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(54) Title: SUSTAINABLE PRODUCTION OF CAMPTOTHECIN IN THE SUSPENSION CULTURE OF THE ENDOPHYTE FROM *NOTHAPODYTES NIMMONIANA*

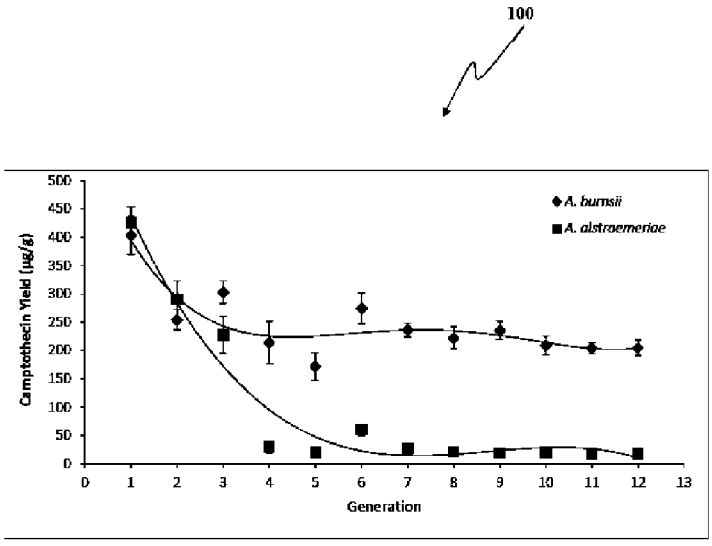


FIG 1

(57) Abstract: Sustainable production of camptothecin in the suspension culture of the endophyte from *Nothapodytes nimmoniana*, is disclosed. A novel high yielding and sustainable, camptothecin producing endophytes (*A. alstroemeriae(NCIM 1408)* and *A. burnsii(NCIM 1409)*)and process for producing camptothecin from the said endophytes from *Nothapodytes nimmoniana*. The endophyte *A. burnsii(NCIM 1409)* is a high yield and sustainable, camptothecin producing endophyte (fungal strain) forming while colony mycelium which turns black during sporulation and forms aerial hyphae. The strain was able to produce 150-200 µg/g DW biomass or 1.5-3 mg/L of CPT titer. The highest yielding endophyte (*A. alstroemeriae (NCIM 1408*) demonstrates a high yield of camptothecin up to 300-400 µg/g DW biomass.

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[Continued on next page]

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— *as to the identity of the inventor (Rule 4.17(i))*
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SUSTAINABLE PRODUCTION OF CAMPTOTHECIN IN THE SUSPENSION CULTURE OF THE ENDOPHYTE FROM *NOTHAPODYTES NIMMONIANA*

TECHNICAL FIELD

[0001] Embodiments are generally related to field of biotechnology. Embodiments are further related to endophytes producing camptothecin. Embodiments are also related to processes for isolation of camptothecin producing endophytes. Embodiments are particularly related to a novel high yielding and sustainable, camptothecin producing endophytes (*A. alstroemeriae (high yield) and A. burnsii*(high yield and sustainable))and process for producing camptothecin from the said endophytes isolated from *Nothapodytes nimmoniana*.

BACKGROUND OF THE INVENTION

[0002] Metabolites produced by plants are of high significance owing to their therapeutic applications in humans. Plants which produce such metabolites are overharvested to meet the increasing demand for metabolites. Endophytes, the microorganisms that reside within the tissues of plants are reported to have the ability to produce their host specific metabolites (Stierle et al., 1993). Scientists around the globe are trying to explore if these endophytes can be used to produce the associated plant metabolites on a commercial scale. However, the limitation of

product yield attenuation with subsequent subculture (generations) of endophytes in axenic conditions is one of the major bottlenecks in their commercial exploitation (Kusari et al., 2014).

[0003] Camptothecin is one of such metabolites produced majorly from two plants, *Camptotheca accuminata and Nothapodytes nimmoniana*. Camptothecin is the third most sought after alkaloid in the globe for their anti-cancer activity (Panneerselvam et al. 2004).

[0004] Camptothecin is a plant derived alkaloid which is of high demand around the world for its anti-cancer activity. Pants producing camptothecin were exploited in large number to meet the demand. Therefore, to prevent such plants from getting extinct and to meet the demand for camptothecin, an alternate method of production is highly required. Endophytes, the microorganisms residing within plants have sometimes demonstated the ability to produce camptothecin, but the problem of product attenuation under axenic conditions remain a drawback for them. The prior art approaches do not discuss on any sustainable camptothecin producing endophytes and processes for producing camptothecin from such endophytes which will limit the dependency for producing the camptothecin directly from the plant source.

[0005] Based on the foregoing a need therefore exists for a high-yielding endophyte from *Nothapodytes nimmoniana* for maximum and sustainable

production of camptothecin in suspension culture using bioprocess optimization, as discussed in greater detail herein.

SUMMARY OF THE INVENTION

[0006] The following summary is provided to facilitate an understanding of some of the innovative features unique to the disclosed embodiment and is not intended to be a full description. A full appreciation of the various aspects of the embodiments disclosed herein can be gained by taking the entire specification, claims, drawings, and abstract as a whole.

[0007] One aspect of the disclosed embodiments is to provide an high yielding and sustainable, camptothecin producing endophytes.

[0008] Another aspect of the disclosed embodiments is to provide high camptothecin yielding endophytes from *Nothapodytes nimmoniana.*

[0009] Further aspect of the disclosed embodiments is to provide an optimized bioprocess for high and sustainable production of camptothecin from the endophytes of *Nothapodytes nimmoniana.*

[0010] The aforementioned aspects and other objectives and advantages can now be achieved as described herein. A novel high yielding and sustainable, camptothecin producing endophytes (*A. alstroemeriae(NCIM 1408)* and *A. burnsii*(*NCIM 1409*)) and process for producing camptothecin from the said endophytes of *Nothapodytes nimmoniana.* The endophyte *A. burnsii*(*NCIM 1409*) is

a high yielding and sustainable, camptothecin producing endophyte (fungal strain) forming while colony mycelium, which turns black during sporulation and forms aerial hyphae. The strain was able to produce 150-200 µg/g DW biomass of camptothecin and 1.5-3 mg/L of camptothecin titer in suspension. The highest camptothecin yielding endophyte (*A. alstroemeriae (NCIM 1408)* demonstrates 300-400 µg/g DW biomass of camptothecin yield.

[0011] The camptothecin producing endophytes are isolated from the plant *Nothapodytes nimmoniana.* The Leaves, Petioles, Stem and bark regions were collected from the plant and washed to remove the dust particles in sterile conditions. Surface sterilization was carried out to remove the surface contaminants. Plant parts were treated with 0.5-4% (v/v) sodium hypochlorite for 1-4 min and then washed with sterile water to remove traces of residual sodium hypochlorite. They were then treated with 50-80% (v/v) ethanol for 1-5 min and then rinsed with sterile water to remove ethanol from the plant parts. The sterile explants were cut into small pieces and placed onto Potato dextrose agar medium. The explants were incubated at 25-30 °C for 5-10 days to allow the microorganisms to emerge from the cut ends. Morphologically distinct fungi were removed and plated on separate plates to obtain pure strains.

[0012] Subsequently, the suspension cultures were initiated for isolation of strains for harvesting the biomass. A loop full of the mycelia was streaked on slants made

with 3-6 ml of Potato dextrose agar and incubated at 25-30 °C for 5-10 days with an initial pH of 5-6. The spores and the mycelia from the slants were washed using 3-6 ml of saline (0.5-1% NaCl) and this was used as inoculum for initiating suspensions. Suspensions were made with 30-60 ml of Potato dextrose broth with an initial pH of 5-6 and 1-5% of the culture broth from slants was added as inoculum.

[0013] Fungus was allowed to grow for a period of 5-10 days at 25-30 °C and at 100-150 rpm. After 5-10 days the biomass was harvested by centrifuging at 8000-12000 rpm for 10-15 min and discarding the supernatant. The pelleted cells were again washed with distilled water to remove traces of media and separated by centrifuging at 8000-12000 rpm for 10-15 min. The fungal biomass was dried in hot air oven at 60-70 °C by spreading the cells on glass petri plates until complete dryness.

[0014] Finally, the dried biomass was dissolved in 15-30 ml of distilled water and homogenized using a mortar and pestle, followed by liquid – liquid extraction, repeated thrice with 40-60 ml of chloroform: methanol. The separated organic layer was further removed using a rotary evaporator. The concentrated extract was re-suspended in 0.5-2 ml of DMSO: methanol and analyzed in HPLC after filter sterilization.

BRIEF DESCRIPTION OF DRAWINGS

[0015] The accompanying figures, in which like reference numerals refer to identical or functionally-similar elements throughout the separate views and which are incorporated in and form a part of the specification, further illustrate the present invention and, together with the detailed description of the invention, serve to explain the principles of the present invention.

[0016] FIG. 1 illustrates a graph representing the yields of camptothecin in the high yielding and sustainable, camptothecin producing endophytes from *Nothapodytes nimmoniana* (*A. alstroemeriae(NCIM 1408) and A. burnsii*(*NCIM 1409*)), in accordance with the disclosed embodiments; and

[0017] FIG. 2 illustrates a graphical representation of HPLC chromatogram of the isolated endophytes (*A. alstroemeriae(NCIM 1408) and A. burnsii*(*NCIM 1409*)) against CPT standard, in accordance with the disclosed embodiments.

DETAILED DESCRIPTION

[0018] The particular values and configurations discussed in these non-limiting examples can be varied and are cited merely to illustrate at least one embodiment and are not intended to limit the scope thereof.

[0019] The embodiments now will be described more fully hereinafter with reference to the accompanying drawings, in which illustrative embodiments of the invention are shown. The embodiments disclosed herein can be embodied in many different forms and should not be construed as limited to the embodiments set forth

herein; rather, these embodiments are provided so that this disclosure will be thorough and complete, and will fully convey the scope of the invention to those skilled in the art. Like numbers refer to like elements throughout. As used herein, the term "and/or" includes any and all combinations of one or more of the associated listed items.

[0020] The terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention. As used herein, the singular forms "a", "an" and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise. It will be further understood that the terms "comprises" and/or "comprising," when used in this specification, specify the presence of stated features, integers, steps, operations, elements, and/or components, but do not preclude the presence or addition of one or more other features, integers, steps, operations, elements, components, and/or groups thereof.

[0021] Unless otherwise defined, all terms (including technical and scientific terms) used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. It will be further understood that terms, such as those defined in commonly used dictionaries, should be interpreted as having a meaning that is consistent with their meaning in the context of the relevant art and will not be interpreted in an idealized or overly formal sense unless expressly so defined herein.

[0022] A novel high yielding and sustainable, camptothecin producing endophytes (*A. alstroemeriae(NCIM 1408) and A. burnsii*(*NCIM 1409*))and process for producing maximum camptothecin from the said endophytes from *Nothapodytes nimmoniana.* FIG. 1 illustrates a graph 100 representing the camptothecin yields of high and sustainable, camptothecin producing endophytes (*A. alstroemeriae(NCIM 1408) and A. burnsii*(*NCIM 1409*))from *Nothapodytes nimmoniana*, in accordance with the disclosed embodiments.

[0023] The endophyte *A. burnsii*(*NCIM 1409*) is a high camptothecin yielding and sustainably producing endophyte (fungal strain) forming while colony mycelium which turns black during sporulation and forms aerial hyphae. The strain was able to produce 150-200 µg/g DW biomass or 1.5-3 mg/L titer of CPT in suspension. The highest yielding endophyte (*A. alstroemeriae (NCIM 1408)* demonstrates a camptothecin yield of 300-400 µg/g DW biomass.

[0024] FIG. 2 illustrates a graphical representation 200 of HPLC chromatogram of the isolated endophytes (*A. alstroemeriae(NCIM 1408) and A. burnsii*(*NCIM 1409*)) against CPT standard, in accordance with the disclosed embodiments.The camptothecin producing endophytes are isolated from the plant *Nothapodytes nimmoniana.* The Leaves, Petioles, Stem and bark regions were collected from the plant and washed to remove the dust particles in sterile conditions. Surface sterilization was carried out to remove the surface contaminants. Plant parts were

treated with 0.5-4% (v/v) sodium hypochlorite for 1-4 min and then washed with sterile water to remove traces of residual sodium hypochlorite. They were then treated with 50-80% (v/v) ethanol for 1-5 min and then rinsed with sterile water to remove ethanol from the plant parts. The sterile explants were cut into small pieces and placed onto Potato dextrose agar medium. The explants were incubated at 25-30 °C for 5-10 days to allow the microorganisms to emerge from the cut ends. Morphologically distinct fungi were removed and plated on separate plates to obtain pure strains.

[0025] Subsequently, the suspension cultures were initiated for isolation of strains for harvesting the biomass. A loop full of the mycelia was streaked on slants made with 3-6 ml of Potato dextrose agar and incubated at 25-30 °C for 5-10 days with an initial pH of 5-6. The spores and the mycelia from the slants were washed using 3-6 ml of saline (0.5-1% NaCl) and this was used as inoculum for initiating suspensions. Suspensions were made with 30-60 ml of Potato dextrose broth with an initial pH of 5-6 and 1-5% of the culture broth from slants was added as inoculum.

[0026] Fungus was allowed to grow for a period of 5-10 days at 25-30 °C and at 100-150 rpm. After 5-10 days the biomass was harvested by centrifuging at 8000-12000 rpm for 10-15 min and discarding the supernatant. The pelleted cells were again washed with distilled water to remove traces of media and separated by centrifuging at 8000-12000 rpm for 10-15 min. The fungal biomass was dried in hot

air oven at 60-70 °C by spreading the cells on glass petri plates until complete dryness.

[0027] Finally, the dried biomass was dissolved in 15-30 ml of distilled water and homogenized using a mortar and pestle, followed by liquid – liquid extraction, repeated thrice with 40-60 ml of chloroform: methanol (1:4 to 4:1). The separated organic layer was further removed using a rotary evaporator. The concentrated extract was re-suspended in 0.5-2 ml of DMSO: methanol (1:50 to 50:1) and analysed in HPLC after filter sterilization.

Sample No	Yield (µg/g)	Sample No	Yield (µg/g)	Sample No	Yield (µg/g)	Sample No	Yield (µg/g)	Sample No	Yield (µg/g)	Sample No	Yield (µg/g)
1	ND	**23**	2.75	**45**	1.45	**67**	19.98	**89**	0.88	**111**	1.67
2	11.23	**24**	2.12	**46**	0.28	**68**	3.35	**90**	2.28	**112**	ND
3	3.69	**25**	1.14	**47**	12.32	**69**	0.03	**91**	0.72	**113**	1.50
4	0.48	**26**	7.47	**48**	2.71	**70**	ND	**92**	3.21	**114**	103.73
5	2.21	**27**	ND	**49**	7.71	**71**	0.13	**93**	ND	**115**	7.45
6	4.30	**28**	1.67	**50**	2.39	**72**	ND	**94**	0.96	**116**	ND
7	3.33	**29**	2.46	**51**	ND	**73**	0.45	**95**	0.20	**117**	0.75
8	0.21	**30**	3.62	**52**	2.01	**74**	0.03	**96**	6.45	**118**	ND
9	1.37	**31**	0.67	**53**	ND	**75**	5.41	**97**	1.62	**119**	0.67
10	4.18	**32**	1.56	**54**	4.35	**76**	0.29	**98**	ND	**120**	ND
11	0.25	**33**	4.40	**55**	1.73	**77**	ND	**99**	ND	**121**	53.67
12	0.37	**34**	5.75	**56**	ND	**78**	0.30	**100**	ND	**122**	ND
13	5.57	**35**	ND	**57**	1.08	**79**	ND	**101**	ND	**123**	1.11

14	ND	**36**	4.57	**58**	1.41	**80**	ND	**102**	0.04	**124**	ND
15	0.20	**37**	5.10	**59**	ND	**81**	ND	**103**	0.56	**125**	426.70
16	2.79	**38**	3.91	**60**	0.16	**82**	ND	**104**	0.83	**126**	ND
17	8.99	**39**	0.86	**61**	0.08	**83**	ND	**105**	403.31	**127**	0.83
18	10.89	**40**	ND	**62**	0.20	**84**	ND	**106**	ND	**128**	ND
19	0.44	**41**	0.17	**63**	0.09	**85**	0.57	**107**	ND	**129**	40.95
20	3.88	**42**	ND	**64**	1.16	**86**	ND	**108**	9.40	**130**	1.90
21	4.51	**43**	ND	**65**	0.42	**87**	0.10	**109**	0.81	**131**	ND
22	1.91	**44**	ND	**66**	0.18	**88**	0.51	**110**	85.50	**132**	ND

TABLE 1

[0028] Table 1 discloses the yield of camptothecin from the fungi isolated from *Nothapodytes nimmoniana.*

[0029] It will be appreciated that variations of the above-disclosed and other features and functions, or alternatives thereof, may be desirably combined into many other different systems or applications. Also, that various presently unforeseen or unanticipated alternatives, modifications, variations or improvements therein may be subsequently made by those skilled in the field.

CLAIMS

1. A process for sustainable production of camptothecin in the suspension culture of the endophyte from *Nothapodytes nimmoniana,* wherein said process produces:

a novel high yielding and sustainable, camptothecin producing endophyte, *A. burnsii*(*NCIM 1409*) from *Nothapodytes nimmoniana,* forming while colony mycelium which turns black during sporulation and forms aerial hyphae wherein the strain produces 150-200 µg/g DW biomass or 1.5-3 mg/L titer of camptothecin in suspension.

2. The process of camptothecin production further comprising a novel high camptothecin yielding endophyte, *A. alstroemeriae(NCIM 1408),* wherein the high yielding endophyte (*A. alstroemeriae (NCIM 1408)* demonstrates a high yield of camptothecin up to 300-400 µg/g DW biomass.

3. The process of claim 1 & 2 further comprising incubation of the fungal strain on a Potato dextrose medium for about 5-10 days at temperature of 20 – 30 °C at 80-200 rpm.

4. The process of claim 1 & 2 further comprising separation of the fungal mycelia by centrifuging at 6000 – 12000 rpm and at 4°C to obtain the fungal pellet.

5. The process of claim 4 further comprising washing the fungal pellet with distilled water (pH-6 to 7).

6. The process of claim 1 & 2 further comprising centrifuging the washed fungal pellet at 6000–12000 rpm and at 4°C to remove water and drying the fungal biomass at 40-70°C to obtain dried biomass.

7. The process of claim 4 and 6 comprising separation of the supernatant from fungal pellet and/ or the dried biomass by homogenization and extraction using organic solvents to identify and estimate camptothecin.

8. The process of claim 3 wherein the potato dextrose medium consists of potato infusion from 200 g/l of potato, Dextrose 20 g/l and Agar.

9. The process of claim 7 wherein the organic solvents consist of a mixture of chloroform and methanol in the ratio of 4:1.

DRAWINGS

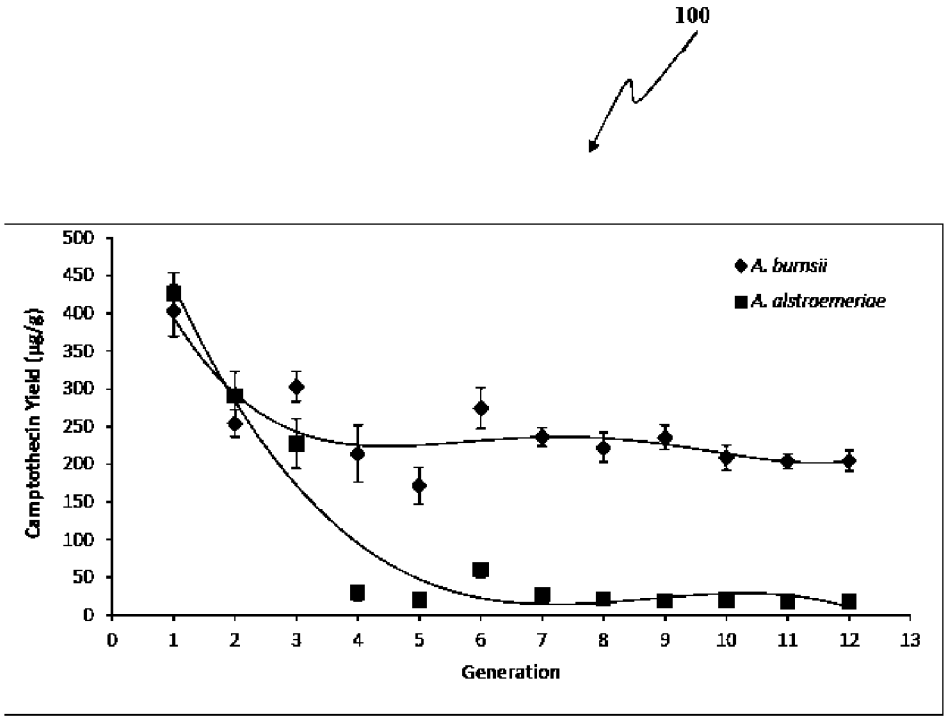


FIG 1

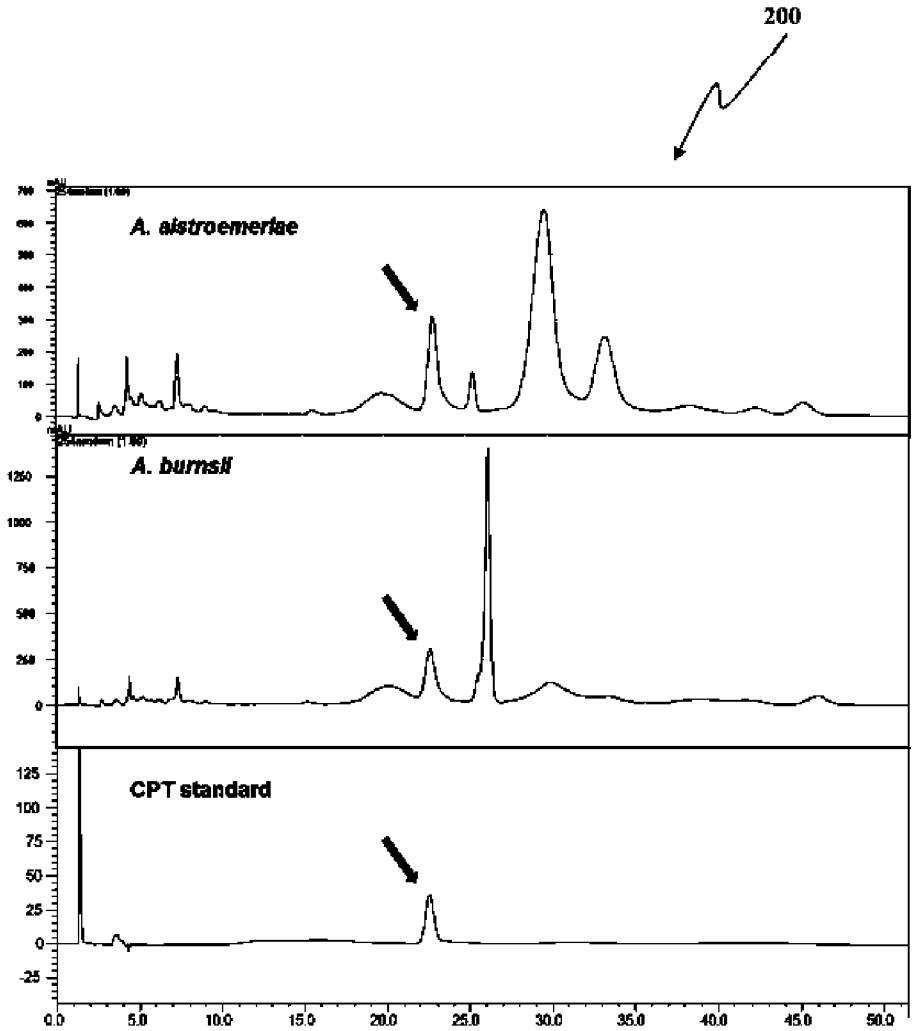

200
A. aistroemeriae
A. burnsii
CPT standard
0.0
5.0
10.0
15.0
20.0
25.0
30.0
35.0
40.0
45.0
50.0

FIG. 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IN2019/050626

A. CLASSIFICATION OF SUBJECT MATTER
C12M3/00,C07D491/22 Version=2019.01

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12M, C07D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

TotalPatent One, IPO Internal Database

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	IN3220DELNP2006A [COUNCIL OF SCIENTIFIC & INDUSTRIAL RESEARCH] 31 AUGUST 2007, 31/08/2007 abstract, claims 1-9	1-9
Y	Pu X, et al. Camptothecin-producing endophytic fungus Trichoderma atroviride LY357: isolation, identification, and fermentation conditions optimization for camptothecin production. Applied microbiology and biotechnology. 2013 Nov 1;97(21):9365-75. abstract	1-9
Y	Paul NC, et al. Characterization and Pathogenicity of Alternaria burnsii from Seeds of Cucurbita maxima (Cucurbitaceae) in Bangladesh. Mycobiology. 2015 Dec 1;43(4):384-91. abstract	1-9

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"D" document cited by the applicant in the international application
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10-12-2019	10-12-2019
Name and mailing address of the ISA/ Indian Patent Office Plot No.32, Sector 14,Dwarka,New Delhi-110075 Facsimile No.	Authorized officer Akash Kumar Telephone No. +91-1125300200

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/IN2019/050626

Citation	Pub.Date	Family	Pub.Date
IN 3220DELNP2006 A	31-08-2007	US 20060134762 A1	22-06-2006