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Reprint of: Effect of fermentation parameters, elicitors and precursors on camptothecin production from the endophyte *Fusarium solani* ☆

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HIGHLIGHTS

- Effect of fermentation parameters on camptothecin production from *Fusarium solani*.
- Effect of elicitor and precursor addition on camptothecin production from *F. solani*.
- Enhancement in CPT productivity (up to 152 fold) under optimized culture conditions.

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ABSTRACT

Volumetric productivity of camptothecin from the suspension culture of the endophyte *Fusarium solani* was enhanced up to ~152 fold (from 0.19 μg l^{-1} d^{-1} to 28.9 μg l^{-1} d^{-1}) under optimized fermentation conditions including initial pH (6.0), temperature (32 °C) and agitation speed (80 rpm) with (5% (v/v)) ethanol as medium component. Among various elicitors and precursors studied, tryptamine (0.5 mM) as precursor and bovine serum albumin (BSA) (0.075 mM) as an elicitor added on day 6 of the cultivation period resulted in maximum enhancement of camptothecin concentration (up to 4.5 and 3.4-fold, respectively). These leads provide immense scope for further enhancement in camptothecin productivity at bioreactor level. The cytotoxicity analysis of the crude camptothecin extract from the fungal biomass revealed its high effectiveness against colon and mammary gland cancer cell lines.

1. Introduction

Traditionally, plants have been the major source of drugs or their lead molecules. However, the indispensability of plant-based bioactive molecules is accompanied by the disadvantages of extremely low yields which vary with environmental conditions, and limited supply leading to over-harvesting and the eventual extinction of a medicinally important plant. Hence, there has been constant search for a sustainable alternative source of well-known high value plant metabolites. This led to the discovery that fungi which reside within medicinally important plants (endophytes) have the ability to mimic the secondary metabolite repertoire of the host. Beginning with the report of Stierle et al. (1993) on taxol production by the endophyte *Taxomyces andreanae*, bioprospecting of endophytes for secondary metabolite production has become an actively researched area spawning numerous patents, publications and reviews every year. Today, a plethora of different endophytes are reported to produce most of the in-demand bioactive molecules including camptothecin, vincristine, vinblastine, huperzine, podophyllotoxin, diosgenin and azadirachtin (Zhao et al., 2011). These endophytes have thus given an impetus to the possibility of using microbial fermentation to meet the ever-growing demand for several life-saving drugs derived from plant sources. Microbial fermentation is a very robust technology involving shorter fermentation periods and relatively inexpensive culture media in comparison to plant/animal cell cultivations. It is a well-established technology in bioprocess industry due to easier scale-up and amenability to yield enhancement strategies like strain improvement, precursor feeding, elicitor addition, appending inhibitors and co-cultivation (Venugopalan and Srivastava, 2015a).

Most studies aimed at the exploitation of endophytes on an industrial scale have focussed on taxol producing endophytes (Flores-Bustamante et al., 2010; Gond et al., 2014; Zhou et al., 2010). Recently, with the first report of a camptothecin (CPT) producing endophyte by Puri et al. (2005), the CPT producing

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endophytes have also attracted a lot of interest with reactor studies (Amna et al., 2006; Rehman et al., 2009) as well as the application of yield enhancement strategies being explored. Examples of various yield enhancement strategies applied to CPT producing endophytes have been listed in Table 1. Recently, endophytic variants of *Fusarium solani* have been reported to produce important anti-cancer drug leads including taxol (Chakravarthi et al., 2008), podophyllotoxin (Nadeem et al., 2012) and CPT (Kusari et al., 2009; Shweta et al., 2010). The fact that some strains of *F. solani* are already being used in industry for production of drug molecules make these endophytic strains of *F. solani* an excellent choice for the development of an optimized bioprocess for *in vitro* production of high value plant metabolites. However, efforts are required to develop strategies to enhance the yield and productivity of these high value plant secondary metabolites from potential strains of *F. solani* for commercial application.

Present study thus involves *in vitro* cultivation of a potential endophyte *F. solani* MTCC 9668 for production of CPT. Ethanol was used as a medium component based on the previous work reported by the authors (Venugopalan and Srivastava, 2015b), and the effect of fermentation parameters including pH, temperature and agitation speed on the biomass and CPT production was studied. An integrated study was carried out under optimized culture conditions to study the synergistic effect of culture parameters, if any, on biomass and CPT production. Further, the effect of exogenous addition of various elicitors and precursors in suspension culture of *F. solani* was also investigated for enhanced production of CPT. A cytotoxicity analysis of the crude CPT extract (obtained from the biomass) was carried out on one human non-cancer cell line and three different human cancer cell lines.

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 as slants until sporulation, and the spore solution ($\sim 10^6$ spores/ml) was collected and stored in the form of glycerol stocks (Venugopalan and Srivastava, 2015b).

2.1.2. Inoculum preparation

The inoculum (2% (v/v)) for each experiment was prepared from the glycerol stocks (Venugopalan and Srivastava, 2015b). Required inoculum size ($\sim 2 \times 10^4$ spores/ml) for all the experiments was obtained by suitably diluting the concentrated spore solution. Experimental inconsistencies due to error in the inoculum spore count measurement were accounted for by initiating fresh control experiments every time which were run in parallel for each comparative study. In all the experiments, the shake flasks were harvested after the cultivation period for the estimation of biomass concentration (g/l), CPT yield (μg/g DW) and CPT concentration (μg/l).

All the experiments were done in duplicate and average values have been reported.

2.2. Effect of fermentation parameters on biomass and CPT production in the suspension culture of F. solani

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)). It was initially established in a previous study that ethanol (2% (v/v)) addition to the growth medium on day 0 of the cultivation period had a positive effect on the biomass and CPT production in the suspension culture of *F. solani* MTCC 9668 (Venugopalan and Srivastava, 2015b). Hence, in the present study, the cultivation conditions used in the experiments (as control) were as follows: initial ethanol concentration in the medium was 2% (v/v), 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. The effect of the fermentation parameters namely pH, temperature and agitation speed (rpm) on biomass and CPT production in the suspension culture of *F. solani* was studied using single factor optimization methodology, where each parameter was varied within a range keeping the other parameters at the same level as used under control conditions for the development of the suspension culture.

The effect of initial pH was studied in the range of 4.0–6.5. Similarly, the effect of temperature was studied in the range of 25–35 °C and the effect of agitation speed was investigated in the range 40–200 rpm. The culture was harvested from the shake-flasks for the estimation of biomass and CPT production after the cultivation period of 14 days. The optimum values of the fermentation parameters were determined from the study to maximize CPT concentration (μg/l), a mathematical product of biomass (g/l) and CPT yield (μg/g DW).

2.3. Kinetics of growth and CPT production (in shake flask) in the suspension culture of F. solani under optimized cultivation conditions

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)). As reported earlier by the authors, in a separate study carried out in parallel to optimize the effect of ethanol in the medium, maximum enhancement (up to 15.5-fold) in CPT concentration was observed when ethanol concentration was increased to 5% (v/v) in the growth medium (Venugopalan and Srivastava, 2015b). Hence, in order to establish the growth and CPT production kinetics under integrated

Table 1
Examples of yield enhancement strategies applied to CPT producing endophytes.

Strategy	Strain	Parameter	Yield enhancement	Reference
Medium and fermentation parameter optimization	*Trichoderma atroviride* LY357	Medium composition, fermentation time, pH, temperature, agitation rate	50–75 folds (including elicitor and adsorbent addition)	Pu et al. (2013)
Elicitation (10-Hydroxy camptothecin)	*Xylaria sp.*	Ce^{3+}, Cr^{3+}, La^{3+}, MJ, SA, Cu^{2+}, Fe^{2+}, Se^{5+}, Mn^{2+}, Ca^{2+}, Li^{+}	2.7 folds (0.1 mM SA) – except MJ all elicitors had positive effect	Liu et al. (2010)
Precursor feeding	*T. atroviride* LY357	Tryptamine Secologanin	Positive Negative	Pu et al. (2013)
Adsorbent resins	*T. atroviride* LY357	HP20 XAD16	11 folds 4.4 folds	Pu et al. (2013)