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Production of camptothecine using whey by an endophytic fungus: standardization using response surface methodology

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Abstract

Fusarium oxysporum kolhapuriensis, a novel endophytic fungi isolated from *Nothapodytes nimmoniana* Mabb. Grahm, was found to produce camptothecine (CPT) using whey as complex medium. Highest production of CPT was attained using statistical methods -Response Surface Methodology (RSM). Central Composite Design (CCD) was used to optimize the complex medium and culture conditions for maximum production of CPT by the fungus. The optimized medium that yielded 283 ± 0.27 mg l^{-1} of CPT contained 70% (v/v) of acid whey and 2% (w/v) malt extract. The other two culture parameters optimized through RSM were temperature (30ºC) and period of incubation (6 days). The production of CPT was confirmed by analytical techniques such as HPTLC, HPLC and LC–HRMS. This cost effective optimized medium using RSM might be useful for the large scale CPT production which will ultimately reduce the further downstream processing cost.

Keywords

Camptothecine, endophyte, *Fusarium oxysporum*, *Nothapodytes nimmoniana*, whey

1. Introduction

Camptothecine (CPT) is a potent anti-cancer quinoline alkaloid, first isolated from the wood of a Chinese tree *Camptotheca acuminata* Decaisne (Family Nyssaceae) [1]. Higher contents of CPT were reported from the plant endemic to the Western Ghats in India, *Nothapodytes nimmoniana* Mabb. Grahm, belonging to family Icacinaceae (formerly known as *N. foetida* (Wights) Sleumer) [2]. CPT and its water soluble derivatives are effective anti-tumor drugs used world-wide due to its inhibitory action to DNA topoisomerase I [3]. In the present scenario, majority of the supply of CPT comes from direct harvesting of the tree bark which has led the elite species to become endangered [4]. Chemical synthesis methods involve extreme conditions which increases the overall cost of the process. Also the supply is insufficient to meet the current market demand of the potent anti-cancer alkaloid. Alternative sources such as endophytic microorganisms isolated from the same plant sources are exploited for production of identical drugs. Endophytes are microorganisms inhabiting living tissues of plant without causing any apparent disease or harm to the host [5]. Currently many research communities are focusing on bioprospecting of endophytes for medicinally important secondary metabolites.

Camptothecine production by different endophytic fungi isolated from different CPT producing plants has been reported previously [6, 7, 8, 9]. These reports strongly support use of endophytic fungi as potent producers of CPT and ultimately curb the extensive harvesting of the natural plant populations. Besides isolation, identification and characterization of the endophytic fungi, optimization of medium for optimum CPT synthesis is also important. Optimization of media and culture conditions for optimum yield of the product involves huge efforts and time but is essential to reduce the cost of process while increasing the product yield. Classical optimization methods involve “one variable at a time” approach where effect of change in one

variable on product yield is determined while keeping the rest of the variables constant. This approach is laborious, time consuming and does not facilitate study of interactive effects among the influencing factors. Recently, optimum substrate concentrations for CPT production by an endophytic *Fusarium* spp. using response surface methodology (RSM) was reported [10]. Statistical methods have gained much attention and importance as they allow interactive studies between different variable parameters at a time. RSM is a combination of statistical and mathematical techniques used for developing, improving and optimizing the process. It can also be used to determine and evaluate several factors affecting the process and their relative significance, even in presence of complex interactions, in a limited number of experiments [11, 12].

Whey - an abundant dairy waste - was used as a medium for CPT production for the first time in the present study. The greenish liquid remaining after milk has been curdled and strained is called whey which has several commercial uses [13]. Sweet whey is the by-product of manufacture of rennet induced hard cheese like cheddar or Swiss cheese while acid whey (or sour whey) is produced during the making of acid coagulated cottage cheese or strained yogurt. Acid whey was used as a raw medium for growth and production of CPT by the endophytic fungus. In the present study, endophytic fungus have been isolated from the leaf and stem segments of the endangered plant *N. nimmoniana*, identified and detected to produce significantly high amounts of CPT. Efforts were made to optimize the media and process conditions for the maximum possible CPT production from the endophytic fungus *Fusarium oxysporum* kolhapuriensis using RSM. Also, effects of independent variables affecting CPT production, alone and in combination with the process were studied. Using the experimental

methodology a mathematical model was developed that describes the biochemical process for CPT production.

2. Materials and methods

2.1 Chemicals

Standard CPT was purchased from Sigma–Aldrich (St. Louis, MO, USA), and remaining chemicals were obtained from Hi-media (India). All chemicals used were of highest purity and of analytical grade.

2.2 Collection of plant material

Plant material was collected from Dajipur forest areas near Kolhapur district, Maharashtra, India. It was identified and authenticated by expertise from Department of Botany, Shivaji University, Kolhapur, India. Fresh and healthy leaf and stem segments of *N. nimmoniana* plant were collected and stored in dry clean polythene bags at 4ºC for further use.

2.3 Isolation of Endophytic fungi

Leaf and stem segments were washed under running tap water for 10 min. This was followed by treatment with 70% ethanol for 1 min and then with Mercuric chloride solution (0.1%) for 2 min, rinsed with sterile distilled water thrice after every treatment. Each plant sample was cut aseptically into 1-2 cm long segments and placed on petri-dishes containing potato dextrose agar (PDA) and Sabouraud's agar (SBA) and incubated at 25°C±2°C. The petri dishes were monitored every day to check the growth of endophytic microbial colonies from the plant segments. After 2-3 days several fungal and few bacterial colonies emerged out from the plant segments, they were isolated and sub-cultured to obtain pure culture by serial sub-culturing. Negative control were maintained using the same procedure without surface sterilization of the

plant samples to prevent false positives from contamination by other microorganisms. Morphological and colony behavior of the isolates were studied. Preliminary identification was carried out using standard staining methods of microscopy. The culture was maintained on SBA slants and stored at 4ºC.

2.4 Identification of Endophytic Fungi

Pure isolates were selected further to check their ability to produce CPT, if any. The most proficient microbial isolate, a fungus, was selected from this screening experiment and used for further studies. Further identification of the endophytic fungus was done based on molecular characterization. The genomic DNA was extracted from the mycelium using modified CTAB method and the flanking ITS regions intervening the 5.8s rDNA and large subunit of rRNA was amplified using universal primers ITS 3 (5′GCATCGATGAAGAACGCAGC3′) and ITS4 (5′TCCTCCGCTTATTGATATGC3′) (GeNei^{TM}, Bengaluru, India). The isolated fungus was identified as *Fusarium oxysporum* kolhapuriensis and nucleotide sequence data was submitted to Genbank. It was subjected to nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and resultant homologous sequences of species were used for phylogenetic analysis. The phylogenetic tree (Neighbor joining method) was constructed with MEGA5.2 software using Jukes-Cantor model at 1000 bootstrap replications (AZ, USA).

2.5 Classical optimization

Parameters for best-suited media and culture conditions–synthetic media, complex supplements like whey, yeast extract, beef extract, malt extract, pH, temperature of incubation, agitation rate (revolution per minute) and incubation period were checked for maximum production of CPT individually. Initial fermentations were carried out at 30ºC for 8 days under shake flask conditions at 110 rpm. Four most influencing factors – whey, malt extract, incubation

period and temperature were further considered for optimization using RSM. The spore suspension was prepared as described earlier [10] and 1 ml of spore suspension was used as inoculum for fermentation in all experiments.

2.6 Experimental design: CCD

In order to determine the optimum levels of significant variables for CPT production, Response Surface Methodology (RSM) using central composite design (CCD) was adopted for augmentation of CPT production by the endophytic fungus. CCD has three groups of design points: two-level factorial or fractional-factorial design points, axial points (sometimes called 'star' points) and center points. CCDs are designed to estimate the coefficients of a quadratic model [14]. A 2^4 factorial central composite experimental design with eight start points ($\alpha = 2$) and six replicates at the central point, which results in 30 experiments, was used to optimize the screened variables grouped as temperature (A), whey (B), incubation period (C), and malt extract (D). Each of the four significant variables was assessed at five coded levels (-2, -1, 0, +1, and +2) and is shown in Table 2, while the detailed experimental design is shown in Table 3. The variables were coded according to the equation:

$$x_i = (X_i - X_0)/ \Delta X_i$$

Where, x_i is the independent variable coded value, X_i the independent variable real value, X_0 the independent variable real value at the center point and ΔX_i the step change value. Statistical analysis and graphs were plotted using Design-Expert software (Trial Version 9.0.4.1, Stat-Ease, Inc, USA). Whey concentrations were varied in the range of 50-90% (v/v), temperature from 20-40 ºC, malt extract 1-3 % (w/v) and incubation period from 4-8 days.

2.7 Extraction of CPT

Filtration was used to separate mycelial biomass and broth. Mycelial biomass was washed thoroughly with distilled water and homogenised in 2 ml methanol. Ultra-sound assisted extraction of CPT from the fungal cultures was performed using a sonication probe (Vibra cell by Sonics and Materials Inc., USA) at 20 kHz at room temperature. Sonicated samples and cell-free broths were further extracted using chloroform and methanol (4:1, v/v). The organic phase was evaporated to dryness and the residue was collected in 1 ml HPLC grade methanol and used for further analysis.

2.8 Analysis of CPT

2.8.1 High Performance Thin Layer Chromatography (HPTLC)

Detection of CPT in the extracted samples was performed using TLC and HPTLC system (CAMAG, Switzerland). Aliquots of standard CPT (100 mg l^{-1}) and the extracted samples, before and after incubation were loaded using Linomat 5 applicator (Camag, Switzerland). The silica gel 60 F_{254}s plate (Merck, Germany) was developed in a pre-saturated TLC chamber (Camag, Switzerland) using solvent system chloroform/ethyl acetate (1:1, v/v). After development, the chromatograms obtained were scanned out using TLC scanner. The results were analyzed using HPTLC Win CATS 1.4.4.6337 software at 254 nm.

2.8.2 High Performance Liquid Chromatography (HPLC)

HPLC was performed to quantify the amount of CPT produced. HPLC was performed on DGU-20A 5R (Shimadzu, Japan) with C-18 column (5 µm x250 mm x 4.6 mm, Enable, Spincotech Pvt. Ltd., Japan) using mobile phase methanol/water (3:2, v/v) at a flow rate of 0.7 ml min^{-1}. The CPT produced by extracted samples was detected using a PDA detector (SPD-M 20 A, Shimadzu, Japan) in dual mode with 1.2 slit width and the chromatograms were extracted at 254 nm. Quantification of the CPT produced was done by spiking the calibration

curve with different concentrations of standard CPT (Sigma-Aldrich) and the validation was determined by performing 5 replicates of each sample. Data acquisition and post analysis were performed using LC solution software (Shimadzu, Japan).

2.8.3 Liquid chromatography – High Resolution Mass spectroscopy

LC-HRMS was performed using Dual AJS ESI ion source on Quadrupole Time of Flight (QTOF) Mass Spectrometer Model-G6540B (Agilent Technologies). Selected reaction monitoring (SRM) was performed using a highly sensitive TSQ quantum ultra AM mass spectrometer (Thermo, Finnigan) equipped with an ESI ion source (Ion Max) operating in positive mode. Nitrogen was employed as both the drying and nebulizer gas. The source parameters used were: Sheath Gas Flow 11, Sheath Gas Temp 300°C, Nebulizer pressure 35 psi, gas flow rate - 10 l/min. Scan source parameters: Octopole RF Peak 750 V, Skimmer1- 65 V, Fragmentors 175 V, Nozzle Voltage 1000 V, VCap 3500 V with positive ion mode. Reference masses (positive) obtained were 922.00979800 and 121.05087300, chromatogram type – TIC with solvent composition – Pump A: 20% water and Pump B: 80% Acetonitrile, signal wavelength at 254 nm, bandwidth 4 nm and mass range from 100-3000 m/z.

2.8.4 CPT production over successive generations

Subculturing of the CPT-producing endophytic fungus was carried out to check the potential of the fungus to produce CPT over successive generations. The pure culture of the fungus was subcultured from the first generation to its eighth generation following the method described by [15]. The cultures of each generation were grown using the optimized culture conditions, extracted, and analyzed for CPT production using the same procedures mentioned above.

3. Results

3.1 Identification of endophytic fungus

Molecular characterization of the isolated fungi was carried out using ITS genotyping technique. ITS rDNA regions generate high nucleotide sequence variations which allow convenient distinction at species and strain level [16]. CPT producing pure colony of the endophytic fungus was isolated and identified as *Fusarium oxysporum* kolhapuriensis (sequence submitted to GenBank accession no. KR259541). The sequence analysis data shows 99.9% identity with genus *Fusarium* and species *oxysporum*. The phylogenetic position of *Fusarium oxysporum* kolhapuriensis in relation to other species of the genus is as depicted in fig. 1.

3.2 Screening of variables by classical method

Classical optimization method was used to study the effect of individual factor of media and culture conditions on CPT production by the fungus. Sabouraud's broth showed high biomass generation and CPT production followed by whey as a sole complex/undefined medium. Temperature of incubation best suited for growth and product formation was found to be 30ºC with incubation period of 6 days and shaker rotation speed at 110 rpm. The variable pH did not show much significant effect on yield but optimum range was around 4-6 pH units. The most influencing factors found to have major impact on CPT production by the fungus were whey, malt extract, incubation period and temperature. These variables were further considered for optimization by RSM experiments to study their interactive effect on CPT production.

3.3 Analysis of CPT

The presence of CPT in the fermentation broths were detected by TLC and HPTLC methods. The HPLC profile showed a sharp peak of standard CPT at a retention time of 9 min. Quantification of the drug was facilitated by HPLC technique using linear calibration curve obtained by a range of different concentrations of the standard compound from Sigma Aldrich

(USA). The broth devoid of fungal inoculum did not show presence of any such compounds. Structural confirmation was provided by the LC-HRMS report. The retention times (1.258 min) and the *m/z* peaks for samples containing CPT were matching to those of the standard compound. Fragmentation pattern of CPT molecular ion $(M+H)^+$ yielded characteristic peak at *m/z* 349.11851. LC-HRMS and MS2 studies reveal the comparable structural identity of the product to that of pure compound and also provides insight into the application of the test compound as a potent and easily available cheaper source of the anti-cancer pro-drug. Fig. 3 depicts the MS2 fragmentation pattern as well as the *m/z* peaks of the TLC extracted compound present in the fungal extract.

3.4 Optimization of medium

From the CCD, experiment of 30 runs was carried out to optimize medium composition and the results were presented in Table 2. By applying multiple regression analysis on the experimental coded data, a second-order polynomial equation for CPT (Y) was obtained as follows:

$$CPT = 283.2 - 5.1625A - 3.67917B - 0.39583C + 1.795833D - 4.03125AB + 7.75625AC + 5.78125AD - 3.93125BC - 5.23125BD + 3.95625CD - 68.224A^2 - 69.1865B^2 - 70.899C^2 - 57.8115D^2$$

Where, Y represents CPT yield (mg l^{-1}), A, B, C and D are coded values of temperature (A), whey (B), incubation time (C), and malt extract (D). The equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. By default, the high levels of the factors are coded as +1 and the low levels of the factors are coded as -1. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients. The capability of the model was checked using ANOVA which was tested using

Fisher's statistical analysis, and the results are showed in Table 3. ANOVA showed that the regression model was statistically good with a lack of fit value of 3.16 (P> 0.05) and an F value of 22705.73 (Table 3). The calculated R^2 value of 0.9999 for CPT production shows improved correlation between the observed and predicted response. Adequate Precision is a statistical measure that quantitates the signal-to-noise ratio, and a ratio greater than value 4 can be anticipated [17]. The ''Adeq Precision'' ratio of 384.9191 obtained in this study indicates an adequate signal. Thus, this model can be used to navigate the design space.

3.5 Interaction of variables

The contour plots and their shapes, circular or elliptical, elucidate the interactions between the variables under study. A circular contour plot indicates that the mutual interactions between corresponding variables are insignificant, whereas the significant interactions between corresponding variables are represented by an elliptical nature of the contour plot [18, 19]. The 3D response surface plots and their respective 2D contour plots generated by the software allow simple and convenient understanding of the interactions between two variables and also to determine their optimum levels.

To observe the interactive effects of variables for CPT production, the response surface and contour plots were generated as graphical representations of the regression equation. The three-dimensional response surface and their corresponding contour plots were obtained for CPT production against any two independent variables by keeping the other independent variable at zero level. The interaction between two variables at a time was shown in Fig. 2-(a) to (f). As the counter plots obtained were elliptical in nature, each graph represents a significant interaction between two variables affecting CPT production. As the concentration of one factor increases to the optimum level, CPT yield also increases up to the maximum. The middle values of variables

under study showed increased CPT level while at the higher and lower ranges decreased CPT levels were obtained. This trend was observed almost similarly in other variables too which represents that every individual factor A, B, C and D have independent effect on CPT production.

3.6 Validation of model

Validation of the model was carried out under conditions predicted by the software. A good correlation can be seen between the experimental and the predicted values, and hence, the model was successfully validated. Validation of the statistical model and regression equation was performed by taking optimum values of temperature (30ºC), whey (70%), incubation period (6 days) and malt extract (2%) in the experiment. In this reaction mixture, the predicted-CPT yield was 283.033 mg l^{-1}, while the experimental yield was found to be 284 mg l^{-1}. The effective concentration of four influencing factors for optimum production of CPT was determined using validated model given by CCD method of RSM.

3.7 CPT production over successive generations

The pattern of CPT production by the endophytic fungus through subsequent generations was studied. CPT was extracted and detected using HPTLC and HPLC techniques. A considerable decrease in the CPT concentration produced by the successive generations of the fungus was observed (Table 4). First generation fungal culture yielded up to 283.2 mg l^{-1} CPT using the optimized operating conditions while second generation subculture grown under same conditions produced ~198 mg l^{-1} CPT which was attenuated to the lowest level ~33 μg l^{-1} CPT in the eighth generation.

4. Discussion

Most of the CPT is obtained either from natural sources or synthesized chemically, but only few reports are available on production of CPT using endophytic fungal strains. Among microbial sources, *Fusarium oxysporum* kolhapuriensis has capacity to produce CPT using whey as a complex medium and showed the possibility of being used as a commercial source for large scale industrial production. High production cost and high commercial value of CPT has given rise to an intensive research for cheaper production methods for this anti-cancer drug precursor. With the view of utilizing the attribute of the endophyte to commercially produce CPT attempts were made to optimize the media and culture conditions for the same. RSM was successfully used to study and figure out the most influencing factors for CPT production by the fungus and their optimum levels for process operation.

Fungal endophytes from a vastly distributed population of vascular plants hold crucial stance in the biosynthesis of medicinally important secondary metabolites. The synthesis of secondary metabolites in plants and fungi is a combined effect of inducing factors from both host and endophyte, respectively [20, 21]. These endophytic fungi harbor similar but distinct biosynthetic pathway as the host plant for production of the secondary metabolites [22]. The high market value of the anti-cancer drug CPT is a consequence of the higher processing cost and/or limited natural resources. Thus it is essential to reduce the cost of processing which can be achieved through biotechnological approaches. The present study was such an attempt wherein expensive or valuable product was synthesized using cheaper substrates by a biological source-endophytic fungi. Cheese-making industries consider whey as a waste product and are interested in disposing it of in most economical ways such as discharge in water bodies, spraying onto farm lands or application as animal feed. Whey is a rich medium with common applications in lactic acid production, bakery products, beverages, as human protein supplements and animal feed. It

contains about 93% water, 5-6% lactose and 1-1.8% proteins while vitamins and fats in trace amounts. Whey is in itself a carbohydrate and protein reservoir that makes it a suitable raw medisum with complex nutritional stock for growth of multiple microorganisms [23]. The composition of whey varies depending upon its source and collection conditions but in general whey contains branched chain amino acids viz. leucine, isoleucine and valine in abundance. Also methionine and cysteine are found to be majorly present in whey which are considered to be vital amino acids for overall growth and repair of the cells [24]. Earlier studies on precursor feeding experiments report that leucine and tryptophan significantly contribute to the ring structures of CPT [25]. These reports suggest the probable effect of whey proteins on CPT synthesis by the endophyte. The endophytic fungi showed optimum growth and CPT yield in 70% whey whereas higher and lower dilutions decreased the product yield. These results suggest the probable role of complex nutritional components of whey in supporting growth and secondary metabolite production by the fungus.

Temperature also played a dominating role in the fermentation process as illustrated, CPT yield reaches maximum at 30ºC while decreases as the temperature shifts from moderate to higher or lower ranges (Fig.2-a,b,c). This indicates that temperature of incubation is an essential factor responsible for optimum growth and following product formation by the fungus under study. Malt extract served as the carbohydrate supply for the increase in mycelial backbone growth and corresponding CPT yield as shown in Fig.2-c,e,f. The elliptical nature of the graph signifies the independent impact of both malt and whey as complex media for CPT production. 2% of malt extract was observed as the optimum concentration for CPT production by the fungus. Malt extract powder is prepared by drying the aqueous extract of sprouted malt grains at low temperature that allows preservation of nutrients present in the form of carbohydrates and

nitrogenous substances (Hi-Media Laboratories-Technical Data). It is considered as a suitable medium supplement for cultivation of fungi and has been found to boost growth associated CPT yield in the present study too. Incubation period also affected the yield as number of days of incubation decreased below 6, the CPT production must not be complete and therefore was not significantly detectable. While above 6 days, the stable production of CPT was detected in lower concentrations due to its conversion into other metabolites. Thus, selecting incubation period of 6 days facilitates desired optimum CPT yield as well as economy of the process. CPT was not detected at all in un-inoculated broth and in the day-zero sample of the fermentation process. This depicts that the endophytic fungus alone is responsible for production of CPT in the provided culture medium.

A maximum CPT yield of 283 ± 0.27 mg l^{-1} was detected as the optimum value produced by the fungus using whey (70%, v/v) and malt extract powder (2%, w/v) as media with incubation parameters: temperature 30ºC and incubation period of 6 days. The results are mean of six experiments. This yield is 1000 times enhanced in comparison to that reported earlier (250 ± 20 µg l^{-1} in broth) by an endophytic fungus *Entrophospora infrequens* isolated from *N. foetida* [7]. A maximum CPT yield of 5.5 mg l^{-1} has been reported from an endophytic fungus belonging to *Neurospora spp.* isolated from the same plant *N. foetida* [8]. CPT yields up to 197.82 µg l^{-1} were reported from *Trichoderma atroviridae*-a fungus isolated from Chinese tree *Camptotheca acuminata* [26]. The yields of CPT from two endophytic fungal isolates of *Fusarium solani* were 37 and 53 µg 100 g^{-1} dry cell mass, respectively, which were isolated from *Apodytes dimidiata* [9]. Whereas **t**he same group has also reported presence of CPT and its derivatives from **e**ndophytic fungi isolated from *Miquelia dentata* Bedd. [27]. More recently, 9.7 µg l^{-1} of CPT has been reported from an endophytic fungus *Fusarium oxysporum* NFX06 isolated from

Nothapodytes foetida using RSM [10]. The present study reports for the first time utilization of whey to produce camptothecine in higher amounts till date.

The attenuation observed in case of CPT production through successive generations of the fungus was also reported by previous researchers in case of endophytic fungi producing pharmaceutically important secondary metabolites [28, 29]. Attenuation trend can be attributed to possibilities such as lack of necessary precursors or due to incomplete or altered transcription mechanism upon subsequent generation. Further insights regarding the metabolic and transcription analysis is required to reveal the vital factors underlying the attenuation trend relative to the biomass production of the secondary metabolite [9].

5. Conclusion

Endophytic fungus *Fusarium oxysporum* kolhapuriensis isolated from the endangered plant *N. nimmoniana* was found to exhibit potent productivity for the anti-cancer drug precursor molecule CPT. Dairy waste product-whey was supplied as a complex medium to the fungus for production of CPT. The highest possible concentration of CPT yield obtained was 283.2±0.27 mg l^{-1} using optimum conditions and concentrations of: whey-70%, malt extract powder-2%, temperature-30ºC and incubation period-6 days. Application of easily available and cheaper substrate for production of a highly expensive drug precursor can be achieved. Optimized medium and parameters will lead to the cost effective and industrially feasible production of CPT.

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Legends to figures

Fig 1. Phylogenetic tree based on ITS rDNA sequence of *F. oxysporum* kolhapuriensis and its closest ITS rDNA matches in the GenBank. Values on the nodes indicate percent bootstrap confidence.

Fig 2-(a). Three-dimensional response surface plot of interactions of variables Whey and Temperature and their effect on CPT production

Fig 2-(b). Three-dimensional response surface plot of interactions of variables Temperature and Incubation period (Time) and their effect on CPT production

Fig 2-(c). Three-dimensional response surface plot of interactions of variables Temperature and Malt extract and their effect on CPT production

Fig 2-(d). Three-dimensional response surface plot of interactions of variables Incubation period (Time) and Whey and their effect on CPT production

Fig 2-(e). Three-dimensional response surface plot of interactions of variables Malt extract and Whey and their effect on CPT production

Fig 2-(f). Three-dimensional response surface plot of interactions of variables Malt extract and Incubation period (Time) their effect on CPT production

Fig 3. Liquid Chromatography – High Resolution Mass Spectroscopy analysis of TLC extracted sample from endophytic fungus *Fusarium oxysporum* kolhapuriensis.

449 **Table 1** Experimental range and levels of the variables for response surface methodological
450 experiments

Variable		Level				
Code	Name	Range				
		-2	**-1**	**0**	**1**	**2**
A	Temperature (ºC)	20	25	30	35	40
B	Whey (%)	50	60	70	80	90
C	Incubation Period (days)	4	5	6	7	8
D	Malt extract (%)	1	1.5	2	2.5	3

451

452

453 **Table 2** Experimental design of RSM studies by using four variables with six center points
454 showing observed and predicted value for CPT production

Std. Order	A	B	C	D	CPT (µg/l) Experimental	Predicted
1	25	60	5	1.5	28	28.82
2	35	60	5	1.5	0	-0.5166
3	25	80	5	1.5	48	47.85
4	35	80	5	1.5	1.9	2.3875
5	25	60	7	1.5	12.1	12.466
6	35	60	7	1.5	13.9	14.154
7	25	80	7	1.5	15	15.77
8	35	80	7	1.5	1	1.33
9	25	60	5	2.5	24.2	23.4
10	35	60	5	2.5	16.2	17.187
11	25	80	5	2.5	20	21.5041
12	35	80	5	2.5	0	-0.833
13	25	60	7	2.5	21.6	22.97
14	35	60	7	2.5	48	47.683
15	25	80	7	2.5	5.2	5.25
16	35	80	7	2.5	13	13.937
17	20	70	6	2	21.9	20.629
18	40	70	6	2	0	-0.02
19	30	50	6	2	14.2	13.8125
20	30	90	6	2	0	-0.9041
21	30	70	4	2	0.5	0.3958
22	30	70	8	2	0	-1.187
23	30	70	6	1	48.9	48.3625
24	30	70	6	3	56.3	55.545
25	30	70	6	2	282.2	283.2
26	30	70	6	2	282.7	283.2

27	30	70	6	2	283.1	283.2
28	30	70	6	2	283.5	283.2
29	30	70	6	2	284	283.2
30	30	70	6	2	283.7	283.2

455

456

Table 3 Analysis of variance for quadratic model for CPT production as provided by Design-Expert software (trial version 9.0.4.1)

Source	Sum of Squares	Degree of freedom	Mean Square	F Value	p-value Prob > F
Model	347036.9	14	24788.35	22705.73	8.17E-30
Residual	16.37583	15	1.091722		
Lack of Fit	14.13583	10	1.413583	3.15532	0.108294
Pure Error	2.24	5	0.448		
Total	347053.2	29			

462 **Table 4** CPT production over successive generations

Subculture generation	**CPT (mg l^{-1})***
First	283 ± 0.27
Second	198 ± 0.12
Third	102 ± 0.87
Fourth	46 ± 0.54
Fifth	0.138 ± 0.24
Sixth	0.260 ± 0.12
Seventh	0.056 ± 0.18
Eighth	0.033 ± 0.16

463 *Values of CPT are mean of five replications; S.E. calculated by GraphPad InStat3 software

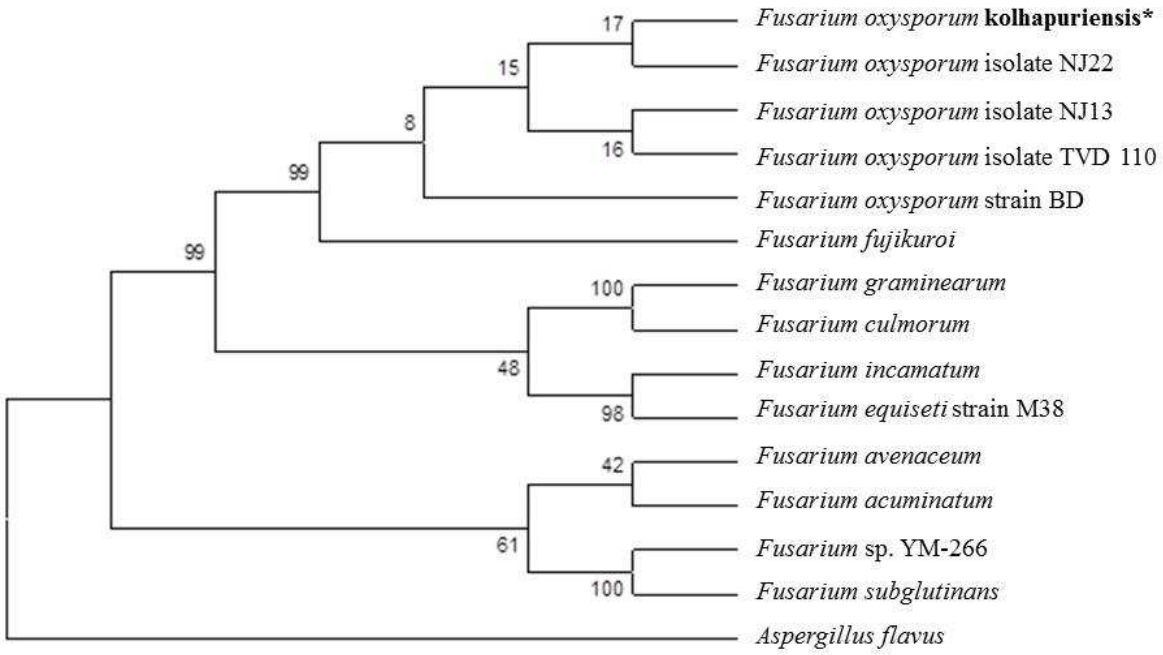
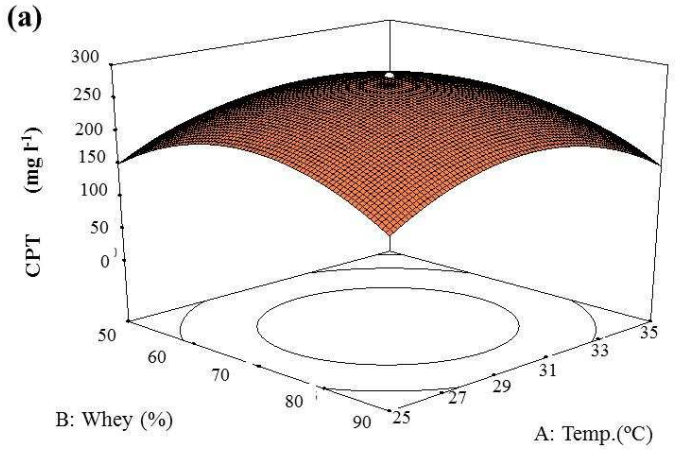
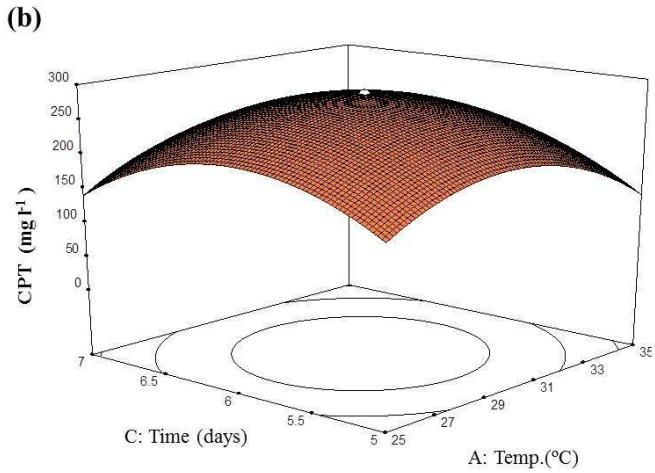
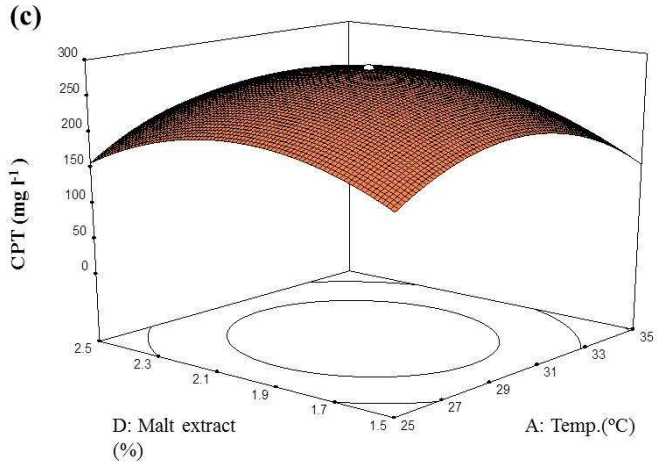

17
15
8
16
99
99
100
48
98
42
61
100
Fusarium oxysporum **kolhapuriensis***
Fusarium oxysporum isolate NJ22
Fusarium oxysporum isolate NJ13
Fusarium oxysporum isolate TVD 110
Fusarium oxysporum strain BD
Fusarium fujikuroi
Fusarium graminearum
Fusarium culmorum
Fusarium incamatum
Fusarium equiseti strain M38
Fusarium avenaceum
Fusarium acuminatum
Fusarium sp. YM-266
Fusarium subglutinans
Aspergillus flavus

Fig. 1.


(a)
CPT (mg l-1)
300
250
200
150
100
50
0
50
60
70
80
90
B: Whey (%)
25
27
29
31
33
35
A: Temp.(°C)


(b)
CPT (mg l-1)
300
250
200
150
100
50
0
7
6.5
6
5.5
5
C: Time (days)
25
27
29
31
33
35
A: Temp.(°C)


(c)
CPT (mg l-1)
300
250
200
150
100
50
0
2.5
2.3
2.1
1.9
1.7
1.5
D: Malt extract (%)
25
27
29
31
33
35
A: Temp.(°C)

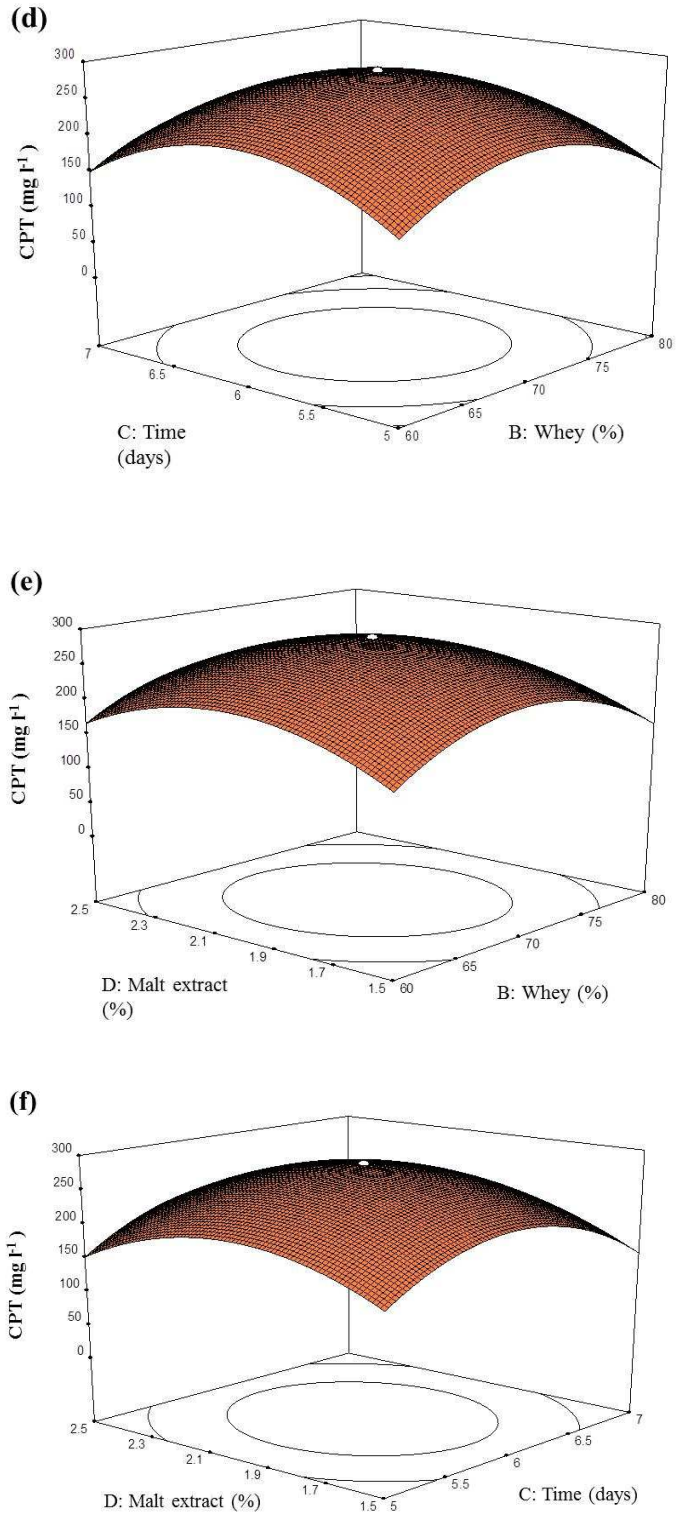

(d)
CPT (mg l-1)
300
250
200
150
100
50
0
7
6.5
6
5.5
5
C: Time (days)
60
65
70
75
80
B: Whey (%)
(e)
CPT (mg l-1)
300
250
200
150
100
50
0
2.5
2.3
2.1
1.9
1.7
1.5
D: Malt extract (%)
60
65
70
75
80
B: Whey (%)
(f)
CPT (mg l-1)
300
250
200
150
100
50
0
2.5
2.3
2.1
1.9
1.7
1.5
D: Malt extract (%)
5
5.5
6
6.5
7
C: Time (days)

Fig. 2. (a) to (f)

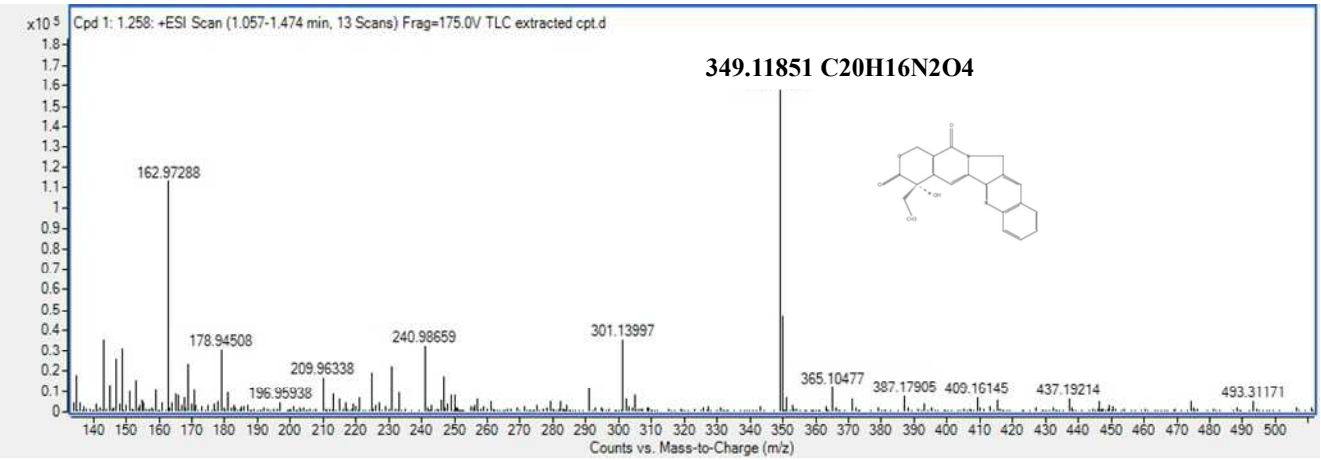

x10 5
Cpd 1: 1.258: +ESI Scan (1.057-1.474 min, 13 Scans) Frag=175.0V TLC extracted cpt.d
349.11851 C20H16N2O4
162.97288
178.94508
196.95938
209.96338
240.98659
301.13997
365.10477
387.17905
409.16145
437.19214
493.31171
Counts vs. Mass-to-Charge (m/z)

Fig. 3.