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Research Article

Random Mutagenesis of Endophytic Fungi for Enhanced Taxol Production

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Abstract: In this study, endophytic fungal strain HM-1 (*Aspergillus* sp.) has been reported to be capable of producing taxol. The wild strain was treated with physical (UV irradiation) and chemical (Nitrous acid and Ethidium bromide) mutagens to enhance taxol production. Mutant strains were subjected to surface culture fermentation at 30°C for 21 days. Taxol production was estimated quantitatively by HPLC while mycelial mass was calculated by the dry weight method. Results revealed that PM-5 mutant strain showed slightly increased production of taxol i.e. 99.44 ± 0.76 mg/L than 87.25 ± 0.5 mg/L of wild strain however, mycelial mass production was enhanced upto 10.25± 0.48 g/L of mutant strain C1 M-46 as compared to 7.75 ± 0.25 g/L of wild strain HM-1. 13 mutant strains gave low taxol production but high mycelial mass production as compared to wild strain HM-1. In addition to this, UV light, ethidium bromide, and nitrous acid had completely inhibited taxol production by 18 mutant strains. Results revealed that mutagens could enhance secondary metabolite production by fungi.

Keywords: Taxol, *Aspergillus* sp., Ethidium bromide, UV light, Nitrous acid, high performance liacid chromatography (HPLC).

1. INTRODUCTION

Taxol (paclitaxel) is an anticancer drug broadly used in the treatment of various types of cancers. The molecule of paclitaxel comprises a tetracyclic core called baccatin III and an amide tail [1]. Taxol was isolated for the first time from a gymnosperm tree *Taxus brevifolia* [2]. Its physical appearance is off-white to a white crystalline structure. Taxol is known to be a highly lipophilic compound that is soluble in organic compounds. Different types of cancers can be treated by taxol which includes breast, lung, ovarian, leukemia, murine, sarcoma, and other diseases [3, 4, 5]. The mechanism of paclitaxel to inhibit the growth of cancerous cells is based on its ability to stabilize the assembly of microtubules [6]. Paclitaxel prevents the disassembling of microtubules thus compelling the mitotic checkpoints to arrest mitosis [7, 8, 9].

Production of Taxol from plant sources is costly and limited due to the slow growth of plants and as well as small yield of Taxol. In addition to plants, taxol could be obtained from some other sources like chemical synthesis, semi-synthesis, tissue culture technique, and fungal endophytes. However, neither of these sources is sufficient to meet the ever-increasing demand for taxol [7,10, 11, 12, 13]. Recent studies depict that approximately 200 endophytic fungal sources of Taxol from around 40 genera have been isolated [14]. With the discovery of a wide range of Taxol producing endophytes, scientists use biotechnological tools and techniques to increase the ability of endophytic microbes to produce Taxol. These techniques include strain improvement through site-directed mutagenesis, genetic engineering, and optimization [15]. The present study reports the first-ever attempt to enhance the taxol yield from an endophytic fungus through the use of random mutagenesis.

Random mutagenesis means the insertion of point mutations at random in the genome of the organism which could increase the production of target metabolite thus, exhibiting an equal probability to produce positive or negative mutations. The technique is preferred because of its simplicity, low cost as well as limited data on the

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function of each amino acid in protein sequences involved in the production of the target metabolite. The mutations can be induced by physical and chemical mutagens [16] which react with nucleotides and produce changes in the genome of organisms [17]. Wild strain could be improved for their metabolite production by exposing it to different mutagens for example ultraviolet (UV) radiations, nitrous acid, ethyl methyl sulphonate (EMS), and ethidium bromide, etc. In the present study, Taxol-producing endophytic fungus was mutated by using UV irradiation, nitrous acid, and ethidium bromide to improve its taxol production ability. Mycelial mass of mutant strains was also analyzed for quantification of taxol per gram of mycelial mass-produced to establish a relationship between the growth and taxol production in the fungal endophytes.

2. METHODOLOGY

2.1 Microorganism

Endophytic fungal strain HM-1 *(Aspergillus* sp.), capable of producing taxol was obtained from the culture bank of Institute of Industrial Biotechnology Government College University, Lahore. The strain was maintained on potato dextrose agar (PDA) slants and stored at 4°C in a cold Cabinet (MPR-1410, SANYO, Japan).

2.2 UV Treatment

Spore suspension of 48-hour old culture slant of endophytic fungal strain HM-1 (*Aspergillus* sp.) was prepared in sterilized distilled water and spores count was set at 1.52 × 10 5 per ml using a hemocytometer. Death curve was prepared to optimize conditions for UV treatment. A hundred microliters of spore suspension were inoculated on a sterilized PDA plate by spread plate method and were incubated at 30° C for 2 hours to initiate germination of spores. Plates were exposed to UV irradiation for varying time intervals i.e. 0-30 minutes with an interval of 5 minutes under a beam (λ=253 nm and 220 V at 50 c/s) of UV lamp. Distance between the UV lamp and the sample was kept constant at 13 cm to attain a 95% death rate. Afterward, incubation of treated plates was carried out at 30° C for 48 hours. The death curve was plotted and mutagenesis experiments were carried out at optimum conditions [18].

2.3 Nitrous Acid Treatment

Endophytic fungal strain HM-1 (*Aspergillus* sp.) was grown on PDA slants for 48 hrs. The spore suspension was prepared using potato dextrose broth (PDB) and incubated at 30° C for 2 hours to initiate germination. Spore count was set at 1.52×10^5 spores per ml by using Hemocytometer. One milliliter of spore suspension was centrifuged to obtain a pellet of spores. The death curve of the fungal strain was prepared to find out the concentration and exposure time of nitrous acid at which mutagenesis would be done. Sodium acetate buffer (0.1 M of pH 4.2) and sodium nitrite were added to the pellet to adjust the final concentration of nitrous acid to 1, 2, 3, 4, 5, 6, 7, and 8 M. Spores were left exposed to nitrous acid from 0-30 minutes. After a specific time interval, nitrous acid was neutralized by adding 0.1 M phosphate buffer (pH 7.0). After centrifugation, the pellet was re-suspended in phosphate buffer and 100 ul was inoculated on PDA plates. Plates were incubated at 30° C for 48 hours. Death curves of nitrous acid concentration and exposure time were plotted and mutagenesis was carried out at optimum conditions [19].

2.4 Ethidium Bromide Treatment

Endophytic fungal strain HM-1 (*Aspergillus* sp.) was grown on PDA slants for 48 hrs. Spore suspension of 48 hr old culture slant of Endophytic fungal strain HM-1 (*Aspergillus* sp.), having spore count of 1.52×10^5 was prepared and germinated in PDB at 30° C for 2 hours. A death curve was prepared to optimize the concentration and exposure time for ethidium bromide treatment. The spore suspension was centrifuged and the pellet was exposed to 1 ml of ethidium bromide in concentrations 1 mg/ml and 2 mg/ml for 10, 20, 30, 40, 50, and 60 minutes. Pellet was washed twice with and resuspended in normal saline from which 100 µl was inoculated onto PDA plates by spread plate method and incubated at 30° C for 48 hours in a static incubator. The death curve of ethidium bromide concentration and the exposure time was plotted and mutagenesis was carried out at optimum conditions [20].

2.5 Selection of Mutants

After treatment with various mutagens, plates were incubated at 30oC and were observed regularly after 2-3 days to study the growth pattern and phenotypic variations. The colonies that differed from the control were picked and allowed to grow on PDA. These colonies were then analyzed qualitatively and quantitatively for the production of taxol using surface culture/static fermentation.

2.6 Inoculum development

Mutated colonies were purified on PDA slants and spore suspension was prepared using 10 ml of autoclaved distilled water with spore count set at 8.16×10^6 spores/ml with Hemocytometer (0630010, Neubauer- improved, Germany). The spore suspension was used as an inoculum for fermentation.

2.7 Fermentation Technique

The spore suspension (1%) was transferred aseptically to a 250mL Erlenmeyer flask containing 30mL of sterilized potato dextrose broth and was incubated at 30° C for three weeks under static conditions.

After fermentation, extraction of taxol was carried out by separating fungal mycelia from culture broth by using four layers of muslin cloth. The filtrate obtained was treated with sodium carbonate (Na_2CO_3) to reduce fatty acids that may cause hindrance in extraction. Two equal volumes of the solvent dichloromethane were added to the culture filtrate. The organic layer formed was evaporated using a rotary evaporator and solid residue obtained was dissolved in methanol for further analysis [21].

2.8 Quantification of Production of Taxol and Mycelial Mass

Taxol quantification was done by using High-Performance Liquid Chromatography (HPLC) method (26). HPLC system (Perkin Elmer) was used having a C18 reverse-phase column (SpherSIL, 5um diameter, column size (250 x 4 mm). Mobile phase containing methanol and water (70:30) was used and the flow rate was adjusted to 1 mL/min. The absorbance was taken at 232 nm. Mycelial mass was quantified by the dry weight method. Fungal mycelia were dried on pre-weighed filter paper and then dry weight was calculated.

3. RESULTS AND DISCUSSION

Taxol production potential of wild strain HM-1 *Aspergillus* sp.) was calculated by comparing HPLC chromatograms of the wild strain and standard paclitaxel sample (100 µg/0.5 ml Fig. 1).

Chromatogram of wild strain HM-1 (*Aspergillus* sp.) is shown in Fig. 1. The area of standard paclitaxel peak and wild strain peak was compared. The concentration of taxol produced by wild strain HM-1 (*Aspergillus* sp.) was calculated to be 87.25 ± 0.5 mg/L with a weighed mycelial mass of 7.75 ± 0.25 g/L. Production of taxol by endophytic fungal strain HM-1 (*Aspergillus* sp.) is following other studies, as cited in the literature, in which taxol was produced by the endophytic fungus *Aspergillus fumigatus* [22] and *Aspergillus* niger [12].

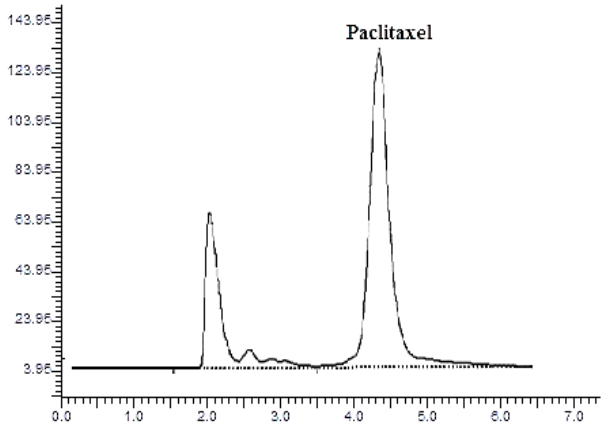

Fig 1. HPLC Chromatogram of Paclitaxel of Wild Strain HM-1 (*Aspergillus sp*.).

Table 1. Taxol and Mycelial Mass Production by UV Treated Mutant Strains

S. No.	Strains	Taxol Production (mg/L)	Mycelial mass (g/L)	S. No.	Strains	Taxol Production (mg/L)	Mycelial mass (g/L)
1	*PM-1	0	6.5 ± 0.25	**14**	PM-14	0	5 ± 0.67
2	PM-2	12.29 ± 0.25	7.8 ± 0.35	**15**	PM-15	0	5.5 ± 0.76
3	PM-3	12.36 ± 0.28	10 ± 0.98	**16**	PM-16	11.25 ± 0.76	8.7 ± 0.54
4	PM-4	16.60 ± 0.87	8.5 ± 0.76	**17**	PM-17	3.91 ± 0.67	5 ± 0.67
5	PM-5	99.44 ± 0.76	7 ± 0.67	**18**	PM-18	3.95 ± 0.56	5.5 ± 0.54
6	PM-6	.58 ± 1.5	6 ± 0.65	**19**	PM-19	67.08 ± 0.45	4.75 ± 0.34
7	PM-7	24.24 ± 1.2	8.9 ± 0.54	**20**	PM-20	29.12 ± 0.34	6.5 ± 0.32
8	PM-8	82.97 ± 1.3	5 ± 0.43	**21**	PM-21	8.66 ± 0.45	6 ± 0.78
9	PM-9	6.15 ± 0.98	9.9 ± 0.34	**22**	PM-22	45.89 ± 0.32	5.5 ± 0.67
10	PM-10	4.62 ± 0.89	9.1 ± 0.38	**23**	PM-23	21.89 ± 0.65	6.75 ± 0.45
11	PM-11	51.34 ± 0.54	4.2 ± 0.12	**24**	PM-24	65.91 ± 0.76	5.5 ± 0.98
12	PM-12	0	3.9 ± 0.32	**25**	PM-25	45.92 ± 0.78	6 ± 0.76
13	PM-13	38.40 ± 0.45	7 ± 0.98	**26**	*HM-1	87.09 ± 0.5	7.5 ± 0.25

*P M-1: Physical mutagen (UV irradiation) induced mutant strains

*HM-1: Wild strain (*Aspergillus* sp.)

±value: Standard deviation

3.1 UV Mutagenesis

To enhance the production of taxol, different colonies were obtained by irradiating spores of HM-1 (*Aspergillus* sp.) with UV light for 25 minutes at a distance of 13 cm. The data of screened colonies after treatment is given in Table 1. Twenty-five colonies were picked based on their difference from parental strain and were subjected to static culture fermentation for the production of taxol.

Among the tested colonies, PM-5 was found to exhibit a slight increase in Taxol production i.e. 99.44 ± 0.76 as compared to 87.09 ± 0.5 of wild strain. Six out of twenty-five tested colonies showed a lower concentration of taxol than the parental strain, which signified the inhibitory effect of UV irradiation on colonies. However, 5 mutant strains produced a low amount of Taxol but an increased amount of mycelial mass.

UV light induces thymine dimers and 6, 4-photoproducts by damaging hydrogen bonding between nucleotide basis and complementary strands of deoxyribonucleic acid (DNA). Induction of mutation by UV light in wild strain HM-1 (*Aspergillus* sp.) is following other studies such as induction of mutation in *Aspergillus* niger by UV light [23]. Mutant strains that showed an equal amount of taxol production as compared to wild strain might be due to the reason that mutation did not occur in genes responsible for taxol production. Strains that produced a low or moderate amount of taxol were possibly due to negative mutations in taxol producing genes. The absence of positive mutation for taxol production in mutant strains might be due to multiple genes involved in the taxol biosynthetic pathway [24] and negative mutation in any of the responsible genes could suppress or completely block the production. Enhanced mycelial mass production in mutant strains with low taxol production, in comparison to wild strain HM-1 (*Aspergillus sp.*), had clearly indicate that mutations had positively affected mycelial mass production but negatively affected Taxol production.

3.2 Nitrous Acid Mutagenesis

Wild strain HM-1 (*Aspergillus* sp.) was exposed to 5M nitrous acid for 20 minutes to obtain mutant colonies.

The colonies with macroscopically distinct character than wild strain were selected. Fifty

colonies were selected in this way and were subjected to Taxol production through surface culture fermentation. Taxol production and mycelial mass production of these mutant strains are shown in Table 2. Out of fifty (50) strains, six (6) strains completely inhibited the taxol production. In comparison to the wild strain, 26 mutant strains showed a lower level of taxol production than the wild strain. Nine strains showed no effect on the amount of taxol production as compared to wild strain. Out of 50 mutant strains, 9 gave an increased amount of mycelial mass production compared to parental strain as shown in Table 2.

Nitrous acid is a deaminating agent that removes the amino group from nucleotide bases i.e. adenine and changes it into hypoxanthine which is an analogous compound of guanine thus leading to transition mutations i.e. A-T to G-C transition [25]. In this way, point mutations at the first or second codon position may change an amino acid in the gene coding for specific products thus affecting its expression. This was how to transition mutation may occur in the genes of Wild strain HM-1 (*Aspergillus* sp.). Mutant strains which had completely inhibited Taxol production may be due to mutations that inhibited the enzymes of important intermediate steps. Mutant strains that produced a low or moderate amount of Taxol may be due to mutations that suppressed the Taxol production. Strains that produced Taxol in an amount equal to wild strain maybe because mutation occurred in the genes other than genes responsible for Taxol production. No mutant strain produced a greater amount of Taxol than produced by wild strain, this may be due to multiple genes involved in the production

Table 2. Taxol and Mycelial Mass Production by nitrous acid Treated Mutant Strains

S. No.	Strains	Taxol Production (mg/L)	Mycelial mass (g/L)	S. No.	Strains	Taxol Production (mg/L)	Mycelial mass (g/L)
1	*C_1 M-1	73.31 ± 0.45	7.5 ± 0.23	**27**	C_1 M-27	0	6.75 ± 0.89
2	C_1 M-2	57.88 ±0.76	3.2 ±0.24	**28**	C $_1$M-28	8.38 ± 0.45	7 ± 0.76
3	C_1 M-3	3.64 ± 0.87	5.3 ± 0.43	**29**	C_1 M-29	68.24 ± 0.23	6.5 ± 0.78
4	C_1 M-4	19.36 ±0.89	9.7 ± 0.54	**30**	C_1 M-30	0	6.25 ± 0.87
5	C_1 M-5	56.45 ±0.78	5.2 ± 0.45	**31**	C_1 M-31	8.38 ± 0.34	5.25 ± 0.67
6	C_1M-6	32.98 ± 0.76	3.5 ± 0.87	**32**	C_1 M-32	11.78 ± 0.45	5.5 ± 0.54
7	C_1M-7	53.52 ± 0.45	4.25 ± 0.98	**33**	C_1 M-33	72.37 ± 0.32	5 ± 0.23
8	C_1 M-8	15.23 ± 0.34	9.8 ± 0.67	**34**	C_1 M-34	36.70 ± 0.34	4 ± 0.43
9	C_1 M-9	13.85 ± 0.23	8.4 ± 0.12	**35**	C_1 M-35	80.35 ± 0.23	5.75 ± 0.56
10	C_1 M-10	71.59 ± 0.23	5.5 ± 0. 34	**36**	C_1 M-36	6.88 ± 0.98	5.75 ± 0.78
11	C_1 M-11	31.88 ± 0.43	5.25 ± 0.23	**37**	C_1 M-37	67.22 ± 0.34	5.75 ± 0.34
12	C_1 M-12	33.01 ± 0.34	5.75 ± 0.43	**38**	C_1 M-38	53.35 ± 0.78	6.5 ± 0.54
13	C_1 M-13	22.46 ± 0.54	4.8 ± 0.34	**39**	C_1 M-39	32.55 ± 0.67	9.25 ± 0.23
14	C_1 M-14	1.64 ± 0.34	5.6 ± 0.32	**40**	C_1 M-40	22.30 ± 0.23	6.25 ± 0.45
15	C_1 M-15	0	4.7 ± 0.56	**41**	C_1 M-41	21.13 ± 0.34	5 ± 0.87
16	C_1 M-16	0	6.8 ± 0.76	**42**	C_1 M-42	5.03 ± 0.87	8 ± 1.01
17	C_1 M-17	38.52 ± 0.78	4.3 ± 0.23	43	C_1 M-43	19.88 ± 0.78	8.9 ± 1.23
18	C_1 M-18	15.23 ± 0.87	9.4 ± 0.56	44	C_1 M-44	38.38 ± 0.12	8.25 ± 0.87
19	C_1 M-19	1.90 ± 0.78	6.4 ± 0.65	45	C_1 M-45	35.77 ± 0.32	7 ± 1.45
20	C_1 M-20	76.87 ± 0.76	7 ± 0.89	**46**	C_1 M-46	20.03 ± 0.23	10.25 ± 1.43
21	C_1 M-21	71.64 ± 0.54	6.25 ± 0.98	**47**	C_1 M-47	19.93 ± 0.43	9.5 ± 0.87
22	C M-22	0	5.75 ± 0.61	**48**	C_1 M-48	76.96 ± 0.45	6 ± 0.90
23	C_1M-23	5.05 ± 0.65	6 ± 1.4	**49**	C_1 M-49	22.95 ± 0.65	6 ± 0.34
24	C_1M-24	11.80 ± 0.56	9.9 ± 1.5	**50**	C_1M-50	21.44 ± 0.34	7.5 ± 0.43
25	C $_i$M-25	53.34 ± 0.43	7.25 ± 0.34	**51**	HM-1	87.13 ± 0.7	7.25 ± 0.26
26	C_1 M-26	0	7.5 ± 0.87				

*C_1M-1: Chemical mutagen 1 (nitrous acid) induced mutant strain 1

of Taxol [24] which made it difficult to mutate the genes in a manner to increase the production of Taxol. However, nitrous acid mutations enhanced the mycelial mass production which depicts nitrous acid had increased mycelial mass production while negatively affected taxol production.

3.3 Ethidium Bromide Mutagenesis

The wild strain of *Aspergillus* sp. HM-1 was exposed to 2 mg/ml ethidium bromide for 60 minutes and as a result of this treatment, 25 visibly distinct colonies were selected which were then tested for their taxol production and mycelial mass. Taxol production and mycelial mass of selected strains are shown in Table 3.

Among them, 7 colonies showed complete inhibition of Taxol production while 8 colonies had produced Taxol in a lesser amount than wild strain. There was no ethidium bromide treated mutant strains that gave enhanced Taxol production than wild strain. Biomass production of 8 mutant colonies showed enhanced mycelial mass than the wild strain. On comparing Taxol production and mycelial mass production, it was found that 5 mutant strains were so that produced low amounts of Taxol but higher amounts of mycelial mass than wild strain HM-1 (*Aspergillus* sp.).

These frameshift mutations in the genes of wild strain HM-1 (*Aspergillus* sp.) affected the expression of specific products. Taxol production was inhibited completely by some strains which might be due to mutations that blocked intermediate steps that are crucial for Taxol production. A low amount or moderate amount of Taxol production might be due to mutations in one or more genes responsible for taxol production as multiple genes are involved in the biosynthetic pathway of Taxol production [24]. This means that more than one gene involvement made the positive induction of mutagenesis a difficult task. But mutations in the genome of wild strain HM-1 (*Aspergillus* sp.) may be confirmed by the increased amount of mycelial production than wild strain. The strains which gave the lesser amount of Taxol production showed an increased amount of biomass production in this experiment thus confirming the above statement.

4. CONCLUSION

It is concluded from the present study that random mutagenesis can be used as an effective technique

Table 3. Taxol and Mycelial Mass Production by ethidium bromide Treated Mutant Strains

S. No.	Strains	Taxol Production (mg/L)	Mycelial mass (g/L)	S. No.	Strains	Taxol production (mg/L)	Mycelial mass (g/L)
1	*C_2 M-1	0	3.5 ± 0.23	**14**	C_2 M-14	38.30 ± 0.34	7 ± 0.99
2	C_2 M-2	16.59 ± 0.78	8.75 ± 0.65	**15**	C_2 M-15	16.67 ± 0.67	9.8 ± 1.0
3	C_2 M-3	24.14 ± 0.76	9.4 ± 0.78	**16**	C_2 M-16	0	3.5 ± 0.67
4	C_2 M-4	0	6.75 ± 0.89	**17**	C_2 M-17	25.87 ± 0.56	9.3 ± 0.56
5	C_2 M-5	46.86 ± 0.34	6.5 ± 0.5	**18**	C_2 M-18	39.73 ± 0.34	7.75 ± 0.87
6	C_2 M-6	0	8.75 ± 0.45	**19**	C_2 M-19	20.68 ± 0.99	6.8 ± 0.56
7	C_2 M-7	0	4.2 ± 0.44	**20**	C_2 M-20	9.41 ± 0.76	7.6 ± 0.54
8	C_2 M-8	47.06 ± 0.36	4.5 ± 0.34	**21**	C_2 M-21	51.44 ± 0.34	3.6 ± 0.34
9	C_2 M-9	37.41 ± 0.46	7.25 ± 0.65	**22**	C_2 M-22	0	7 ± 0.54
10	C_2 M-10	0	6.25 ± 0.78	**23**	C_2 M-23	11.01 ± 0.67	9.9 ± 0.87
11	C_2 M-11	47.77 ± 0.89	5.75 ± 0.98	**24**	C_2 M-24	8.62 ± 0.56	9 ± 0.98
12	C_2 M-12	58.10 ± 0.34	5.75 ± 0.78	**25**	C_2 M-25	0	7.7 ± 0.77
13	C_2 M-13	77.21 ± 0.98	6.5 ± 0.43	**26**	HM-1	87.24 ± 0.46	7.75 0.24

*C_2 M-1: chemical mutagen 2 (ethidium bromide) induced mutant strain-1

to induce mutations which may result in enhanced production of the secondary metabolites by an organism. This study also revealed that the enhancement of production in wild strain could be achieved by conducting a greater number of experiments through a variety of physical and chemical mutagens. It is therefore recommended that such experiments should be carried out in the future to engineer strains that would give unusually higher production of taxol.

5. REFERENCES

1. D.M. Cassandra, C.D. Churchill, M. Klobukowski and J.A. Tuszynski. Elucidating the mechanism of action of the clinically approved taxanes: a comprehensive comparison of local and allosteric effects. *Chemical Biology and Drug Design,* 86(5): 1253-1266. (2015)
2. M.C. Wani, H.L. Taylor, M.E. Wall, P. Coggon, and A.T. McPhail. Plant antitumor agents VI. The isolation and structure of Taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*. *Journal of American Chemical Society*., 93: 2325–2327. (1971).
3. M.E. Wall, M.C. Wani, C.E. Cooke, K.T. Palmer, A.T. McPhail and G.A. Sim. Plant Antitumor Agents. I. The Isolation and Structure of Camptothecin, a Novel Alkaloidal Leukemia and Tumor Inhibitor from Camptotheca acuminate. *Journal of American Chemical Society*., 105: 3732-3734 (1966).
4. M.D. Bethesda, National Cancer Institute Clinical Brochure: Taxol (NSC1259973). *Division of Cancer Treatment and diagnosis*, 3: 6-12 (1983).
5. Jacrot, M.J. Roindel, F. Picot, D. Leroux, C. Mouriquand, H. Beriel, P. Potier, C.R. Seances. Action of Taxol on human tumors transplanted in athymic mice. Comtes Rendus de I Academie des Sciences, Series III, Sciences de la vie 3(247) 597-600 (1983).
6. P.B. Schiff, J. Fant and S.B. Horwitz Promotion of microtubule assembly in vitro by Taxol. *Nature*., 277: 665–667(1979).
7. B.J. Kim, D.M. Gibson and M.L. Shuler Effect of subculture and elicitation on instability of Taxol production in Taxus sp. suspension cultures. *Experimental Cell Research.,* 243(1): 129–141(2004).
8. P. Lara-Gonzalez, F.G. Westhorpe and S.S. Taylor. The spindle assembly checkpoint. *Current Biology* 22: 966–980 (2012).
9. E.A. Foley, and T.M. Kapoor, T.M. Microtubule attachment and spindle assembly checkpoint signaling at the kinetochore. *Nature reviews molecular cell biology,* 14: 25–37 (2013).
10. G. X. Strobel, Yang, J. Sears, R. Kramer, R.S. Sidhu and W.M. Hess Taxol from Pestalotiopsis microspora, an endophytic fungus of *Taxus wallichiana. Nature Microbiology*., 142(43): 5-440 (1992).
11. K.C. Nicolaou, W.M. Dai, and R.K. Guy. Chemistry and Biology of Taxol. *Angenwandte Chemie International Edition in English. Chem. lnt. Ed. Engl*., 33: 15-44 (1994).
12. D. Li, D. Fu, Y. Zhang, X. Ma and L. Gao. Isolation, Purification, and Identification of Taxol and Related Taxanes from Taxol-Producing Fungus *Aspergillus niger* subsp. taxi. Journal of Microbiology and Biotechnology., 27(8): 1379-1385 (2017).
13. Y. Li, G. Zhang and B.A. Pfeifer Current and Emerging Options for Taxol Production. *Advances in Biochemical Engineering and Biotechnology*, 7: 1547–1558 (2014).
14. S. Kusari, S. Singh and C. Jayabaskaran. Rethinking production of Taxol (paclitaxel) using endophyte biotechnology. *Trends in Biotechnology*., 32(6): 304-311 (2014).
15. K. Zhao, D.P. Zhou and W.X. Ping Study on the mutagenesis of Taxol-producing fungus *Nodulisporium sylviforme* protoplast. *Chinese journal of. Biotechnology*., 24(9): 64-68 (2004).
16. C.A. Hutchison-III, C.A., S. Phillips, M.H. Edgell, S. Gillam, P. Jhanke and M. Smith. Mutagenesis at a specific position in a DNA sequence. *The Journal of Biology and Chemistry*., 253: 6551-6560 (1990).
17. S.T. Yang. Bioprocessing for value added products from renewable sources: new technologies and applications, *Elsevier science,* United States (2007).
18. J. G. S.Mala, R. Numbi, Kamini and R. Puvanakrishnan (2001). Strain improvement of *Aspergillus* niger for enhanced lipase production. The *Journal of General and Applied Microbiology*, 47: 181–186 (2001).
19. I.U. Haq, A. Nawaz. H. Mukhtar, Z. Mansoor, M. Riaz, M. Ahmed and S.M. Ameer. (2014) Random mutagenesis of *Aspergillus* niger and process optimization for enhanced production of glucose oxidase. *Pakistan Journal of botany*., 46(3): 1109-1114 (2014).
20. B. Stackowiak, Efficiency of selected mutagens in generating Xanthophyllomyces dendrorhous strains hyperproducing astaxanthin. *Polish Journal of*

Microbiology., 62(1): 67-72 (2013).

21. M. Pandi, R.S. Kumaran, Y.K. Choi, H.J. Kim, and J. Muthumary. Isolation and detection of taxol, an anticancer drug produced from *Lasiodiplodia theobromae*, an endophytic fungus of the medicinal plant *Morinda citrifolia African. Journal of Biotechnology*., 10(8): 1428-1435 (2011).
22. D. Sun, X. Ran and J. Wang. Isolation and identification of a taxol-producing endophytic fungus from Podocrapus. *Chinese Journal of Biotechnology*., 48(5): 589-95 (2008).
23. O.O. Olaniyi, I.O Folasade, T.V. Familoni, and E.O. Bankefa. (2015). UV Mutagenesis of *Aspergillus flavus* for Enhanced Mannanase Synthesis and Catabolite Activation Studies. *Research. Journal of Microbiology,* 10, 542-550 (2015).
24. R. Croteau, E.B. Raymond, Ketchum, M.L. Robert, R. Kaspera and M.R. Wildung Taxol biosynthesis and molecular genetics, *Phytochemistry Reviews.,* 5(1): 75–97 (2006).
25. M.F. Sanders and J.L. Bowman *Genetic analysis: an integrated approach*. 2nd Edition. *Pearson education limited, England* (2016).
26. J. Wang, G. Li, H. Lu, Z. Zheng, Y. Huang and W. Su. Taxol from *Tubercularia* sp. strain TF5, an endophytic fungus of *Taxus mairei. FEMS Microbiol. Letters*., 193: 249-253 (2000).