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Camptothecine production by mixed fermentation of two endophytic fungi from *Nothapodytes nimmoniana*

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ABSTRACT

Two endophytic fungi isolated from the endangered plant *Nothapodytes nimmoniana* (Grah.) Mabb. were found to effectively synthesize CPT independent of their host plant under submerged fermentation conditions. Molecular characterization of fungi revealed their identity as *Colletotrichum fructicola* SUK1 (F1) and *Corynespora cassiicola* SUK2 (F2). Mixed fermentation experiments were carried out to study the effect of microbial signalling between the two fungal species on camptothecine production. Effect of culture parameters on CPT production was studied for both mono-cultures (F1 and F2) separately as well as for the mixed fermentation (F1 + F2). Further the most influencing ones were optimized statistically using response surface methodology. Statistical model based optimized parameters were whey (70 %), agitation rate (110 rpm), temperature (30 °C), and incubation period (7 d) for the mixed fermentation. Monocultures of the two fungal species F1 and F2 yielded CPT up to 33 ± 1.1 mg $l^{-1}$and 69 ± 1.1 mg l^{-1}, respectively; while their mixed fermentation under the optimized conditions yielded up to 146 ± 0.2 mg l^{-1}. HPLC and LC-MS/MS techniques were used to analyze the products obtained.

Introduction

Endophytic microorganisms are potential sources of many therapeutically important secondary metabolites produced by their original plants which have turned endangered or endemic owing to their excessive exploitation by mankind (Musavi *et al.* 2015). Bioprospecting of anticancer natural products, both novel and existing ones, from endophytic microbial sources particularly inhabiting the endangered plants has attained special significance in recent research arenas. Several endophytic fungi have been explored to study their potential for synthesis of CPT outside their plant hosts. Scale-up methods for production of CPT has been reported earlier (Rehman *et al.* 2009; Musavi *et al.* 2015). However, cost-effective and commercially feasible production of CPT by endophytic fungi still remains a challenging task. The major obstacle in this aspect is the attenuation of secondary metabolite production over successive generations under axenic monoculture conditions (Gurudatta *et al.* 2010; Shweta *et al.* 2010; Bhalkar *et al.* 2015). The existing monoculture strategies used generally for production of such secondary metabolites needs to be manipulated in order to maintain the *in vitro* biosynthetic activity of endophytes (Kusari & Spiteller 2011).

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Recently, various strategies have been suggested to enhance the potential of these endophytic microorganisms such as manipulation of media and culture conditions, metabolic engineering, epigenetic modulation, chemical induction, mixed fermentation, and fermentation technology (Qadri et al. 2013). An interesting way of inducing the secondary metabolite production in these endophytes is simulating their natural habitat by co-cultivation of microorganisms from the same plant or ecosystem. Such an approach where in two or more microorganisms are grown together under same confined conditions using solid medium is termed as mixed fermentation while the same is addressed as mixed fermentation when liquid medium is used (Bertrand et al. 2014). Mixed fermentation experiments not only help in discovering novel secondary metabolites but also activate the known productivity of the microorganisms (Brakhage & Schroeckh 2011). Earlier research proposes that such modifications are necessary to bring about induction in production of the secondary metabolites via certain genetic modulation which otherwise remain inactive in typical monoculture conditions. It is well known that the culture parameters vitally affect the metabolic processes of microorganisms (Scherlach & Hertweck 2009). Mixed fermentation of two or more microorganisms is routinely practiced and has limited but significant practicability in discovery of novel pharmaceutically or agriculturally important compounds (Brakhage 2013; Moody 2014; Netzker et al. 2015). Significance of co-cultivation was reported initially in 1982 by Watanabe et al. where coculture of *Gluconobacter* sp. W-315 with the fungi *Neurospora crassa* or *Aspergillus oryzae* resulted in synthesis of the antibacterial polyketide enacyloxin.

Synthesis of camptothecine (CPT), precursor for the lead anticancer drugs Topotecan and Irinotecan, has been reported from endophytic microbial sources including most fungi and a few bacterial species isolated from their respective host plants (Puri et al. 2005; Rehman et al. 2008; Musavi et al. 2015). Production of CPT by mono-cultures of endophytic fungi has been described but very little attention has been paid towards their mixed fermentation. An effort was made to isolate endophytic fungi from *Nothapodytes nimmoniana* (Grah.) Mabb., an endangered elite species found in Western Ghats of India which is well-known for its high CPT content (Govindachari & Viswanathan 1972). Mixed fermentation strategies have been applied to enhance the ability of known potential endophytic fungi as well as discover new bioactive compounds. Mixed fermentation has its own advantages over mono-cultures which facilitate expression of gene clusters responsible for metabolite production that are considered to remain silent under unnatural laboratory conditions (Chagas et al. 2013). HPLC, GCMS, and LCMS provide better separation of metabolites from crude extracts and allow partial or full spectroscopic identification of compounds (Bertrand et al. 2013).

A complete optimization of the operational conditions for CPT production by co-cultivation of two endophytic fungi using RSM method was carried out in the present study.

Materials and methods

Chemicals

Reference compound CPT was purchased from Sigma–Aldrich (USA). Extraction chemicals, chloroform, ethyl acetate, and methanol, used were of analytical grade and with highest purity obtained from Hi-media (India). Synthetic media for culturing microorganisms were also obtained from Hi-media (India). HPLC-grade solvents obtained from Sigma–Aldrich (USA) were used for HPLC and GC–MS.

Plant material

Plant samples were collected from Dajipur forest areas surrounding Kolhapur district, Maharashtra state, India. Identification and further authentification of the collected plant material was done by expertise from Department of Botany, Shivaji University, Kolhapur, India. Fresh and healthy leaf and stem cuttings of *Nothapodytes nimmoniana* plant were collected and stored in clean dry polythene bags at 4 °C for further use.

Isolation and screening of potential endophytic fungi

Surface sterilization of the excised leaf and stem segments was carried out aseptically. Isolation protocol was followed as described by Bhalkar et al. (2015). Pure culture of the isolated fungi were maintained on Sabouraud's agar (SBA) and incubated at 25 °C. Explants without surface sterilization were treated as negative controls to prevent false positives from contamination by other microorganisms. The isolates obtained were screened for their potential to produce CPT in the absence of their host plant. The fungi were subcultured to liquid Sabouraud's media and incubated under shaking conditions (120 rpm) at 30 °C for 7 d. The suspension cultures were extracted separately for biomass and cell free broth and checked for presence of CPT using TLC and HPTLC methods. Only those isolates capable of producing significant quantities of CPT as detectable through HPTLC method were selected as potent isolates and processed further.

Molecular characterization of potent isolates

Out of seven (five fungal and one bacterial) isolates obtained in the present study, only three of the fungal isolates [Isolate 1, isolate 5 (F1), and isolate 6 (F2)] showed CPT production which were further identified using DNA sequencing techniques. Modified CTAB method was used to isolate the genomic DNA from the fungal mycelia and further purified using PCR technique. Universal primers ITS 1 (5′TCCGTAGGTGAACCTGCGG3′) and ITS4 (5′TCCTCCGCTTATTGATATGC3′) (GeNei™, Bengaluru, India) were used to amplify the flanking internal transcribed spacer (ITS) regions intervening the 5.8s rDNA and large subunit of rRNA. The resulting sequences were subjected to nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the homologous sequences of species obtained were used for phylogenetic analysis. Neighbour joining method was applied to construct the phylogenetic tree using MEGA5.2 software with Jukes–Cantor model at 1000 bootstrap replications (AZ, USA).

Mono- and mixed fermentation of potential fungi

Endophytic fungi were cultivated under standard laboratory conditions individually (monoculture) as well as together in