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Bioprocess-related, morphological and bioinformatic perspectives on the biosynthesis of secondary metabolites produced by *Penicillium solitum*

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ABSTRACT

The qualitative and quantitative analysis of the secondary metabolic repertoire of *Penicillium solitum* CBS 288.36 in submerged and surface liquid cultures was performed with the use of UPLC–MS. The examination of culture filtrates led to the identification of nine previously described molecules (compactin, 4a,5-dihydrocompactin, 3α-hydroxy-3,5-dihydro ML-236C, ML-236A, cyclopenin, cyclopenol, cyclopeptin, dehydrocyclopeptin and atlantinone A). The production of secondary metabolites was observed to be inhibited under submerged conditions in the standard growth media. In order to induce the underlying pathways in the submerged cultures, several cultivation-related strategies were applied, including the supplementation with glycerol, mannitol, calcium chloride or rapeseed oil, adjusting the initial concentration of nitrogen sources and subjecting the growing mycelia to various aeration intensities and mechanical stress caused by the impeller in a stirred tank bioreactor. The correlation between compactin concentration, dissolved oxygen level and mycelial morphology was observed. On the basis of sequence similarity analysis, the genes involved in the biosynthesis of benzodiazepine alkaloids and meroterpenoids were detected in the genomes of several *P. solitum* strains. This work describes a novel attempt to quantitatively analyse the metabolic spectrum of *P. solitum* in shake flask and stirred tank bioreactor cultures with the use of a diverse set of media.

1. Introduction

Penicillium solitum is a filamentous fungus mostly known for contaminating apples and pears [1]. It is associated with the decay of pome fruits during storage and therefore leads to significant economic losses, reduced fruit quality and is a food safety concern [1–3]. This species has also been isolated from other food products, including meat [4,5] and cheese [6,7]. While the physiology of *P. solitum* was primarily addressed in the context of food microbiology, this microorganism was shown to biosynthesize a myriad of secondary metabolites (SMs), including compactin, benzodiazepine alkaloids and meroterpenoids [8–11]. Compactin (also referred to as mevastatin or ML-236B), a fungal SM obtained on an industrial scale with the use of *Penicillium citrinum*, is utilized for the production of pravastatin, an important cholesterol-lowering drug [12–15]. Unlike the primary metabolites, the SMs are not essential for growth and their role in fungal biology is often unclear. The biosynthetic pathways leading to SMs are triggered in response to certain environmental cues and some of them were shown to remain inactive under standard laboratory conditions [16]. In such cases, both cultivation-based and genetic approaches have been applied to induce the production of SMs [17–19]. The common strategy involves the adjustment of culture conditions and media composition to unlock the metabolic spectrum of a given organism [20].

While the biotechnological manufacturing of fungal metabolites typically relies on the bioreactor-based liquid cultivation, the catalogue of SMs biosynthesized by *P. solitum* was previously examined mostly with regard to agar media [8–11]. To the best of our knowledge, the work of Nielsen et al. [21] is the only published report addressing the variety of SMs produced by *P. solitum* in various liquid cultures. The corresponding experiments were conducted in 25 ml and 50 ml bottles containing less than 10 ml of growth medium. These authors observed that the submerged cultivation of *P. solitum* IBT 21838 and IBT 21545 led to relatively poor metabolite production compared to surface (static) liquid cultures, however the conclusions were formulated on the basis of chromatographic signals, with no concentration values reported for the detected SMs [21]. Importantly, the quantitative analysis regarding the metabolic spectrum of *P. solitum* has not been documented so far. Furthermore, the landscape of SMs displayed by this species has never been investigated in the context of bioreactor cultivations.

Recently, the genomes of three *P. solitum* strains have been

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sequenced, assembled, annotated and deposited in the NCBI database, namely *P. solitum* NJ1 [22], *P. solitum* RS1 [23] and *P. solitum* IBT 29525 [24]. By providing the essential genomic data, these facilitated the analysis of biosynthetic gene clusters (BGCs) responsible for the formation of SMs in this species. This was recently addressed by Nielsen et al. [24], who identified the gene clusters associated with the production of fungisporins and YWA1 (a precursor of melanin) in the genome of *P. solitum* IBT 29525. However, most of the genetic inventory underlying the SMs formation in *P. solitum* remains to be elucidated. Notably, the genes of *P. solitum* responsible for the biosynthesis of compactins, benzodiazepine alkaloids and meroterpenoids have not been yet characterized.

The aim of the current study was to quantitatively characterize the secondary metabolic spectrum of *P. solitum* in various liquid cultures, induce the formation of SMs by applying the cultivation-based methods and identify the genes and gene clusters involved in the biosynthesis of SMs in this species.

2. Materials and methods

2.1. Strain

P. solitum CBS 288.36 (type strain of *P. solitum* Westling) was used throughout the study.

2.2. Growth media

The sporulation medium was composed as follows: malt extract: 20 g l^{-1}; peptone: 5 g l^{-1}; agar: 25 g l^{-1}.

The following standard liquid media were used for the cultivation of *P. solitum* [25–27]:

- CYA (Czapek yeast autolysate): sucrose: 30 g l^{-1}; $NaNO_3$: 3 g l^{-1}; K_2HPO_4: 1 g l^{-1}; KCl: 0.5 g l^{-1}; $MgSO_4{\cdot}7H_2O$: 0.5 g l^{-1}; $FeSO_4{\cdot}7H_2O$: 0.01 g l^{-1}; $ZnSO_4{\cdot}7H_2O$: 0.01 g l^{-1}; $CuSO_4{\cdot}5H_2O$: 0.005 g l^{-1}; yeast extract 5 g l^{-1};
- YES (yeast extract sucrose): sucrose: 150 g l^{-1}; yeast extract 20 g l^{-1}; $MgSO_4 \cdot 7H_2O$: 0.5 g l^{-1}; $ZnSO_4{\cdot}7H_2O$: 0.01 g l^{-1}; $CuSO_4{\cdot}5H_2O$: 0.005 g l^{-1};
- YME (yeast malt extract): glucose: 10 g l^{-1}; malt extract: 3 g l^{-1}; yeast extract: 3 g l^{-1}; peptone: 5 g l^{-1};
- LACYE (lactose yeast extract): lactose 30 g l^{-1}; KH_2PO_4: 1.51 g l^{-1}; yeast extract: 5 g l^{-1}; $MgSO_4{\cdot}7H_2O$: 0.52 g l^{-1}; $ZnSO_4{\cdot}7H_2O$: 1 mg l^{-1}; $Fe(NO)_3{\cdot}9H_2O$: 2 mg l^{-1}; biotin 0.04 mg l^{-1}; NaCl: 0.4 g l^{-1}; 1 ml of trace element solution of the following composition: H_3BO_3: 65 mg l^{-1}; $MnSO_4{\cdot}7H_2O$: 43 mg l^{-1}; $CuSO_4{\cdot}5H_2O$: 250 mg l^{-1}; $Na_2MoO_4{\cdot}2H_2O$: 50 mg l^{-1}.

The following modifications of the CYA medium were suggested to induce the production of SMs (other ingredients were as in the CYA medium);

- CYAoca: $CaCl_2$: 0.5 g l^{-1}; rapeseed oil: 1% *v/v*,
- CYAsg: glycerol: 15 g l^{-1}; sucrose: 20 g l^{-1},
- CYAsm: mannitol: 15 g l^{-1}; sucrose: 20 g l^{-1},
- CYAg: glycerol: 15 g l^{-1}; sucrose: 0 g l^{-1},
- CYAm: mannitol: 15 g l^{-1}; sucrose: 0 g l^{-1}.

Ultimately the CYAsg medium was modified to achieve the CYAsgn medium containing: $NaNO_3$: 1.5 g l^{-1}; yeast extract: 2.5 g l^{-1}; other ingredients were as in the CYAsg medium.

The pH level of the growth media was adjusted to 7.3. The media were autoclaved at 121 °C. The time of sterilization was equal to 20 min for the flask culture media and 90 min for the bioreactor media.

2.3. Inoculum

Sporulation was induced by cultivating the fungus for 10 days on malt agar slants. The inoculum was prepared by washing the spores from slants with sterile physiological salt solution. The volume of suspension used for inoculation was chosen to achieve the initial number of spores in the culture equal to approximately 10^9 spores per litre.

2.4. Cultivation in flasks

The cultures were propagated in the shaken and static flasks (total volume: 500 ml, working volume: 150 ml). The submerged shake flask culture was incubated in a rotary shaker at 110 min^{-1}. After 7 days of cultivation at 25 °C the culture filtrate samples were collected and examined for the presence of secondary metabolites.

2.5. Cultivation in a stirred tank bioreactor

The stirred tank bioreactor BIOSTAT® B Plus (Sartorius, Germany) was used to perform two independent experimental runs (C1 and C2). The working volume of the bioreactor was equal to 5.4 l. The initial and at the same time lower setting values of *vvm* (air flow rate per bioreactor volume) and the rotary speed of the impeller were set respectively to 0.28 $l_{air}\,l^{-1}\,min^{-1}$ and 170 min^{-1} in the C1 run, 0.74 $l_{air}\,l^{-1}\,min^{-1}$ and 400 min^{-1} in the C2 run. Dissolved oxygen saturation was controlled in both runs at 15% by the automatic adjustment of aeration rate and impeller speed. The upper setting values of *vvm* and the rotary speed of the impeller were respectively 0.92 $l_{air}\,l^{-1}\,min^{-1}$ and 300 min^{-1} in the C1 run, 1.11 $l_{air}\,l^{-1}\,min^{-1}$ and 600 min^{-1} in the C2 run. The cultures were propagated for 7 days at 25 °C.

2.6. Chemical analysis

The identification and quantitative analysis of SMs was performed with the use of ultra-high performance liquid chromatography (UPLC® Acquity) coupled with high-resolution mass spectrometry (SYNAPT G2, Waters, USA) and comprised both ESI+ and ESI− ionization modes, as previously described [25]. The following authentic standards were used in the course of the study: cyclopenin, cyclopeptin, dehydrocyclopeptin (Santa Cruz Biotechnology, USA), compactin (Sigma-Aldrich, USA) and cyclopenol (Adipogen, USA). The calibration curves and the quantitative analysis were performed with the use of TargetLynx™ software (Waters, USA). The β-hydroxyacid form of compactin was obtained via the chemical transformation of the purchased compactin lactone (Sigma-Aldrich, USA) according to the procedure presented by Casas-Lopez et al. [28]. The database of natural products “Antibase 2014: The Natural Compound Identifier” [29] was used for the tentative identification of SMs for which the standards were not available.

2.7. Image analysis

The light microscope OLYMPUS BX53 (OLYMPUS, Japan) was used for microscopic observations of the growing mycelia. The image processing and analysis software cellSens Dimension ver. 1.16 (OLYMPUS, Japan) was used to capture the microscopic images and add the scalebars.

2.8. In silico analysis of putative biosynthetic gene clusters

The antiSMASH software [30] was used to detect the putative biosynthetic gene clusters in the genomes of *P. solitum* strains. The genomic sequences of *P. solitum* NJ1 [22], RS1 [23] and IBT 29525 [24] were downloaded in FASTA format from the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov). The BlastP software [31] was applied for the comparative analysis of