried out in the dark, as exposure to light leads to lower levels of secondary metabolites (Patakova et al. 2015).

Recently, a number of studies have focussed on the production of increased lovastatin and/or pigment levels, with lower levels of the toxic citrinin. This can be achieved through: (i) selection of non-toxigenic strains (Chen et al. 2008); (ii) modification of environmental factors (Mostafa and Abbady 2014); and (iii) modification of substrate nutritional conditions (Mostafa and Abbady 2014). Indeed, many *M. purpureus* strains and cultivation substrates have been screened in the past, with a view to selection of strains with high production of lovastatin and/or pigments and low production of citrinin (Carvalho et al. 2005; Mostafa and Abbady 2014).

It was reported recently that different cereals (Ivaništova et al. 2017), and millet in particular (Zhang et al. 2018), show a good potential for *M. purpureus* cultivation and lovastatin production. Furthermore, germinated finger millet compared to finger millet resulted in higher yield of total statin (lovastatin and pravastatin content) production (Venkateswaran and Vijayalakshmi 2010). Further investigations into the influence of cereal-based substrates on lovastatin, citrinin, ergosterol and pigment production are thus of great interest. On this basis, the present study investigated the lovastatin, citrinin and pigment production by six *M. purpureus* strains cultivated over 21 days on two types of substrate: rice and millet. To better define the mechanisms of fungal growth and performance, further parameters were measured as well as lovastatin, citrinin and pigment levels, including substrate pH and dry matter loss, mycelium growth rate through ergosterol analysis, and α-amylase, lipase and protease activities.

Materials and methods

Fungal strains

Monascus purpureus (red mould) strain 'NRRL 1596' was from the Agricultural Research Service Culture Collection, National Centre for Agricultural Utilization Research (Peoria, USA). The *M. purpureus* strains 'EBY2', 'EBY3', 'EBY4' and 'MOPU GS1' were from the culture collection of Mycomedica d.o.o. (Podkoren, Slovenia). *M. purpureus* strain 'BCRC 31,615' was from the Food Industry Research and Development Institute (Hsinchu City, Taiwan). These *M. purpureus* strains were stored on potato dextrose agar (PDA; Difco, New Jersey, USA) in test tubes at 3 °C, transferred onto PDA plates, and incubated for 14 days at 27 °C. They were then transferred to the grain-based substrates. For strain determination, the protocol described by Ravnikar et al. (2015) was used, according to which all of these *M. purpureus* strains were taxonomically confirmed with 99.9% accuracy.

Chemicals

Reagents for bicinchoninic acid (BCA) protein assays were from Pierce (Washington, USA), beech wood xylan was from Apollo Scientific (Stockport, UK), carboxymethyl

cellulose sodium salt was from Thermo Fisher Scientific (Waltham, USA), potassium sodium tartrate and Folin and Ciocalteu reagent were from Merck (New Jersey, USA), 3,5-dinitrosalicylic acid was from Fluka-AG (New Jersey, USA), maltose and starch were from Kemika (Zagreb, Croatia), and casein, L-tyrosine, *p*-nitrobuthyrate, citrinin standard, lovastatin and ergosterol (≥ 75%) were from Sigma (St. Louis, USA). For HPLC analysis, methanol [Liquid chromatography–mass spectrometry (LC–MS) Chromasolv; 99.9%] and acetonitrile (Chromasol; 99.9%) were from Honeywell (New Jersey, USA), and trifluoroacetic acid was from Merck (New Jersey, USA).

Solid-state cultivation of fungi

Organic brown rice and a mixture of unhulled and hulled Kornberg variety of proso millet (*Panicum miliaceum*) in 2:1 (w:w) ratio were used as cultivation substrates. Grains (130 g) were initially steamed at 90 °C for 20 min (to prevent clumping), and then put into 400 mL glass jars with lids equipped with high efficiency particulate air (HEPA) membranes (diameter, 12 mm; Ecotip d.o.o., Slovenjske Konjice, Slovenia). Water was added to 40% moisture content. The substrates were sterilised at 121 °C, for 20 min, and when cooled inoculated with 5 mL liquid inoculum. The liquid inoculum was prepared by blending one petri dish of PDA medium overgrown with *M. purpureus* into 300 mL sterile deionised water. Inoculated substrates were incubated at 27 °C in the dark in an incubator, connected to a constant air flow source replacing total air volume of an incubator every hour.

The classical Chinese process of rice fermentation with *M. purpureus* lasts 20 day (Blanc et al. 1997). Based on literature data, the levels of lovastatin are significantly higher after 21 days of fermentation compared to 14 days (Ganrong et al. 2004; Patakova et al. 2015; Mousa et al. 2018). Taking in consideration these data, we decided to perform samplings on 7th, 14th and 21st day after inoculation of the substrate with *M. purpureus*, to follow the trends of metabolite (lovastatin, citrinin, pigment) production and substrate degradation during the cultivation period. Three jars were sampled randomly for each substrate/strain combination. There were three biological repeats for every sample (i.e., two substrates, six strains), and 2 controls for each substrate for every time point, with 120 samples in total (i.e., rice, 60; millet, 60).

For each sample, the substrate pH was determined as described by Adinarayana et al. (2004), as well as the moisture content (drying at 103 °C for 24 h), and loss of dry substrate weight through fungal degradation. Fresh overgrown substrate was used for enzyme extraction and part of the material was dried at 35 °C for 48 h, and then ground into smaller particles using a coffee grinder. The material obtained was used for extraction of secondary metabolites.

Preparation of extracts

For preparation of aqueous extracts, 20 g overgrown moist substrate (control, 20 g non-inoculated moist substrate) was mixed with 20 mL 20 mM trisaminomethane (TRIS) buffer, pH 6.5. After 2 h of extraction at 4 °C with shaking on an orbital shaker, the samples were centrifuged at 5000 × *g* for 10 min at 4 °C, with the supernatants then frozen and stored at − 20 °C. Prior to analysis, the supernatants were thawed and centrifuged again at 10,000 × *g* for 10 min at 4 °C. The total protein in the samples was determined using BCA protein assay kits. These supernatants were also assayed for α-amylase, lipase and protease activities. Organic extracts were obtained according to a procedure described by Drvarič Talian (2015). Briefly, 1 g ground dried material was mixed with 5 mL 80% methanol and extracted on a shaker for 1 h at 25 °C. Following centrifugation at 3000 × *g* for 10 min, the supernatants were filtered through 0.45-µm filters (Chrom 4; Suhl, Germany). The lovastatin, citrinin and ergosterol levels were determined using HPLC analysis, as described in the next chapter. We prepared 3 biological replicates for selected strain, grown on chosen solid medium at selected time point and 2 biological replicates for every control (uninoculated solid medium at selected time point).

Determination of lovastatin, citrinin and ergosterol contents in organic extracts

HPLC was performed for simultaneous determination of lovastatin (as lactone and methyl ester), citrinin and ergosterol. The limits of detection and limits of quantification for these analytes are given in Online Resource Table S1. The analyses were performed using a HPLC system (2965 HPLC system; Waters, Milleford, USA) equipped with a degasser, a quaternary pump, an autosampler, a photodiode array detector (237 nm) and a fluorescence detector (excitation, 331 nm; emission, 500 nm). A C18 HPLC column was used (150 × 4.6 mm, particle size 5 µm; Kinetex XB; Phenomenex, Torrance, USA). The flow rate of the mobile phase was 1 mL/min and injection volume was 10 µL. The separation of lovastatin (as lactone and methyl ester), citrinin and ergosterol in organic extracts of the substrates overgrown with the *M. purpureus* strains, and the separation of the standards, was carried at 30 °C out using two eluents: A, 0.1% trifluoroacetic acid in ultra-pure water; B, acetonitrile. A discontinuous gradient was run as follows: 0–0.5 min, 30% B; 0.5–2.0 min, 30–50% B; 2.0–14.0 min, 50% B;

14.0–17.5 min, 50–70% B; 17.5–20.5 min, 70% B; 20.5–22.5 min, 70–100% B; 22.5–62.5 min, 100% B; 62.5–63.0 min, 100–30% B; 63.0–65.0 min, 30% B. The retention times were identified by injection of citrinin, lovastatin and ergosterol standards. Before HPLC analysis the ergosterol standard was purified according to Nylund and Wallander (1992). The retentions times for citrinin, lovastatin, and ergosterol were: 6.7 (citrinin), 18.2 (lovastatin lacton), 18.7 (lovastatin methyl ester) and 33.2 min (ergosterol). The peaks of the analysed compounds in the samples were also confirmed by spiking the samples with commercial standards.

Pigment extraction and estimation

For pigment extraction and estimation, the procedure described by Dikshit and Tallapragada (2012) was used. We prepared 3 biological replicates for selected strain, grown on chosen solid medium at selected time point and 2 biological replicates for every control (uninoculated solid medium at selected time point). One gram of fermented substrate was mixed with 10 mL 96% ethanol and agitated on a rotary shaker at 200 rpm for 24 h. The extracts were allowed to settle at 25 °C and were filtered through filter paper (S&S Rundfilter, Schleicher & Schüll, Germany). The pigment levels were analysed spectrophotometrically at 490 nm using a microplate reader (Kinetic; Dynex Technologies, Chantilly, USA). The absorbances were converted into colour value units using the following formula:

$$\text{Colour value} = \text{Optical density(O.D.)} \times \text{dilution} \times \text{volume of extract (mL)/amount of sample(g)} \quad (1)$$

Ethanol extracts of unfermented substrates were used as blanks.

Determination of enzymatic activities in aqueous extracts

The α-amylase activities of the fungal aqueous extracts were measured using a spectrophotometric method with the 3,5-dinitrosalicylic acid (DNS) reagent as substrate (12 g sodium potassium tartrate tetrahydrate in 8 mL 2 M NaOH and 96 mM DNS). For each sample, 100 µL of fungal extract was incubated with 100 µL 1% (w/v) starch solution in distilled water for 10 min at 37 °C. A blank sample was prepared with buffer and starch only. We performed 3 technical repetitions for each sample. The reaction was stopped by adding 1 mL DNS reagent and heating at 100 °C for 5 min. The production of the reducing sugar (released from starch) was monitored at 540 nm using a microplate reader (Kinetic; Dynex Technologies, Chantilly, USA). The product was quantified as maltose equivalents using standards. Different concentrations of maltose were used (0–40 mM) for the calibration curves. Specific enzyme activities are defined as the release of maltose (µmol) in 1 min per 1 mg soluble protein.

The protease activities of the fungal extracts were assayed using a spectrophotometric method with Folin and Ciocalteu reagent as substrate (1 mL diluted in 4 mL distilled water). We performed 3 technical repetitions for each sample. Twenty microlitres of the fungal extracts were incubated with 100 µL casein (0.65% in 50 mM K-phosphate buffer, pH 7.5) for 10 min at 37 °C. Then, 100 µL 110 mM trichloroacetic acid was added and the solution incubated for additional 30 min at 37 °C and centrifuged at 25 °C at 10,000 × *g* for 10 min. For the negative control, the casein was incubated for 10 min at 37 °C and then 100 µL of it was added to 100 µL of trichloroacetic acid and 20 µL fungal extract. The supernatants (40 µL) were transferred to 96-well plates and 100 µL Na_2CO_3 and 20 µL Folin and Ciocalteu reagent were added. After 30 min incubation at 37 °C, the plates were cooled to 25 °C and the absorbance was measured at 600 nm using a microplate reader (Kinetic; Dynex Technologies, Chantilly, USA). For the calibration curves, the enzyme extracts were replaced with L-tyrosine (0–1.1 mM). The specific enzyme activities are defined as the release of tyrosine (µmol) in 1 min per 1 mg soluble protein.

The lipase activities of the enzyme extracts were assayed using a spectrophotometric method with *p*-nitrophenyl butyrate (*p*NPB) as substrate. We performed 3 technical repetitions for each sample. Briefly, 450 µL buffer (100 mM sodium phosphate buffer, supplemented with 150 mM NaCl, 0.5% [v/v] Triton X-100, pH 7.2) and 5 µL 50 mM *p*NPB (dissolved in acetonitrile) were mixed and incubated for 10 min at 37 °C. After incubation, 45 µL of the enzyme extracts were added to the mixtures, and incubated for an additional 10 min at 37 °C. The reactions were stopped by adding 750 µL acetone. All the samples were centrifuged at 5000 × *g* for 10 min at 25 °C. The supernatants (200 µL) were transferred to 96-well plates and the production of *p*-nitrophenol (*p*NP) was monitored at 405 nm using a microplate reader (Kinetic; Dynex Technologies, Chantilly, USA). The specific enzyme activities are defined as the release of *p*NP (µmol) in 1 min per 1 mg soluble protein.

The protein concentration in all of the extracts were determined using BCA protein assay kits, as a modification of the bicinchoninic acid protein assay (Pierce, Rockford, USA). For each sample we conducted 3 technical repetitions.

Statistical data analysis

Two-way ANOVA was applied to test differences between different strains and growth media. For pair-wise comparison Student–Newman–Keuls (SNK) post hoc test at $P < 0.05$ was applied. All tests were performed in R v3.2. with Agricolae v1.2-8 library.

Results and discussion

The traditional substrate for cultivation of *M. purpureus* is rice, and this was compared here to millet as substrate, as rice is not extensively cultivated in Europe and millet might represent a good gluten-free substitute for *M. purpureus* cultivation. We hypothesised that the smaller particle size and lower viscosity of millet when compared to rice and other cereals would enable more rapid colonisation by *M. purpureus*, consequently shortening the production cycle and potentially producing higher lovastatin and lower citrinin levels due to larger growth surface area and an increased aeration rate in the substrate.

This hypothesis was in line with Zhang et al. (2018), who recently suggested that millet represents a promising alternative for lovastatin production by a related fungus, *M. ruber*. Of note, direct comparisons of the data from the present study with those of Zhang et al. (2018) are not possible, mainly because of the use of a different *Monascus* species and the lack of information on the physical state of the substrate used by Zhang et al. (2018) (e.g., the use of hulled or unhulled millet). However, both studies arrive at similar conclusions regarding substrate selection and optimisation of other parameters that are important for enhanced lovastatin production by *Monascus* fungi. It is indicative that millet represents a better choice for lovastatin production in comparison to other grain substrates analyzed so far. This might be at least partially attributed to physical characteristics of the millet grains (e.g. to its smaller size and consequently larger specific surface area, as well as its lower viscosity after autoclaving which prevents the substrate agglomeration enabeling higher aeration), all these features allowing better interaction with the fungus and substrate utilization. Further, main chemical components of millet are very different from rice (higher fat, protein, fiber, mineral and carbohydrate content) (Verma et al. 2015), and these might have an important role in secondary metabolites production.

Effect of cultivation duration and substrate on growth of Monascus purpureus

The loss of dry matter for both the rice and millet substrates inoculated with *M. purpureus* increased with fermentation time (Fig. 1). After 21 days of fermentation, most of these *M. purpureus* strains showed 54–60% dry matter loss on rice and 46–48% dry matter loss on millet. This higher loss of dry mass on rice compared to millet might be attributable to the difference in chemical composition of these grains, and because the millet substrate included husks, which are resistant to degradation by the fungal enzymes. Among these *M. purpureus* strains, the only exception was 'MOPU GS1'. The dry matter loss of both substrates after 21 days of growing this strain (i.e., rice, 18%; millet, 17%) was significantly ($P < 0.05$) lower when compared to other *Monascus* strains, which indicated slower growth and lower metabolic rate on both substrates. This was also reflected in the changes in and moisture content (Online Resource 1) during the 21-day cultivation of the *M. purpureus* strains. The moisture content was again significantly ($P < 0.05$) lower with 'MOPU GS1'. For 'MOPU GS1', the initial pH for both substrates (pH 6.7) decreased to pH 5.5 to pH 6.0 and remained at this value to day 21, while for the other strains, the pH decreased to around 5.0 after 7 days of cultivation, and then increased to pH 6.0 to pH 8.0 after 21 days. Furthermore, significantly ($P < 0.05$) lower amounts of ergosterol were extracted from the rice and millet substrates inoculated with 'MOPU GS1', in comparison with the other *M. purpureus* strains (Fig. 2). Of note also, for the rice and millet inoculated with 'MOPU GS1', the ergosterol content continued to increase with time, and was highest after 21 days, while the other *M. purpureus* strains generally reached maximal growth after 14 days, as defined by the ergosterol contents of the substrate (Nylund and Wallander 1992). Finally, if the increase in substrate water content due to a combination of respiration and biomass synthesis (Oriol et al. 1988) is taken into account (Online Resource 1), these data indicate that compared to millet, rice supports higher respiration activity and/or more vigorous growth of these strains.

Effects of substrate and incubation time on lovastatin, citrinin and pigment production by Monascus purpureus

Using HPLC for simultaneous determination of lovastatin, citrinin and ergosterol, differences in the production of lovastatin and citrinin were seen across these different *M. purpureus* strains (Figs. 3, 4). Here, again, 'MOPU GS1' significantly ($P < 0.05$) differed from the other *M. purpureus* strains, in that it was the only to show detectable levels of lovastatin. These levels of lovastatin increased with cultivation time and were always higher with millet, with significantly higher yields after 21 days of cultivation (1.3 mg/g rice substrate; 1.6 mg/g millet substrate; Fig. 3).

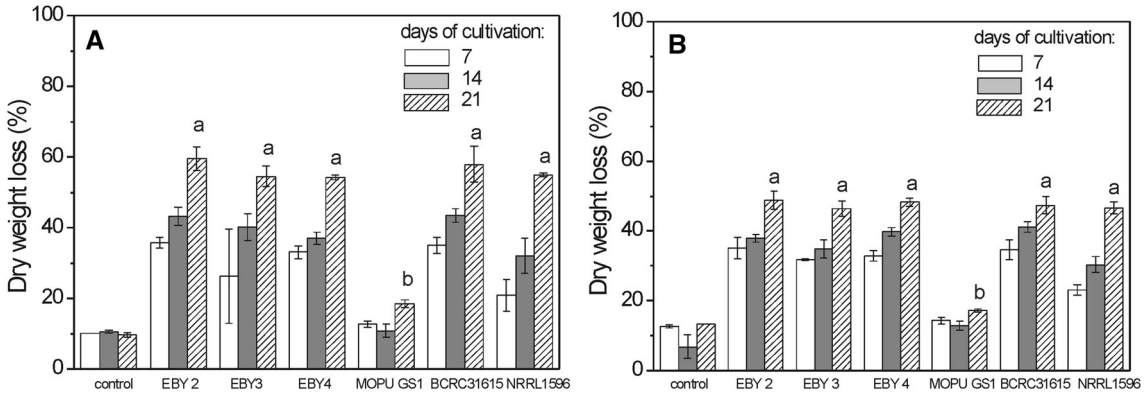

Fig. 1 Loss of dry matter of rice (**A**) and millet (**B**) substrates after 7, 14 and 21 days of fermentation with selected *Monascus purpureus* strains. Data are mean ± standard deviation (n = 3). Mean values with the same letter on the bar are not significantly different ($P < 0.05$), according to the SNK test

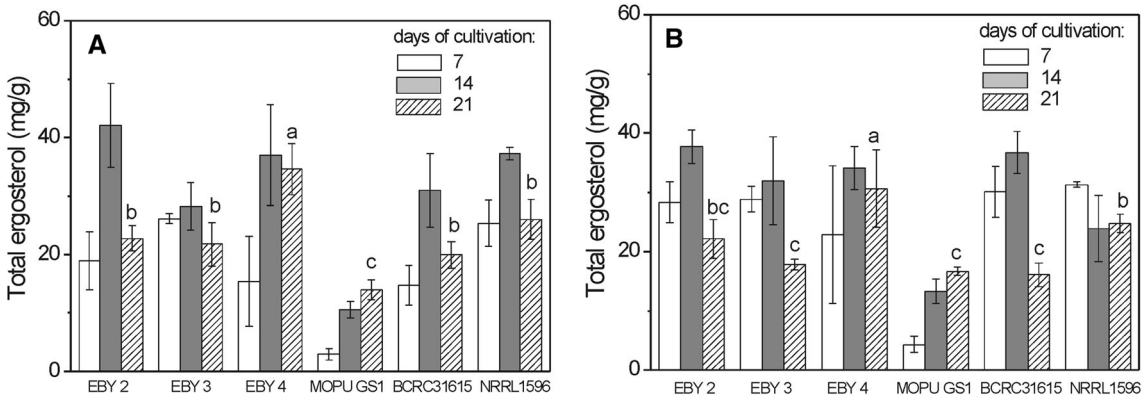

Fig. 2 Influence of rice (**A**) and millet (**B**) substrates on production of ergosterol by selected *Monascus purpureus* strains after 7, 14 and 21 days of fermentation. Data are mean ± standard deviation (n = 3). The amount of ergosterol was estimated based on the dry mass of the fermented rice and millet substrates. Mean values with the same letter on the bar are not significantly different ($P < 0.05$), according to the SNK test

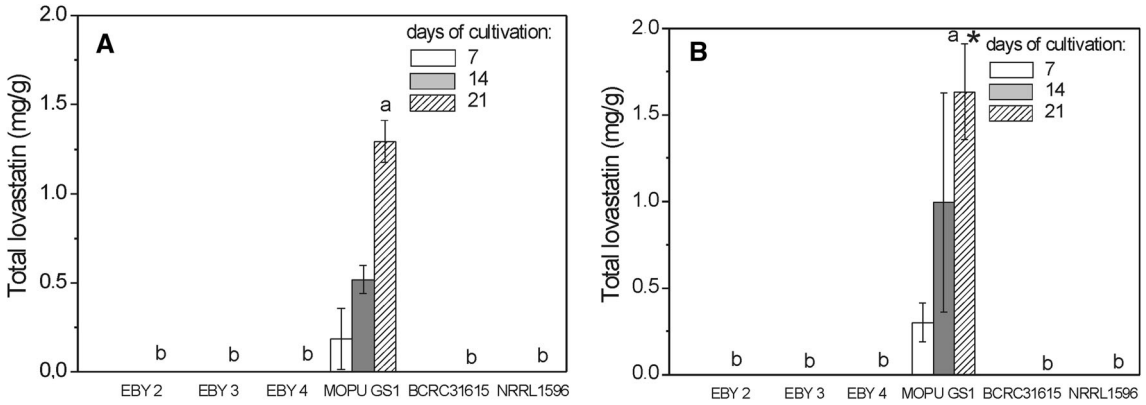

Fig. 3 Influence of rice (**A**) and millet (**B**) substrates on production of lovastatin by the selected *Monascus purpureus* strains after 7, 14 and 21 days of fermentation. Data are mean ± standard deviation (n = 3). The amount of lovastatin was estimated based on the dry mass of the fermented rice and millet substrates. Mean values with the same letter on the bar are not significantly different ($P < 0.05$), according to the SNK test. Asterisk (*) denotes significant difference ($P < 0.05$) between rice (**A**) and millet (**B**) according to the SNK test

As discussed by Zhang et al. (2018), various factors influence the production of lovastatin in the related fungus *M. ruber*, with 'charge amount' having the greatest impact in their study. The charge amount represents the ratio between solid medium and free space in the jar, whereby the optimal of 10% was proposed by Zhang et al. (2018),

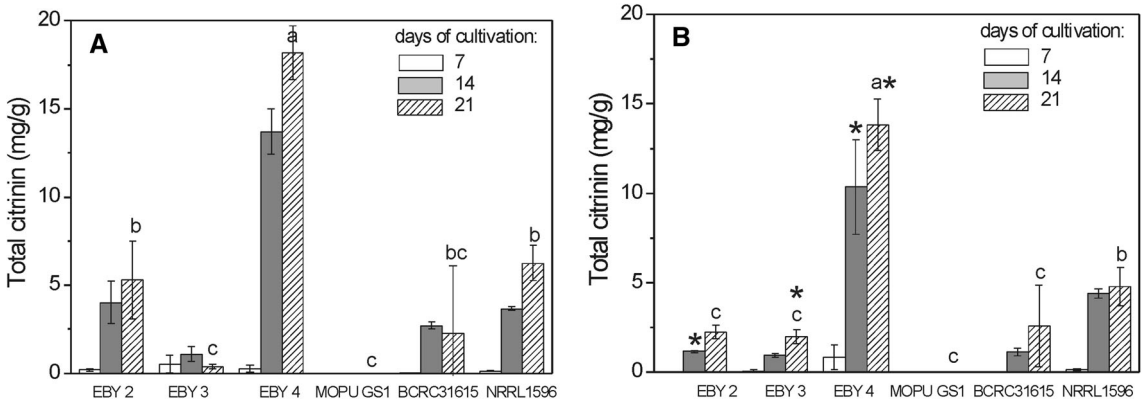

Fig. 4 Influence of rice (**A**) and millet (**B**) substrates on production of citrinin by the selected *Monascus purpureus* strains after 7, 14 and 21 days of fermentation Data are mean ± standard deviation (n = 3). The amount of citrinin was estimated based on the dry mass of the fermented rice and millet substrates. Mean values with the same letter on the bar are not significantly different ($P < 0.05$), according to the SNK test. Asterisk (*) denotes significant difference ($P < 0.05$) between rice (**A**) and millet (**B**) according to the SNK test

which resulted in 8 mg lovastatin/g substrate dw after 20 days of cultivation on millet. They also indicated that higher charge amounts had a negative impact on lovastatin production, which appeared to be due to the quantity of fungal biomass that decreased the oxygen content and increased heat build-up in the bottle. Here, it would be interesting to know what kind of filter material Zhang et al. (2018) used to ensure the aeration of their test bottles. In the present study, the charge amount was 32.5%, and therefore reducing this would be expected to provide greater production of lovastatin. Furthermore, Zhang et al. (2018) showed that the initial 40% moisture content of the substrate (as also used in the present study) resulted in lower lovastatin levels, which indicates that the optimal moisture content for all of the *M. purpureus* strains examined in the present study should be determined in the future, to potentially enhance lovastatin production.

Furthermore, 'MOPU GS1' was also the only strain that did not produce the toxic polyketide citrinin when grown on either the rice or millet (Fig. 4).

Although lovastatin was not detectable in the organic extracts obtained from the other five *M. purpureaus* strains, they showed generally higher citrinin levels when cultivated on rice as compared to millet (Fig. 4). We can notice that yields of citrinin are increasing during fermentation time in majority of selected *M. purpureus* strains. Citrinin levels were significantly higher ($P < 0.05$) in extracts from the 'EBY4' strain when cultivated on rice (21 days: 18.2 mg/g rice substrate; 13.0 mg/g millet substrate), and also in comparison with other strains, which reached 0.3 mg citrinin/g to 6.3 mg citrinin/g substrate dw over the same period. For its high production of citrinin, we do not recommend strain EBY4 to be used in food industry. As discussed further later in this section, one of the important factors that might influence the production of citrinin during this solid-state fermentation of *M. purpureus* cultures is oxygen. A lack of oxygen during the cultivation would lead to production of ethanol and CO_2, and consequently decreased pigment production and increased production of citrinin (Carvalho et al. 2003).

Monascus purpureus pigments are biosynthesized during the stationary phase of fungal growth, together with organic acids and esters. In our case, as pigment production was still increasing in majority of strains after 21 days, we assume that selected *M. purpureus* strains were still in stationary phase. Although according to total ergosterol content (Fig. 2) the growth of new mycelium was stopped in all strains except MOPU GS1, substrate degradation was still taking place (Fig. 1), and pigments were still actively produced in most of the strains (Fig. 5). There are many factors that can influence this pigment production, including solid substrate type, water activity, oxygen access, temperature, pH, exposure to light, inhibitors, and cultivation technique, although the main effector is the strain of *Monascus* species (Carvalho et al. 2005; Mostafa and Abbady 2014; Patakova et al. 2015). In the present study, solid-state cultivation was used due to indications of higher pigment yields (Patakova et al. 2015) and because of lower citrinin levels when compared with submerged fermentation procedures (Zhang et al. 2013). As indicated above, the initial pH of the solid media in the samples in the present study was 6.7, although during fermentation this varied from pH 5 to pH 8 after 7 days and 21 days of fermentation, respectively. This is favourable for the pigment production here, as these pigments have been shown to be less stable in acid environments, and more stable at higher pH (Mostafa and Abbady 2014). Indeed, in the present study, the increase in pH after 21 days of fermentation correlated with increased yields of pigment (Fig. 5). The 'EBY3' and 'BCRC 31615' strains were the most

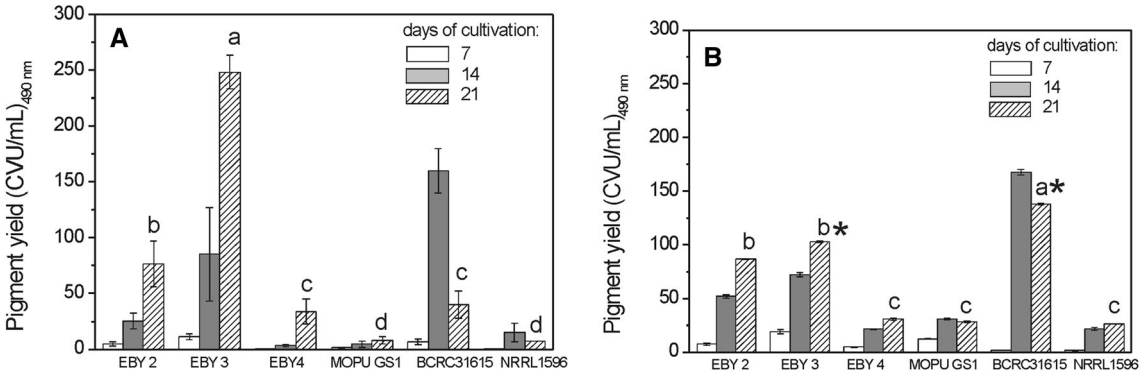

Fig. 5 Influence of rice (**A**) and millet (**B**) substrates on pigment production by the selected *Monascus purpureus* strains after 7, 14 and 21 days of fermentation. Data are mean ± standard deviation (n = 3). CVU, colour value units. Mean values with the same letter on the bar are not significantly different ($P < 0.05$), according to the SNK test. Asterisk (*) denotes significant difference ($P < 0.05$) between rice (**A**) and millet (**B**) according to the SNK test

promising regarding this pigment production, showing statistically ($P < 0.05$) higher levels of pigment after 21 days cultivation on rice or millet, respectively, in comparison with other strains. In most cases, the pigment production increased over time, and with the exception of the extract from 'EBY3' grown on rice for 14 days, the cultivation substrate did not have large effects on the final pigment levels. During the first week of growth production of pigments was generaly higher on millet in comparison to rice substrate. In the third week the only statistically significant differences were observed for isolate EBY3 with higher production of pigments on rice, and isolate BCRC31615 with higher production of pigment on millet. However, these strains also produced citrinin at levels generally higher than the official European Commission Regulation (No 212/2014) for the citrinin limit of 2 µg/g food substrate (i.e., 5.3, 1.1, 18.2, 2.7, 6.3 mg/g substrate for 'EBY 2', 'EBY 3', 'EBY 4', 'BCRC 31615', 'NRRL', respectively).

These data for the 'EBY 3' strain in the present study are in line with data from Moharram et al. (2012), who reported that the type of solid medium has an important role in pigment production, and that the metabolic products, including the pigments, are strain-dependent and culture-medium-dependant. Furthermore, Teng and Feldheim (2000) showed that CO_2 accumulation leads to inhibition of pigment production. *Monascus* strains cannot grow under anaerobic conditions using glucose as substrate, although they can grow under oxygen-limiting conditions. Under such circumstances, they show higher production of ethanol and CO_2, and for that reason lower production of pigments. While with greater/improving aeration, ethanol production decreases during fermentation process and total pigment production increases (Carvalho et al. 2003). For this reason, it would be interesting to monitor the CO_2, O_2 and ethanol levels inside these solid media fermentations to enable better evaluation of selected fermentation parameters.

The present data are, however, in contrast to the conclusions of Dikshit and Tallapragada (2012) in terms of a positive correlation between pigment production and α-amylase activity (Fig. 5 and Online Resource 2). Previously, fungi from the genus *Monascus* have been shown to produce many hydrolytic enzymes, such as α-amylase and β-amylase, proteases, lipases, β-glucosidase and glucoamylase (Zoppas et al. 2013; Chen et al. 2015; Le Bloc'h et al. 2015). *Monascus* rice products have also been used as preservatives for meat and fish, due to the presence of the hydrolytic enzymes (Le Bloc'h et al. 2015). In the present study, protease and lipase activities were not detected in the aqueous extracts of these overgrown substrates, which might be due to the use of the carbohydrate-based substrates. α-amylase activity was detected here, which was higher and more variable when the *M. purpureus* strains were grown on millet (Online Resource 2). As indicated by Manan et al. (2017), the nutrient content of such solid substrates influences the fermentation process. Various carbon and nitrogen sources can be used by fungi due to their secretion of various enzymes for degradation of polymers into small molecules. For this reason, the types and concentrations of carbon and nitrogen sources, carbon-to-nitrogen ratio, and vitamins are important factors in medium formulation for enhancement of fungal growth and sporulation (Ajdari et al. 2011).

In conclusion, although the *M. purpureus* 'MOPU GS1' strain showed slower growth in comparison to the other strains tested in the present study, it can be considered as the most promising strain here in terms of the production of the pharmacologically important secondary metabolite lovastatin. This can also be achieved without contamination with the hepatotoxic citrinin. It would also be

interesting to monitor the production of these two metabolites by 'MOPU GS1' over prolonged periods of time.

Millet was also shown here to be a potential alternative substrate to rice for *M. purpureus* cultivation. The strains cultivated on millet showed comparable growth to the same strains grown on rice, but in general they produced more lovastatin and less citrinin. As mentioned before, Moharram et al. (2012) reported that solid growth medium type has an important role on *Monascus* metabolites production. For example, Venkateswaran and Vijayalakshmi (2010) analyzed two different millet-based substrates, and found that the use of germinated finger millet resulted in higher yield of total statins (lovastatin and pravastatin) production compared to ungerminated finger millet. It is important to mention that there are many varieties of millets. The four main groups comprise pearl millet (*Pennisetum glaucum*), foxtail millet (*Setaria italica*), proso millet or white millet (*Panicum miliaceum*), and finger millet (*Eleusine coracana*). Less frequently used millets include barnyard millet (*Echinochloa spp.*), kodo millet (*Paspalum scrobiculatum*), little millet (*Panicum sumatrense*), guinea millet (*Brachiaria deflexa*), browntop millet (*Urochloa spp.*), teff (*Eragrostis tef*) and fonio (*Digitaria exilis*), sorghum (*Sorghum spp.*) and Job's tears (*Coix lacrima-jobi*) (Sarita 2016). Amadou et al. (2013) compared four main millet groups (foxtail, proso, pearl and finger millet) and found differences in amino acid profiles, as well as in total protein, fat, crude fiber and carbohydrate content among mentioned millets. Sarita (2016) also reported differences among pearl, finger, foxtail and proso millet regarding the mineral, iron and calcium content. It is thus plausible that using different millet types would bring different results. Furthermore Katare (2018) presented differences among sorghum, pearl millet, finger millet, little millet, proso millet and kodo millet regarding protein, fat, minerals, crude fiber, carbohydrates, calcium, phosphorus, iron, thiamine, riboflavin and niacin content. Beside that there are also variabilities among same species of millet. Kamatar et al. (2015) reports significant differences among foxtail millet (*Setaria italica*). Total of 75 elite foxtail millet germplasms were collected from different sources and differences of all their nutritional parameters reported, among them: moisture, protein, fat, crude fiber, carbohydrate, total minerals, and total energy based on their genotype.

Rice-based substrates would however appear to remain the first choice for the production of *M. purpureus* pigments. Among the strains selected in the present study, 'EBY 3' might be considered as the choice for high pigment production when grown on rice, although it contains some citrinin, which might be a limiting factor in the food industry or during large-scale production.

Further optimisation of the secondary metabolite production on millet-based substrates should consider different ways of fermentation as well as those used in the present study. This can also include cultivation at different temperatures, which has the potential to influence the growth and production of secondary metabolites and enzymes across the different *M. purpureus* strains.

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