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Enhanced camptothecin production by ethanol addition in the suspension culture of the endophyte, *Fusarium solani*

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HIGHLIGHTS

- Ethanol as carbon and energy source for the endophyte, *Fusarium solani*.
- Ethanol as an elicitor to enhance camptothecin production by the endophyte *F. solani*.
- Using extract of a non-camptothecin producing plant for camptothecin production.

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ABSTRACT

Ethanolic extract of a non-camptothecin producing plant, *Catharanthus roseus* when added in the suspension culture of the endophyte *Fusarium solani* known to produce camptothecin, resulted in enhanced production of camptothecin by 10.6-fold in comparison to that in control (2.8 μg/L). Interestingly, addition of pure ethanol (up to 5% v/v) in the suspension culture of *F. solani* resulted in maximum enhancement in camptothecin production (up to 15.5-fold) from that obtained in control. In the presence of ethanol, a reduced glucose uptake (by ~40%) and simultaneous ethanol consumption (up to 9.43 g/L) was observed during the cultivation period (14 days). Also, the total NAD level and the protein content in the biomass increased by 3.7- and 1.9-fold, respectively, in comparison to that in control. The study indicates a dual role of ethanol, presumably as an elicitor and also as a carbon/energy source, leading to enhanced biomass and camptothecin production.

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1. Introduction

Camptothecin (CPT) is a pentacyclic quinoline alkaloid ((S)-4-ethyl-4-hydroxy-1H-pyrano[3′,4′:6,7]indolizino[1,2-b]quinoline-3,14-(4H,12H)-dione) which serves as the lead molecule for Topotecan and Irinotecan, two of the FDA-approved, most in demand anti-cancer drugs. It is a highly effective anti-cancer molecule as it acts by binding to the topoisomerase I–DNA complex in cancer cells, thus preventing DNA replication and triggering cell death (Shweta et al., 2010). Several reports also suggest that CPT derivatives are effective in the treatment of AIDS (Sadaie et al., 2004), as anti-viral agents (Liu et al., 2010a), antifungal agents (Zhang et al., 2008), radiation sensitizers (Kvols, 2005), anti-parasitic agents against falciparum malaria (Chauhan and Srivastava, 2001) and trypanosomiasis (Bodley and Shapiro, 1995), and as insecticides (Liu et al., 2010b). Despite the ever-increasing demand, the only commercially available source of CPT is the natural plant extract mainly from *Camptotheca acuminata* and *Nothapodytes nimmoniana* (with maximum CPT yield reported up to 0.3% dry weight (DW)) (Padmanabha et al., 2006). Owing to extensive harvesting and habitat loss, *N. nimmoniana* population has declined by 50–80% in India (Khan, 2010). In case of *C. acuminata*, over-harvesting by the pharmaceutical industry has decimated the population of the endemic trees in China with less than 4000 of the trees remaining in the wild (Balasubramanian and Narayanan, 2012). Considering these facts, search for alternative sources of the drug has become inevitable. Plant cell cultures (Fulzele et al., 2001; Pi et al., 2010; Saito et al., 2001) and total chemical synthesis (Chavan et al., 2004; Yu et al., 2011) have already been explored but have not been extensively exploited at industrial scale due to limitations like complex and uneconomical production, difficulty in scale-up, slow growth and genetic/biochemical instability of plant cells in culture, etc.

Under these circumstances, the discovery of endophytic fungi capable of producing CPT has offered microbial fermentation route as an exciting and potential alternative for production of CPT at commercial level (Zhao et al., 2010). Despite the extensive documentation of various endophytes capable of producing CPT

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(Table 1), very few studies have been undertaken on bioprocess development and subsequent optimization for yield and productivity enhancement of CPT from endophytic fungi (Amna et al., 2012, 2006; Musavi et al., 2014; Pu et al., 2013; Rehman et al., 2009). Hence, there is an immense scope for exploring bioprocess optimization strategies and developing an optimized microbial fermentation based production platform for CPT. However, the potential of endophytes as sustainable alternative production platforms has been severely hampered due to the widely reported problem of subculture based attenuation in product yield, resulting in inconsistent and low production. Lack of host stimuli in culture medium and genomic instability or silencing of the biosynthetic genes in axenic cultures have been hypothesized as possible reasons for the attenuation (Sachin et al., 2013). Strategies to overcome the attenuation problem including re-inoculation into the host followed by re-isolation, supplementing the axenic culture with host plant extracts, have been reported to be successful in some cases (Soliman and Raizada, 2013; Zhao et al., 2013) and not so in other cases (Sachin et al., 2013; Kusari et al., 2011). Thus, in order to improve CPT production in potential endophytes, exogenous addition of possible elicitors including small molecules and non-CPT producing plant extracts were explored as yield enhancement strategies. In the present study, the effect of addition of *Catharanthus roseus* leaf extract and a small molecule elicitor (ethanol) in the growth medium was investigated on biomass and CPT production in the suspension culture of the endophyte, *Fusarium solani* MTCC 9668.

2. Methods

2.1. Fungal culture used

The endophytic fungal strain used in the present study was, *F. solani* MTCC 9668 (Shweta et al., 2010), procured from Microbial Type Culture Collection and GenBank, Chandigarh, India.

2.1.1. Maintenance of the culture

The culture was maintained on potato dextrose agar (PDA) medium having 2.4% (w/v) potato dextrose complex medium (HiMedia Laboratories Pvt. Ltd., Mumbai) and 1.5% (w/v) agar. The culture was allowed to grow on the solid medium as slants at 28 °C for 7 days until sporulation. The spores were collected by washing with 5 ml of saline (0.1% (w/v) NaCl) and the solution was filtered through cheese cloth to remove any hyphae, if present in the solution. The spore solution ($\sim 10^6$ spores/ml) was stored at −80 °C as 16% (v/v) glycerol stocks in 1.5 ml microcentrifuge tubes for subsequent use in experiments.

2.1.2. Inoculum preparation

A glycerol stock of the spore solution (10 μl) was streaked on to the solid growth medium (PDA) for growth up to 7 days at 28 °C under static and dark conditions. Spore solution ($\sim 10^6$ spores/ml) obtained from the freshly grown culture was then used as inoculum (2% (v/v)) for each experiment. The spore count was established using a haemocytometer. The concentrated spore solution was diluted accordingly with distilled water to obtain the required inoculum size ($\sim 2 \times 10^4$ spores/ml) in all the experiments. In order to account for the experimental inconsistencies due to error in the measurement of the spore count in the inoculum, the control experiments were freshly initiated every time and run in parallel for each comparative study.

2.2. Development of suspension culture of F. solani MTCC 9668

The suspension culture of *F. solani* MTCC 9668 was initiated in 250 ml Erlenmeyer flask(s) by adding the spore inoculum in 50 ml potato dextrose medium (2.4% (w/v)). The initial pH of the medium was adjusted to 5.6 and the suspension culture was grown at 28 °C in an incubator shaker rotating at 120 rpm for a cultivation period of 14 days (as control conditions). The shake flask(s) were harvested after the cultivation period for the estimation of biomass (g/L) and CPT production (yield (μg/g DW) and concentration (μg/L)). All the experiments were duplicated and average values have been reported.

2.3. To study the effect of exogenous addition of non-CPT producing plant extract on biomass and CPT production in suspension culture of F. solani

Supplementing the axenic culture with easily available plant extracts as a rich source of precursor(s) and/or elicitor(s) for CPT biosynthesis can be a cost effective strategy to enhance CPT production in the impeded strain of *F. solani*. Strictosidine is not unique to CPT biosynthesis but is actually the precursor to over 1800 different monoterpene indole alkaloids (Sachin et al., 2013). Hence, in an attempt to enhance the CPT yield in the axenic culture of *F. solani* MTCC 9668, ethanolic extracts of *C. roseus* leaves were used as a presumable source of strictosidine and other precursor molecules for CPT biosynthesis. The plant is an ornamental shrub which grows like a weed in the tropics and is the source of about 130 different monoterpene indole alkaloids (but not CPT), all of which use strictosidine as a precursor (van Der Heijden et al., 2004). Moreover, the leaf extract of *C. roseus* was expected to play a dual role as the strictosidine in the leaf extracts could be taken up by the culture as a precursor, while the cytotoxic alkaloids including vincristine and vinblastine might trigger stress responses in the

Table 1
List of recently discovered camptothecin producing endophytic fungi.

Endophytic fungus	Fungal strain	Host plant	CPT	Reference
Entrophospora infrequens	MTCC 5124	*Nothapodytes foetida*	49.6 μg/g	Amna et al. (2006)
Neurospora sp.	ZP5SE	*N. foetida*	+	Rehman et al. (2008)
Nodulisporium sp.	–	*N. foetida*	5.5 μg/g	Rehman et al. (2009)
Fusarium solani	INFU/CA/KF/3	*Camptotheca acuminata*	+	Kusari et al. (2009)
F. solani	MTCC 9667	*Apodytes dimidiata*	0.37 μg/g	Shweta et al. (2010)
F. solani	MTCC 9668		0.53 μg/g	
Alternaria alternata	MTCC 5477	*Miquelia dentata*	73.9 μg/g	Shweta et al. (2012)
Fomitopsis sp.	MTCC 10177		55.49 μg/g	
Phomopsis sp.	JX178957		42.06 μg/g	
Aspergillus sp.	LY341	*C. acuminata*	7.93 μg/L	Pu et al. (2013)
Aspergillus sp.	LY355		42.92 μg/L	
Trichoderma atroviride	LY357		197.82 μg/L	
Fusarium oxysporum	NFX06	*N. foetida*	610.1 ng/g	Musavi et al. (2014)

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