Title: PRODUCTION OF MONASCUS-LIKE PIGMENTS

Abstract: The present invention relates to a method for producing one or more Monascus-like pigment composition from Penicillium species comprising: a) providing a cultivation medium comprising a high concentration of C- and N-sources and a high C/N molar ratio, b) adjusting pH to about 5 to 8, c) inoculating the cultivation medium with an inoculum of Penicillium to form a cultivation composition; d) cultivating the inoculated cultivation composition of (c); e) separating the one or more produced pigment compositions. The method of the invention may be used for producing Monascus-like pigment compositions for use as colouring agents in food items or non food items. The inventions further relates to Monascus-like pigment composition obtainable by a method of the inventions as well as use of the pigments.
— with (an) indication(s) in relation to deposited biological material furnished under Rule 13bis separately from the description (Rules 13bis.4(d)(i) and 48.2(a)(viii))
Production of *Monascus*-

**Technical field of the invention**

The present invention relates to the field of biotechnological production of polyketide based colorants from filamentous fungi, in particular a method for producing *Monascus*-WWe pigment composition from a non-toxigenic and non-pathogenic fungal source and for optimising media conditions in the production of specific, desired pigment compositions. The present invention further relates to the *Monascus*-WWe pigment compositions and use of the *Monascus*-WWe pigment composition as a colouring agent in food items and/or non-food items, and in cosmetic compositions.

**Background of the invention**

Colour has in centuries enabled the user of food-products to have forehand expectations on properties like texture, taste and quality. Likewise, colorants have been added by the manufacture to increase the appeal of their product and thereby enhancing the urge to purchase the item. An increased knowledge in the bulk population of today’s consumers and massive focus on some detrimental effects of synthetic additives has created an increasing demand of natural colorants to be used in foodstuff. Various sources of feedstock is used in the making of coloring agents e.g. fruit skins, roots, berries, insects, crustaceans and plants, which makes it easy recognizable that certain problems could be linked to the making of the final pigment product.

Currently, the European Union has authorized approximately 43 colorants as food additives, while approximately 30 colour additives are approved for food use in the US, and several of the listed colour additives are derived from natural sources typically by physical and/or chemical extraction.
The existing authorized natural food colorants are mostly of plant origin and have numerous drawbacks such as chemical instability and low water solubility. For instance betanins, carotenoids, and chlorophyll pigments contain labile hydrogen, and are therefore easily decolourised by oxidation, and hence sensitive to light, heat and oxygen. These features limit the robustness of these colour additives during the processing, storage, and display of the foods to which they have been added. Naturally derived colorants are usually extracted from sources such as fruit skins, seeds, or roots, which are often not available throughout the year. This means that the colour manufacturers are dependent on the availability and external supply of raw materials for the colour extraction.

Fungi provide an alternative source of natural colours, since they can be produced all year round. Considering the extraordinary diversity of fungal pigments and the potential to produce them in higher yields e.g. by metabolic engineering, fungi could be a promising source of colorants with improved functionality over existing natural food colorants with similar or additional colour hues in the red and yellow spectra.

Fungal pigments have mostly been studied from a taxonomic perspective and earlier work on their application for food use has focused on carotenoids. Many fungi have been reported to produce polyketide pigments but only a few of those have been explored as possible food colorants. Some of these include yellow-red compounds produced by *Penicillium herquei*, blue-green and dark green pigments of *Penicillium atrovenetum*.

*Monascus* is a well studied polyketide-pigment producing genus of the fungi world. The reason for this pronounced attention is the long history of use in the orient cuisine and the excellent dyeing properties of their pigment, even
when subjected to environments with large pH and heat variations i.e. relevant stability in foodstuff. It is well known that strains of Monascus are producing azaphilone pigments like monascorubrin, rubropunctatin, monascusones, ankaflavin, monascin and monascorubramine which all appear as red water soluble colors after chemical reactions with amino acids (aminophiles). These pigments of polyketide origin are commercially available today and are e.g. sold as a dried powder after fermentation of rice and soybeans and then extraction of the colorants.

Earlier work by the inventors has suggested Penicillium subgenus Biverticillium as potential producers of Monascus-like pigments because of their obvious ability to produce pigment in the orange to red spectrum and the wide spread polyketide pathway in the fungi world. To determine if this was true or not the inventors used intelligent screening techniques that excluded toxic/pathogenic strains and selected strains with the desired pigment profile.

WO 2009/026923 relates to a method for producing a Monascus-Wke pigment by inoculation of a cultivation composition with an inoculum of Penicillium spores; a number of strains and examples of Monascus-WWe polyketide based pigments are disclosed.

Based on knowledge from screening results and the fact that they have been proven positive as soluble pigment-producers in liquid media, the main aim of the present invention is related to regulation of polyketide red-pigment production by altering process conditions.

Accordingly, it is an object of the present invention to provide an improved method for producing Monascus-WWe pigment compositions from Penicillium strains. It is a further object to provide a method which allows the production of specific pigment compositions in high yields.
Summary of the invention

The present invention relates to a method for producing one or more Monascus-WWe pigment composition from Penicillium sp. comprising:

- providing a cultivation medium comprising a high concentration of C- and N-sources and a high C/N molar ratio,
- adjusting pH to about 5 to 8,
- inoculating the cultivation medium with an inoculum of Penicillium to form a cultivation composition;
- cultivating the inoculated cultivation composition of (c);
- separating the one or more pigment compositions.

According to the invention the polyketide pigment production, e.g. a red-pigment production, can be regulated by altering media and/or culture conditions (pH, C- and N-sources) in a predefined way. Previous studies has shown that a growth medium composition for cultivating Monascus strains, comprising monosodium glutamate (MSG) as the sole N-source promotes production, excretion (extracellular pigments) and the formation of amino acid/polyketide-complex. This media-composition with MSG as the sole nitrogen source has been shown to produce fewer pigments but with higher yield (and mainly hydrophile) than if the media was complex with lots of nitrogen sources.

The inventors have found that it is possible to regulate and optimize polyketide pigment production from Penicillium strains by altering media and/or culture conditions (pH, C- and N-sources) in a predefined way.

By the present invention it is possible to significantly enhance the concentration of pigment or pigments in the liquid medium.
According to the invention, simple C- and N- sources are selected; the sources are used in high concentrations with a high C/N molar ratio; and pH is selected between 5 and 8 depending on the pigment-producing strain selected. This selection of cultivation conditions leads to higher yield of polyketide pigment from the selected *Penicillium* strains and to a reduced number of molecular components in the pigment composition.

Polyketides are a structurally diverse family of secondary metabolites from bacteria, fungi, plants and animals. They are biosynthesized by the polymerization of activated primary metabolites, acetyl-CoA and Malonyl-CoA, in process very similar to the fatty acid elongation. This process is aided by enzymes known as PKS (polyketide-synthases). Different PKS’s exist with various numbers of catalytic domains, but common for all of them is that they produce molecules that unlike fatty acids, has not been fully reduced and therefore tremendous varieties of polyketide compounds exist. In fungi, the type 1 (iterative) PKS is responsible for the production of many polyketides which often results in compounds with aromatic moieties due to type 1 PKS’s specific active domains. It is among these compounds that a great palette of pigment-hues are found and it turns out that these polyketide pigments, after reaction with amino acids, have more sustainable properties compared with other natural colorants of non-polyketide origin (e.g. carotenoids and chlorophyll pigments have unstable H-atoms that make them susceptible for oxidation which in turn allows discoloration by light, oxygen and heat).

The invention further relates to a method for producing a *Monascus-WWe* pigment composition comprising (IOZ)-3-(9a-methyl-3-octanoyl-2,9-dioxo-2,7,9,9a-tetrahydro-furo[3,2-g]isoquinolin-6-yl)-acrylic acid (PP-V) from *P. purpurogenum*, which method comprises:
a) providing a cultivation medium comprising at least 0.2 % glucose and at least 0.2 % monosodium glutamate and a high C/N molar ratio,
b) adjusting pH to 5-6.5,
c) inoculating the cultivation medium with an inoculum of the _P. purpurogenum_ spores to form a cultivation composition;
d) cultivating the inoculated cultivation composition of (c),
e) optionally analysing samples from the cultivation composition of (d) for pigment production at time intervals during the cultivation until maximal pigment production has been reached,
f) optionally terminating the cultivation when maximal pigment production has been reached, and
g) separating (IOZ)-3-(9a-methyl-3-octanoyl-2,9-dioxo-2,7,9,9a-tetrahydro-furo[3,2-g]isoquinolin-6-yl)-acrylic acid.

The invention also relates to a _Monascus-Wke_ pigment composition obtainable by a method of the invention.

The inventors have found that by the present invention it is possible to detect the number and nature of the pigment compounds in the growth medium and thereby be able to determine whether or not a certain composition of medium enables the fungi to produce a desired, specific _Monascus-Wke_ pigment. This is illustrated by example in figure 3, where PP-V is identified as the main component in the sample obtained after termination of the cultivation.

By the term "high concentration of C- and N-sources" is meant at least 2 g/L of cultivation medium of C-sources or N-sources, respectively, and dependently or independently.

By the term "high C/N molar ratio" is meant a C/N molar ratio of at least 4:1.
Brief description of the drawings

The invention is further illustrated by the drawings.

Figure 1: Kinetics of red pigment production by inoculation with *Penicillium purpurogenum* IBT 11181 spores (5 x 10^5 spores/ml) in different cultivation media having varying composition (N1 to N11). The optical density OD (500nm), an indicator of pigment production, is measured for different samples collected at time intervals between 116 and 308 hours.

Figure 2: Kinetics of red pigment production by inoculation with *Penicillium purpurogenum* IBT 3645 spores (5 x 10^5 spores/ml) in different cultivation media having varying composition (N1 to N11). The optical density OD (500nm), an indicator of pigment production, is measured for different samples collected at time intervals between 118 and 307 hours.

Figure 3: The result of Mass Spectroscopy of a *Penicillium purpurogenum* IBT 3645 sample obtained after cultivation - as illustrated in figure 2 - in a medium with the composition N2 after previous LC-DAD. The compound found in the sample had m/z 412.1661. PP-V has the formula C23-H25-NO6 and a calculated m/z 412.1760. The similarity of the two values indicates that PP-V is the pigment produced in this test.

Figure 4: Contour plot, interaction model of OD (500 nm) in terminated batches of *Penicillium purpurogenum* IBT 3645 spores inoculations with the composition N1 to N11. Values in square boxes indicate g/L of Monascus red equivalent (MoE) in relation to monosodium glutamate-glucose interaction, i.e. the optimal conditions in cultivation medium composition should be found in the bottom right corner.
Figure 5: Contour plot, interaction model of OD (500 nm) in terminated batches of *Penicillium purpurogenum* IBT 11181 spores inoculations with the composition N1 to N11. Values in square boxes indicate g/L of *Monascus* red equivalent (MoE) in relation to monosodium glutamate-glucose interaction, i.e. the optimal conditions in cultivation medium composition should be found in the bottom right corner.

Figure 6: Structures of exemplary *Monascus* and *Monascus-Wke* polyketide based pigments.

Detailed description of the invention

The method of the invention may further comprise

f) analysing samples from the cultivation composition for pigment production at time intervals during the cultivation until maximal pigment production has been reach,

g) optionally terminating the cultivation when maximal pigment production has been reach.

Accordingly, in this embodiment the invention relates to a method for producing one or more *Monascus-Wke* pigment composition from *Penicillium* sp. comprising:

a) providing a cultivation medium comprising a high concentration of C- and N-sources and a high C/N molar ratio,

b) adjusting pH to about 5 to 8,

c) inoculating the cultivation medium with an inoculum of *Penicillium* to form a cultivation composition;

d) cultivating the inoculated cultivation composition of (c);

e) separating the pigment composition;
f) analysing samples from the cultivation composition for pigment production at time intervals during the cultivation until maximal pigment production has been reach;

g) optionally terminating the cultivation when maximal pigment production has been reach.

The *Penicillium* strain may be any strain that is able to produce *Monascus*-like polyketide pigments without co-production of mycotoxins, such as citrinin, and equivalents and homologs thereof, independent of the actual name of the strain. Accordingly, the term *Penicillium* strain and *Penicillium* species is intended to embrace also those strains, which may acquire other names after a future revision of the *Penicillium* subgenus *Biverticillium*. Examples of such strains are

- *P. purpurogenum* (IBT 11181, CBS 123796)
- *P. purpurogenum* (IMI 90178, IBT 4428, IBT 3645, CBS 113154)
- *P. purpurogenum* (NRRL 1136, IBT 3458, CBS 113153)
- *P. purpurogenum* (CBS 364.48, IBT 4529)
- *P. purpurogenum* (NRRL 1748, IBT 3933)
- *P. purpurogenum* (FRR 75, IBT 4454)
- *P. aculeatum* (FRR 2129, IBT 14259, IBT 4185)
- *P. aculeatum* (IMI 133243, IBT 14129)
- *P. aculeatum* (FRR 2005, IBT 14256)
- *P. aculeatum* (FRR 1664, IBT 14254)
- *P. pinophilum* (IMI 114993, IBT 3757)
- *P. pinophilum* (ATCC 9644, IBT 13104)
- *P. minioluteum* (CCRC 32646, IBT 18368)
- *P. purpurogenum* (RMF 81.01, IBT 23082)
- *P. funiculosum* (NRRL 2119, IBT 3954)
- *P. purpurogenum* (IMI 147406, IBT 21723)
- *P. funiculosum* (WSF 3955, IBT 14065)
They can be obtained from one or more of the following culture collections:

The IBT Culture Collection of Fungi, Centre for Microbial Biotechnology, DTU Systems Biology, Technical University of Denmark; Centraalbureau voor Schimmelcultures (CBS); CABI Bioscience (IMI); Agricultural Research Service Culture Collection (NRRL); FRR culture collection (FRR); American Type Culture Collection (ATCC); Culture Collection and Research Centre, Food Industry Research & Development Institute, Hsinchu, Taiwan (CCRC); Rocky Mountain Fungi collection (RMF); Wisconsin Soil Fungi collection (WSF).

In one embodiment of the invention the *Penicillium* is derived from a *P. purpurogenum* strain, e.g. *P. purpurogenum* IBT 11181 (CBS 123796), IBT 3645 (CBS 127571), IBT 4454 (CBS 127570) and IBT 4529 (CBS 127572). The first-mentioned strain is already available at CBS before the filing date of the present application. The other strains have been deposited with Centraalbureau voor Schimmelcultures (CBS) under the Budapest Treaty on 13 August 2010 (IBT 4454) and 19 August 2010 (IBT 3645 and IBT 4529).

In another embodiment of the invention the *Penicillium* is derived from a *P. funiculosum* strain, e.g. *P. funiculosum* IBT 3954 (CBS 127568). The strain has been deposited with Centraalbureau voor Schimmelcultures (CBS) under the Budapest Treaty on 13 August 2010.

In other embodiments the *Penicillium* is derived from a *P. aculeatum* strain, e.g. *P. aculeatum* IBT 14256 (CBS 127567), or from a *P. minioluteum* strain, e.g. *P. minioluteum* IBT 18368 (CBS 127569). These strains have been deposited with Centraalbureau voor Schimmelcultures (CBS) under the Budapest Treaty on the 13 August 2010.
Examples of Monascus-Wke pigments that can be produced according to the method of the invention are shown in figure 6.

The concentration of C-sources is at least 0.2 %, viz. 2 g/l of the total cultivation composition. Examples of concentration is 0.4 %, 0.5 %, 0.7 %, 1 %, 1.5 %, 2 %, 2.1 %, 2.5 %, 3 %, 3.5 % and 4 %.

The concentration of N-sources is at least 0.2 %, viz. 2 g/l of the total cultivation composition. Examples of concentration is 0.4 %, 0.5 %, 0.7 %, 1 %, 1.5 %, 2 %, 2.1 %, 2.5 %, 3 %, 3.5 % and 4 %.

In one embodiment the N-sources are selected from glutamates, e.g. monosodium glutamate, amino acids, e.g. glycine or nitrates, e.g. ammonium nitrate or sodium nitrate, or mixtures thereof or complex sources such as corn steep liquor and soy bean meal.

In another embodiment the C-sources are selected from hexoses, e.g. glucose, fructose or sucrose, pentoses, e.g. xylose, and trisaccharides, e.g. raffinose, or mixtures thereof including crude sources, such as molasses or whey.

The sources may be combined by selection of the appropriate C- and N-sources as exemplified in any combination, e.g. the cultivation medium comprises glucose and monosodium glutamate.

The molar ratio of carbon to nitrogen is at least 4:1; examples are 4.82:1; 5:1; 8:1; 10:1; 20:1; 50:1; 96:1 and 100:1.

The pH may be e.g. 5, 6.5 and 8. The optimal pH within the range 5-8 varies and can be determined for each specific strain in each case by experimentation.
The inoculated cultivation composition of (c) may be cultivated for e.g. 100 to 400 hours and samples from the cultivation composition may be taken out for analysis and analysed by OD measuring at time intervals during this cultivation period. When maximal pigment production is reached, the cultivation may be terminated, and the samples and the final batch analysed. The duration of cultivations depends on choice of *Penicillium* strain. Some typical time intervals are shown in Figures 1 and 2.

In one embodiment of the invention the concentration of pigment composition in the medium can be enhanced 5-40 fold. The yield of pigment composition is typically measured in terms of g/L *Monascus* pigment equivalents (MoE)

The cultivation can be performed in fermentors, such as classical shake flasks. In the fermentors glass beads can be added to prevent pellet-formation of the mycelia and thereby ensuring homologous morphology, i.e. better mycelia/media surface area ratio.

The pigment composition can be further isolated by purification and/or concentration steps, such as microfiltration through a size selective semi-permeable membrane or freeze-drying. Optionally, the pigment composition can be extracted from biomass produced in step (d).

The *Penicillium* inoculum being spores or mycelia preferably comprises 5 x 10^5 spores per ml of liquid medium to be inoculated. The spores are derived from a *Penicillium* strain capable of producing one or more *Monascus-WWe* pigment as exemplified above.

*Penicillium* strains in general can be cultivated in many ways, such as by submerged cultivation, solid-state fermentation. Using available cultivation technology, *Penicillium* strains provide higher yields of pigment (*Monascus-
like azaphililone pigments) than plant-based colorants (pigments). Furthermore, *Penicillium* strains are shown to secrete copious amounts of extracellular pigments, which facilitate their subsequent extraction and/or isolation, as compared to *Monascus* spp., where pigments accumulate within the *Monascus* biomass.

Cultivation of a *Penicillium* strain inoculum and pigment production would normally comprise the following steps: Cultivation in a suitable minimal well-defined medium or complex medium comprising a liquid phase; separating the liquid phase by centrifugation or filtration to remove biomass from the liquid phase; dissolving the pigment in a suitable solvent, such as ethanol, and evaporating the solvent to obtain a coloured powder. Additionally, the biomass may be extracted using an aqueous alcohol, such as ethanol, to recover pigment that is endogenous or trapped in the biomass.

Exemplary types of growth media are Yeast extract sucrose (YES) agar; Malt extract agar (MEA), Potato dextrose (PD) agar and Czapek-Dox yeast autolysate (CYA) agar (Frisvad, J. C.; Thrane, U. Mycological media for food- and indoor fungi. In Introduction to Food- and Airborne Fungi. 6th ed.; Samson, R. A., Hoekstra, E. S., Frisvad, J. C., Filtenborg, O., Eds.; Centraalbureau voor Schimmelcultures: Utrecht, The Netherlands, 2002; p 378). Liquid mediums such as Czapek-Dox (CZ) broth would be suitable to use as well. The media type can be changed to provide one or more advantages in the culturing of the *Penicillium* strains, such as for example higher yield or different colour hues. For example, the growth medium may be supplemented with one or more amino acids, such as D- (Ala, Cys, Asp, Glu, Phe, Gly, His, lle, Lys, Leu, Met, Asn, Pro, Gin, Arg, Ser, Thr, Val, Trp, Tyr), to produce amino acid derivatised *Monascus*-WWe polyketide based azaphilone pigments with different hues, and increased light stability.
Depending on the cultivation method, the pigment composition can be either harvested as the resultant biomass by a conventional procedure such as scraping off of the pigment rich mycelium part from the medium, or recovered by grinding and/or extracting the biomass and the pigmented substrate.

Samples from the cultivation medium are analysed e.g. by using OD. OD can be measured over the course of the experiments, and the data from these measurements can be implemented in a regression program (MODDE 7) to be explained further in the examples. The results from this regression enable to approach optimal settings for pigment production.

The method of the invention may be used for producing a pigment composition comprising (IOZ)-3-(9a-methyl-3-octanoyl-2,9-dioxo-2,7,9,9a-tetrahydro-furo[3,2-g]isoquinolin-6-yl)-acrylic acid (PP-V), and the cultivation composition may for this purpose be inoculated with an inoculum of e.g. *P. purpurogenum* IBT 3645 (CBS 127571).

The pigment composition obtained by the present invention may be used as a colouring agent for a food product or non-food product and, accordingly, the invention also relates to such uses. Example of food products are baked good, baking mix, beverage and beverage base, breakfast cereal, cheese, condiment and relish, confection and frosting, fat and oil, meat and meat products, frozen dairy dessert and mix, gelatine, pudding and filling, gravy and sauce, milk product, plant protein product, processed fruit and fruit juice, and snack food, and of non-food product are textile, cotton, wool, silk, leather, paper, paint, polymer, plastic, inks, tablet.

The pigment may also be used in a cosmetic composition and, accordingly, the invention also relates to a cosmetic composition comprising a pigment obtained according the method of the invention, e.g. in the form of a free, poured or compacted powder, a fluid anhydrous greasy product, an oil for the
body and/or the face, a lotion for the body and/or the face, or a hair product, such as a make-up composition.

**EXAMPLES**

5

*Penicillium strains*

The pigment producing strains used in the following experiments to illustrate the invention are *Penicillium purpurogenum* IBT 3645 (CBS 127571), IBT 11181 (CBS 123796), IBT 4454 (CBS 127570) and IBT 4529 (CBS 127572), *P. funiculosum* IBT 3954 (CBS 127568) and *P. minioluteum* IBT 18368 (CBS 127569) obtained from the IBT Culture Collection at the Technical University of Denmark (DTU) and deposited at Centraalbureau voor Schimmelcultures (CBS) under the Budapest Treaty.

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*Methods*

**EXAMPLE 1**

**Media and Culture conditions**

The basal medium that was used contained (in g/L of distilled water):

\[
\begin{align*}
K_2HP0_4 & : 5; \\
KH2HPO4 & : 5; \\
CaCl_2 & : 0.1; \\
MgSO_4 & : 0.5; \\
FeSO_4 & : 0.01; \\
ZnSO_4 & : 0.01; \\
MnSO_4 & : 0.03.
\end{align*}
\]

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Glucose and monosodium glutamate (MSG) was added to basal medium in 300 ml Erlenmeyer shake flasks in the proportions given in table 1, to a total volume of 75 ml. The working volume in the shake flasks is 60 ml.
Table 1: pH values and concentration of MSG and Glucose in % of the total volume of media

<table>
<thead>
<tr>
<th>Exp. No</th>
<th>Glucose (%)</th>
<th>MSG (%)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0,2</td>
<td>0,2</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
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<td>4</td>
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<td>9 - 11</td>
<td>2,1</td>
<td>2,1</td>
<td>6,5</td>
</tr>
</tbody>
</table>

Glass beads (4-5mm), equivalent to the filling of two 1.5 ml Eppendorf-tubes, 381 Ox, were added to each shake flask.

pH was measured by pHM 210 Meterlab® and adjusted with HNO3 and NaOH.

Shake flasks were autoclaved at 121°C for 20 min. in a Falcon 30 (LTE scientific LTD.) autoclave. To prevent caramelization, glucose was added subsequently to auto-clavation in experiments 4, 6, 7 and 8.

EXAMPLE 2
Collection of spores
Spores were harvested after 7 days growth on CYA-plates and added to spore suspension to determine spore-concentration. This was done in Assistant® counting chamber 0.0025mm². In both runs with spores (3645 and 11181) the inoculum concentration was 5x10⁵ spores/ml.

Shake flasks with spores and mycelia were placed on shaking table with agitation 150 rpm in 25°C dark incubation room.

Experiments ran between 7-14 days.

EXAMPLE 3
Sampling
Throughout the experiment sampling were done to establish the overall picture of pigment secretion. This was done by aseptic removal of 2ml broth into a 5ml Sarstedt® plastic tube (62.547.254). All sterile work was performed in a Holten® Lamin Air MS 2000 flow bench.

The samples was taken at different times as shown in figures 1 and 2 for two of the tested strains and OD-measured on HeAios® Thermo scientific spectrophotometer at wavelength 500nm using pre-inoculation media as reference.

EXAMPLE 4
Analysis (MODDE)
The data from OD measurements was introduced to the Modde 7 software (Umetrics, an MKS company, Sweden). Three factors, pH, glucose and MSG were characterized by full factorial (2-level) interaction modeling with red pigment production as our response value. Each factor was tested at two levels. A total of 11 experimental runs were carried out with three of them being replicate runs of the center point values. Contour-plots were created to visualize the optimal composition, within an empirical chosen low-high level,
of MSG and Glucose at a fixed pH level. The fitting quality was evaluated by the coefficient of determination.

EXAMPLE 5

Solid Phase Extraction

Upon reaching maximal pigment production, the experiments were terminated. Both samples and terminated batch were treated with 96% ethanol and centrifuged in a Eppendorf® centrifuge 5804 at 7000 rpm for 10 min. Supernatant was removed and solid phase extraction was carried out in the following way:

Oasis® MAX loc (30mg) extraction cartridges were washed with 1ml MeOH prior to washing with 1ml MQ water (22 μm).

Samples were mixed in 1:1 ratio with 4% HNOs and loaded in cartridges. Cartridges were treated with 1ml, 2% NH₄OH ; 1ml, 100% MeOH ; 1ml, 100% MeOH + 2% Formic Acid in this successive order. The extraction fluid gathered in the last step, were subject to High-resolution liquid chromatography-diode array detection-mass spectrometry (LC-DAD-MS).

EXAMPLE 6

Chromatographic analysis

LC-DAD-MS was performed as described by Sameer AS Mapari in an earlier publication (WO 2009/026923 A2).

High-resolution liquid chromatography-diode array detection-mass spectrometry (LC-DAD-MS) was performed on an Agilent HP 1100 LC system with a DAD and a 50 mm × 2 mm i.d., 3 μm, Luna C 18 2 II column (Phenomenex, Torrance, CA). The LC system was coupled to a LCT orthogonal time-of-flight mass spectrometer (Waters-Micromass, Manchester, United Kingdom) with a Z-spray electro spray ionization (ESI) source and a LockSpray probe and controlled by the MassLynx 4.0 software.
RESULTS

EXAMPLE 7

Kinetics of red pigments secretion by *P. purpurogenum* (IBT3645, IBT 11181, IBT 4454 and IBT 4529), by *P. aculeatum* (IBT 14256) and by *P. minioluteum* (IBT 18368)

The media composition that was used as the starting environment for the different runs (N1-N11) is given in Table 1 above.

The amount of extracellular red pigment was measured until presumed maximal production and beyond. In all cases no significant increase in the amount of red pigment was detected before approx. 120 h, see figures 1 and 2 (IBT3645 and 11181). The microbial lag phase and the biomass/extrolite relation (red pigment production is expected to be proportional to biomass) is a very likely cause of these "delayed" tendencies.

For all the tested strains, except *P. purpurogenum* IBT 11181 and *P. purpurogenum* IBT 3645 whereby pigment production phase was studied, the pigment yields were measured by the end of 7 days. The yields were measured in terms of g/L *Monascus* pigment equivalents (MoE). This was done by extrapolating the absorbance vs. concentration (g/ml) calibration curve of commercially available *Monascus* red colorant (Riken vitamin Co. Ltd., Japan). The results are shown in Table 2 below.
Table 2: Pigment production for 6 *Penicillium* strains

<table>
<thead>
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<th>EXP</th>
<th>C.- Glucose g/L</th>
<th>N-MSG g/L</th>
<th>pH</th>
<th>C/N molar ratio</th>
<th>Pigment yield (g/L) in terms of <em>Monascus</em> pigments equivalent (MoE)</th>
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<td><em>P. purpureogenum</em> IBT 11181*</td>
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*Estimated at the end of 13 days, **Estimated at the end of 7 days

5 **EXAMPLE 8**

Kinetics of red and yellow pigments secretion by *P. funiculosum* IBT 3954

The media composition that was used as the starting environment for the different runs (N1 - N11) is given in Table 1 above. The results are shown in Table 3 below. It appears that this strain produces both a red and a yellow pigment, under specific media and/or culture conditions, which can be demonstrated by the optical density at 500 nm and 400 nm, respectively. The yield of red pigment was determined in terms of g/L *Monascus* pigment equivalents (MoE).
Table 3
Red and yellow pigment production by *Penicillium funiculosum* IBT 3954*

<table>
<thead>
<tr>
<th>Exp</th>
<th>C-. Glucose g/L</th>
<th>N-. MSG g/L</th>
<th>pH</th>
<th>C/N molar ratio</th>
<th>O.D. @ 400 NM (A)</th>
<th>O.D @ 500 nm (B)</th>
<th>Red pigment yields (g/L) in Terms of MoE</th>
<th>Red colour index O.D_500/400 nm</th>
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<td>5</td>
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<td>0.06</td>
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<td>-0.036</td>
<td>0</td>
<td>-0.06</td>
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* Estimated at the end of 7 days

Example 9

Detection of (IOZ)-3-(9a-methyl-3-octanoyl-2,9-dioxo-2,7,9,9a-tetrahydro-

furo[3,2-g]isoquinolin-6-yl)-acrylic acid

The 3645 batch from N2 media was subjected to solid phase extraction and subsequently LC-DAD-MS. This is illustrated in figure 3.

The sole pigment compound found in the experiment was PP-V (IOZ)-3-(9a-
methyl-3-octanoyl-2,9-dioxo-2,7,9a-tetrahydro-furo[3,2-g]isoquinolin-6-yl)-

acrylic acid. This compound is considered to be synthesized via the polyketide pathway. PP-V has absorbance around 560 nm as shown in figure 3.

PP-V is a monascorubramine homologue only different in the c_{12} position where CH$_3$ is substituted with COOH and in the stereo configuration (Z instead E) at the c_{10} position. In an experiment by Jun Ogihara et al.
(Ogihara, J; Kato, J; Oishi, K; Fujimoto, Y; (2000) Biosynthesis of PP-V, a monascorubramine homologue, by Penicillium sp A, Journal of Bioscience and Bioengineering 90(6): 678-680) where they added labeled acetate (1-13C and 2-13C) to a culture of growing Penicillium sp. they found that the alternate incorporation pattern coincided with the pattern found in the catabolism of Monascus pigment. Based on this information it can be deduced that the PP-V compound found in the present experiment, is indeed Monascus-WWpigment.

**EXAMPLE 10**

**Contour plots**

From the graphs depicted in figures 1 and 2 it is clear that a pH around 5 is the optimal acid/base-condition for some of the tested strains. Hence, contour-plot were made at a fixed pH 5-level. The contour plots are in relation to the present invention used to illustrate the outcome when changing two parameters in a $2^3$ factorial design keeping one fixed. In this case the two parameters that were subjected to alterations were monosodium glutamate (MSG) and glucose.

The contour plots of both IBT 3645 and IBT 11181 (figures 4 and 5) clearly indicate, by moving towards the steepest accent, that excess glucose is of some importance in relation to pigment production. At the maximal glucose value chosen for this experiment, 40 g/l, the highest yield of soluble pigment is recognized. At the same time it shows that the MSG concentration must be kept at a relative low (5g/l or below) to obtain the best results within these settings (i.e. large 40 g/l glucose concentration and pH 5).

A pH around 8 is the optimal acid/base-condition for others of the tested strains, e.g. IBT 4454 and 4529. The optimal pH can be decided by testing the strain at different conditions.
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| 0-3  | Applicant’s or agent’s file reference XIV  
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| 1-2  | line                                                                                                             |
| 1-3  | Identification of deposit                                                                                       |
| 1-3-1| Name of depository institution                                                                                  |
| 1-3-2| Address of depository institution                                                                               |
| 1-3-3| Date of deposit                                                                                                  |
| 1-3-4| Accession Number                                                                                                 |
| 1-4  | Additional Indications                                                                                           |
| 1-5  | Designated States for Which Indications are Made                                                                |
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|      | Dobbelaere, Julie                                                                                               |
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Uppsalaalaan 8, NL-3584 CT Utrecht, The Netherlands / P.O. Box 85167, NL-3508 AD Utrecht, The Netherlands  
13 August 2010 (13.08.2010)  
CBS 12757113082010  
Penicillium purpurogenum IBT 3645  
All designations
C L A I M S

1. Method for producing one or more Monascus-Wke pigment composition from Peniciiiium species comprising:

   a) providing a cultivation medium comprising a high concentration of C-and N-sources and a high C/N molar ratio,
   b) adjusting pH to about 5 to 8,
   c) inoculating the cultivation medium with an inoculum of Peniciiiium to form a cultivation composition;
   d) cultivating the inoculated cultivation composition of (c);
   e) separating the one or more produced pigment compositions.

2. The method of claim 1 further comprising:

   f) analysing samples from the cultivation composition for pigment production at time intervals during the cultivation until maximal pigment production has been reach,
   g) optionally terminating the cultivation when maximal pigment production has been reached.

3. The method of any one of the claims 1 and 2, wherein the Peniciiiium is a P. purpurogenum strain.

4. The method of claim 3, wherein the Peniciiiium is selected from P. purpurogenum IBT 11181 (CBS 123796), P. purpurogenum IBT 3645 (CBS 127571), P. purpurogenum IBT 4454 (CBS 127570) or P. purpurogenum IBT 4529 (CBS 127572).

5. The method of any one of the claims 1 and 2, wherein the Peniciiiium is a P. funiculosom strain.
6. The method of claim 5, wherein the Penicillus is *P. funiculosum* IBT 3954 (CBS 127568).

7. The method of any of the claims 1 and 2, wherein the Penicillus is a *P. aculeatum* strain.

8. The method of claims 7, wherein the Penicillus is *P. aculeatum* IBT 14256 (CBS 127567).

9. The method of any of the claims 1 and 2, wherein the Penicillus is a *P. minioluteum* strain.

10. The method of claims 9, wherein the Penicillus is *P. minioluteum* IBT 18368 (CBS 127569).

11. The method of any of the claims 1-10, wherein the concentration of pigment composition in the medium can be enhanced 5-40 folds.

12. The method of any of the claims 1-11, wherein the concentration of C-sources is from 0.2 % to 4 %, and the concentration of the N-sources is from 0.2 % to 4 % of the total cultivation composition.

13. The method of any of claims 1-10, wherein the N-sources are selected from glutamates, e.g. monosodium glutamate, amino acids, e.g. glycine, or nitrates, e.g. ammonium nitrate or sodium nitrate, or mixtures thereof or complex sources, e.g. corn steep liquor and soybean meal.

14. The method of claim 13, wherein the N-sources are monosodium glutamate.
15. The method of any of the claims 1-14, wherein the C-sources are selected from hexoses, e.g. glucose, fructose or sucrose, pentoses, e.g. xylose, and trisaccharides, e.g. raffinose, or mixtures thereof including crude sources such as molasses or whey.

16. The method of claim 15, wherein the C-sources are glucose.

17. The method of any of the claims 1-16, wherein the C/N molar ratio is at least 4:1.

18. The method of any of the claims 1-17, wherein pH in step b) is adjusted to about 5, 6.5 or 8.

19. The method of any of the claims 2-18, wherein the inoculated cultivation composition of (c) is cultivated for 100 to 400 hours, and the samples are analysed by OD measuring at selected time intervals during this cultivation period.

20. The method of any of the claims 1-4 or 7-19, wherein the pigment composition comprises (IOZ)-3-(9a-methyl-3-octanoyl-2,9-dioxo-2,7,9,9a-tetrahydro-furo[3,2-g]isoquinolin-6-yl)-acrylic acid (PP-V), and the cultivation composition is inoculated with an inoculum of *P. purpurogenum* IBT 3645 (CBS 127571).

21. A method for producing (IOZ)-3-(9a-methyl-3-octanoyl-2,9-dioxo-2,7,9,9a-tetrahydro-furo[3,2-g]isoquinolin-6-yl)-acrylic acid from *P. purpurogenum* comprising:
   a) providing a cultivation medium comprising at least 0.2 % glucose and at least 0.2 % monosodium glutamate and a high molar C/N ratio,
   b) adjusting pH 5-8,
c) inoculating the cultivation medium with an inoculum of the *P. purpurogenum* spores to form a cultivation composition,

d) cultivating the inoculated cultivation composition of (c),

e) optionally analysing samples from the cultivation composition of (d) for pigment production at time intervals during the cultivation until maximal pigment production has been reach,

f) optionally terminating the cultivation when maximal pigment production has been reach, and

g) isolating (IOZ)-3-(9a-methyl-3-octanoyl-2,9-dioxo-2,7,9,9a-tetrahydro-furo[3,2-g]isoquinolin-6-yl)-acrylic acid.


23. Use of the *Monascus-UWe* pigment composition obtained by a method of any of the claims 1-21 as a colouring agent for a food product and/or non-food product.

Pigment production by *Penicillium purpurogenum* IBT 11181 as influenced by media and pH.

![Graph showing pigment production over time](image)

Figure 1
Pigment production by *Penicillium purpureogenum* IBT 3645 as influenced by media and pH.

Figure 2
Figure 3
Investigation: 3645 spores (MLR)
Contour Plot

pigment production

pH = 5

Figure 4
Investigation: glumsgpH (MLR)
Contour Plot

pigment production

pH = 5

Figure 5
Figure 6
INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2011/064152

A. CLASSIFICATION OF SUBJECT MATTER
INV. C09B61/00 A23L1/275 C12M1/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
C09B A23L C12M

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal , CHEM ABS Data, WPI Data, BEI LSTEIN Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>JUN OGIHARA ET AL: &quot;Biosynthesis of PP-V, a monascoribramine homologue, by Penicillium sp. AZ&quot;. JOURNAL OF BIOSCIENCE AND BIOENGINEERING, ELSEVIER, AMSTERDAM, NL, vol. 90, no. 6, 1 January 2000 (2000-01-01), pages 678-680, XP009143588, ISSN: 1389-1723, DOI: 10.1016/0138-1723(00)90017-3 [retrieved on 2009-09-13] page 678, left-hand column, paragraph 3 figure 1 page 679, last paragraph ----- <em>/</em></td>
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Further documents are listed in the continuation of Box C.
See patent family annex.

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Date of the actual completion of the international search
17 October 2011

Date of mailing of the international search report
03/11/2011

Name and mailing address of the ISA/
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2380 HV Rijswijk Tel: (+31-70) 340-2040, Fax: (+31-70) 340-3016

Authorized officer
Durand-Oral, 1 knur
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<td>TZANN F LIN AND ARNOLD L DEMAIN: &quot;Effect of nutrition of Monascus sp. on formation of red pigments&quot;, APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, SPRINGER VERLAG, BERLIN, DE, vol. 36, no. 1, 1 January 1999 (1999-01-01), pages 70-75, XP009143714, ISSN: 0175-7598, DOI: DOI: 10.1007/BF00164701 the whole document page 70, column summary Monosodium glutamate as sole N-source</td>
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