Biocatalysis and Agricultural Biotechnology 18 (2019) 100996

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Bioprocess optimization using response surface methodology for production of the anticancer drug paclitaxel by *Aspergillus fumigatus* and *Alternaria tenuissima*: Enhanced production by ultraviolet and gamma irradiation

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ARTICLE INFO

Keywords:
Paclitaxel
Endophytic
Gamma
UV
Radiation
Response surface methodology

ABSTRACT

Paclitaxel is the most profitable anticancer drug in history; however, the yield of paclitaxel from fungal fermentation is still far from the commercial purpose. In this study, two local fungal endophytes of *Aspergillus fumigatus* TXD105 and *Alternaria tenuissima* TER995 were used as promising sources for the production of paclitaxel under submerged fermentation. In order to improve the paclitaxel magnitude, response surface methodology optimization program was applied to medium constituents that showed the high contribution to paclitaxel production. Moreover, improvement of paclitaxel production by UV and gamma irradiation was studied. The use of response surface methodology resulted in significant increases in the paclitaxel production of the two strains by 16.25 and 19.86%, respectively. Furthermore, the production of paclitaxel by the two strains was greatly enhanced by the application of UV and gamma irradiation. Using 0.75 KGy of gamma irradiation, paclitaxel concentrations were intensified to 495.31 and 195.41 $\mu g\,L^{-1}$, respectively. The obtained paclitaxel concentrations by the two strains irradiated by UV for 30 min were 1.16 and 1.20 fold, respectively of their controls. These findings showed a great potential for production of paclitaxel by fungal cultures in industrial scale that indicate the future possibility to reduce the cost of producing fermentation-based drugs.

1. Introduction

Paclitaxel, also known as taxol, is an isoprenoid natural product of plant origin that posses potent anticancer activity (Li et al., 2014). The drug has been approved by the FDA for the treatment of ovarian cancer in 1992 (Goodman and Walsh, 2001). Its use extended for the treatment of many types of cancers including lung, bladder, breast, prostate, head and neck cancer as well as AIDS Kaposi's sarcoma. Furthermore, paclitaxel has potential therapeutic properties against non-cancerous diseases, including neurodegenerative diseases (Woo et al., 1994), polycystic kidney disease (Zhang et al., 2005) and for the prevention of restenosis (Herdeg et al., 2000). Production of paclitaxel was carried out by extraction processes from the bark of the Pacific yew trees (*Taxus brevifolia*). However, the product yield of these methods is inefficient and environmentally costly. For these reasons, several alternative strategies have been developed for paclitaxel production. Plant tissue culture is successfully developed for large-scale paclitaxel production as an environmentally sustainable method, but long incubation time and low yield render it an economic infeasibility (Yukimune et al., 1996). Chemical synthesis and semi-synthesis have been also achieved. However, these methods are expensive and time-consuming processes, in addition to difficulty in the purification process (Patel, 1998). Thus, finding alternative sources of paclitaxel including endophytic fungi gained considerable interest by researches all over the world (Kusari et al., 2012).

In 1993, the first paclitaxel producing endophytic fungus, *Taxomyces andreanae*, was isolated from yew trees (Stierle et al., 1993). Then, more than 50 paclitaxel-producing fungal isolates were described by groups from all over the world (Hao et al., 2013). Production of paclitaxel by fungal fermentation offers the best hope for an efficient and sustainable process. Compared to plant cell culture, fungal fermentation technology is classical and well known (Malik et al., 2011). Designing a fermentation medium is of critical importance as the medium cost can substantially affect overall process economically. A

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https://doi.org/10.1016/j.bcab.2019.01.034
Received 24 December 2018; Received in revised form 20 January 2019; Accepted 21 January 2019
Available online 29 January 2019
1878-8181/

medium composition such as carbon, nitrogen and phosphate sources as well as micronutrients can affect product concentration, yield, and volumetric productivity as well as the ease and cost of downstream product separation (Kennedy and Krouse, 1999). Moreover, application of strain improvement techniques for overproduction of industrial products has been the hallmark of all commercial fermentation processes. Such improved microbial strains can reduce the cost of the processes with increased product yield (Irum and Anjum, 2012). Irradiation by UV-light and gamma rays are recommended as the mutagens of the first choice (Cadet et al., 1999; Chopra, 2005).

Unfortunately, paclitaxel yield from fungal fermentation is still far from the commercial purpose. Moreover, the physiology of paclitaxel production in fungi remains a mystery (Flores-Bustamante et al., 2010). Information and experience about the paclitaxel biosynthetic pathway in fungi are rare (Chi et al., 2008). So, enhancement of paclitaxel production process by fungal fermentation becomes an essential target. In this paper, optimization of nutritional conditions and media requirements for enhanced paclitaxel production by two promising endophytic fungal strains were studied. Furthermore, the use of UV and gamma irradiation for improvement of paclitaxel production by the two strains was also investigated.

2. Materials and methods

2.1. Fungal strains and media

Aspergillus fumigatus TXD105 and *Alternaria tenuissima* TER995, isolated from the bark of *Taxodium distichum* and *Terminalia arjuna*, respectively; were used as sources for paclitaxel production. The two strains were identified and selected among 60 endophytic fungi from different plant samples from our previous study (Ismaiel et al., 2017). The two fungal strains were deposited in the Culture Collection of "Assuit University Mycological Center, http://www.aun.edu.eg/aumc/aumc.htm" under numbers AUMC 10623 and AUMC 10624, and deposited in the GenBank under accession numbers LC171332 and LC170562, respectively.

M1D medium was composed of (g L^{-1}): glucose 1, fructose 4, sucrose 6, $MgSO_4$ 0.36, yeast extract 0.5, $ZnSO_4$ 0.0025, $FeCl_3{\cdot}6H_2O$ 0.0027, $MnSO_4{\cdot}H_2O$ 0.9967, $Ca(NO_3)_2{\cdot}4H_2O$ 0.00065, sodium benzoate 0.05, sodium acetate 0.1, phenylalanine 0.0017 (Stierle et al., 1993). FBM medium was composed of (g L^{-1}): glucose 80, NH_4NO_3 5.0, $MgSO_4$ 0.5, KH_2PO_4 0.5, $ZnSO_4$ 0.001, $Cu(NO_3)_2$ 0.001, $FeCl_3$ 0.002, sodium acetate 1.0, vitamin B1 0.05, L-tyrosine 0.005 (Xu et al., 2006).

2.2. Inoculum preparation

Fungal spores from 5–day old cultures of *A. fumigatus* TXD105 and 5–day old cultures of *A. tenuissima* TER995 were harvested separately by the flooding of the slants with sterile distilled water containing 0.1% Tween 20 and gently scrapping off the spores with a sterile glass rod. Then, the spore concentration was adjusted to 10^6 spores/mL using haemocytometer.

2.3. Fermentation conditions

For *A. fumigatus* TXD105, sterile M1D broth (50 mL/250 mL Erlenmeyer, pH 6.0) was inoculated with 2 mL of the 5–day old freshly prepared spore suspensions (10^6 spores/mL). The inoculated flasks were then incubated at 25 °C for 10 days at 120 rpm. For *A. tenuissima* TER995, sterile FBM broth (50 mL/250 mL Erlenmeyer, pH 6.0) flasks were inoculated with 4 mL of the 7–day old freshly prepared spore suspensions (10^6 spores/mL). After which, they were incubated at 25 °C for 14 days at 120 rpm. These conditions were found the favorable culture conditions for maximum paclitaxel production by the two fungal strains from our previous study (Ismaiel et al., 2017).

2.4. Experimental design

Basic media constituents (carbon , nitrogen , and phosphorous sources) were screened and optimized by one factor at a time approach. Then, minor media constituents were screened by a two level Plackett-Burman (PB) design to determine the significance of each constituent. Finally, a Box-Behnken (BB) design was selected to find the optimum levels for the screened constituents given by response prediction.

2.5. Effect of carbon, nitrogen and phosphorous sources on growth and paclitaxel production

Sucrose, glucose, and fructose of the M1D broth and glucose of the FBM broth were substituted with one of the following compounds; sucrose, galactose, starch, maltose, mannitol, lactose, glucose, cellulose, glycerol, and fructose (Table 1). All were used at a concentration equal to the same that used in the original medium (10 g L^{-1} for M1D and 80 g L^{-1} for FBM). Moreover, the most effective carbon source for each fungal strain was further tested in different concentrations in the range from 10 to 120 g L^{-1}.

For both media (M1D and FBM), different nitrogen sources were tried individually as substitutes for yeast extract (Table 1). These nitrogen sources were yeast extract, urea, KNO_3, $NaNO_3$, NH_4Cl, $NH_4H_2PO_4$, beef extract, $(NH_4)_2HPO_4$, peptone, NH_4NO_3, $(NH_4)_2SO_4$ and casein. All were used at a concentration equal to the same that used in the original medium (0.5 g L^{-1} for M1D and 5.0 g L^{-1} for FBM). Moreover, different concentrations of the most suitable nitrogen source varied from 0.5 to 15 g L^{-1} were also tested.

The effect of various phosphorous sources on growth and production of paclitaxel was studied. Broth media (M1D and FBM) were supplemented with one of the following phosphorous compounds: NaH_2PO_4, KH_2PO_4, Na_2HPO_4, K_2HPO_4, $NH_4H_2PO_4$ and $(NH_4)_2HPO_4$. All were used at a concentration of 0.5 g L^{-1}. Then, different concentrations of the best phosphorous source varied from 0.1 to 1.0 g L^{-1} were also tested.

2.6. Plackett–Burman (PB) design

PB design was used to optimize other medium components (micronutrients) for each broth medium individually (M1D and FBM). This design is used to identify the most significant constituents and their levels from a group for further optimization. It allows the investigation of up to N–1 variable with N trials (Plackett and Burman, 1946).

In the case of TXD105 strain, M1D medium components (zinc sulphate, ferric chloride, calcium nitrate, magnesium sulphate, manganese sulphate, sodium acetate, sodium benzoate, and phenylalanine) were evaluated, while other components (sucrose, ammonium nitrate and potassium dihydrogen phosphate) were kept at constant concentrations. Regarding the TER995 strain, FBM medium components (zinc sulphate, ferric chloride, cupper nitrate, magnesium sulphate, L-tyrosine, Vitamin B1, sodium acetate and phenylalanine) were evaluated, while other components for each fungal isolate were kept constant. The screened variables were tested at two levels of concentration; high (+) and a low (−) level. The higher level was chosen to equal four times their lower levels (Table 2).

Twelve experiments were designed using PB design for each fungal strain and the response was measured in terms of paclitaxel production (Table 3). M1D broth medium (50 mL, pH 6.0) was prepared, sterilized, cooled and then inoculated with 2 mL of the 5–day old freshly prepared spore suspensions (10^6 spores/mL) of the TXD105 strain. The inoculated flasks were then incubated at 25 °C for 10 days at 120 rpm. Also, FBM broth medium (50 mL, pH 6.0) was prepared, sterilized, cooled and then inoculated with 4 mL of the 7–day old freshly prepared spore suspensions (10^6 spores/mL) of the TER995 strain. After which, they were incubated at 25 °C for 14 days at 120 rpm.

Design–Expert version 8.0.7.1 software (Stat–Ease Corporation,

Table 1
Dry biomass (g L^{-1}) and paclitaxel production (μg L^{-1} culture filtrate) of *A. fumigatus* TXD105 and *A. tenuissima* TER995 grown on different carbon, nitrogen, and phosphorous sources.

C, N, P sources	*A. fumigatus* TXD105		*A. tenuissima* TER995	
	Dry biomass (g L^{-1})	Paclitaxel conc. (μg L^{-1})	Dry biomass (g L^{-1})	Paclitaxel conc. (μg L^{-1})
Sucrose	8.85 ± 0.87^{cd}	317.84 ± 19.03^{a}	11.48 ± 0.82^{c}	120.54 ± 19.39^{ab}
Galactose	7.64 ± 0.59^{e}	296.22 ± 16.55^{c}	10.71 ± 0.25^{d}	103.51 ± 12.47^{c}
Starch	9.41 ± 0.36^{b}	173.51 ± 17.43^{g}	13.32 ± 0.22^{a}	55.14 ± 17.93^{f}
Maltose	8.57 ± 0.11^{cd}	280.54 ± 15.47^{d}	11.65 ± 0.44^{c}	102.97 ± 11.35^{c}
Mannitol	8.98 ± 0.41^{c}	268.11 ± 13.21^{e}	10.63 ± 0.28^{d}	101.35 ± 14.86^{c}
Lactose	6.44 ± 0.45^{f}	264.86 ± 24.11^{e}	11.51 ± 0.86^{c}	85.14 ± 13.27^{d}
Glucose	10.33 ± 0.29^{a}	312.43 ± 12.48^{b}	13.41 ± 0.67^{a}	124.05 ± 13.24^{a}
Cellulose	8.16 ± 0.66^{d}	120.00 ± 15.58^{h}	12.29 ± 0.24^{b}	50.27 ± 17.29^{g}
Glycerol	5.71 ± 0.78^{g}	228.65 ± 19.73^{f}	9.61 ± 0.73^{e}	73.51 ± 14.47^{e}
Fructose	8.35 ± 0.89^{d}	314.59 ± 23.24^{b}	11.71 ± 0.25^{c}	118.65 ± 12.48^{b}
Yeast extract	8.91 ± 0.84^{c}	321.08 ± 12.24^{b}	13.15 ± 0.99^{b}	115.95 ± 15.32^{c}
Peptone	9.59 ± 0.76^{b}	301.08 ± 11.39^{c}	13.07 ± 0.61^{b}	131.27 ± 12.98^{a}
Casein	10.30 ± 0.52^{a}	292.43 ± 19.35^{d}	13.83 ± 0.55^{a}	104.05 ± 15.69^{d}
Beef extract	9.69 ± 0.27^{b}	304.86 ± 19.02^{c}	13.27 ± 0.91^{b}	101.62 ± 17.79^{d}
Urea	4.72 ± 0.32^{g}	102.97 ± 22.88^{k}	5.35 ± 0.19^{f}	42.16 ± 10.35^{h}
$NaNO_3$	8.12 ± 0.83^{d}	200.54 ± 19.91^{e}	11.06 ± 0.96^{d}	98.92 ± 13.53^{de}
KNO_3	6.45 ± 0.45^{f}	174.05 ± 10.8^{g}	12.04 ± 0.78^{c}	91.35 ± 9.47^{d}
NH_4Cl	7.55 ± 0.23^{e}	186.49 ± 12.97^{f}	9.46 ± 0.92^{e}	87.03 ± 14.89^{e}
NH_4NO_3	8.63 ± 0.59^{cd}	327.57 ± 17.43^{a}	12.12 ± 0.83^{c}	124.14 ± 11.69^{b}
$(NH_4)_2SO_4$	7.41 ± 0.21^{e}	166.49 ± 18.93^{h}	12.15 ± 0.70^{c}	110.81 ± 6.99^{c}
$(NH_4)_2HPO_4$	6.61 ± 0.94^{f}	140.54 ± 18.79^{i}	13.10 ± 0.51^{b}	60.27 ± 8.97^{g}
$NH_4H_2PO_4$	6.34 ± 0.76^{f}	121.08 ± 14.17^{j}	13.03 ± 0.33^{b}	77.57 ± 6.09^{f}
NaH_2PO_4	8.69 ± 0.31^{b}	301.08 ± 17.94^{c}	12.16 ± 0.89^{c}	97.57 ± 11.82^{c}
KH_2PO_4	9.47 ± 0.91^{a}	364.86 ± 27.43^{a}	13.47 ± 0.36^{a}	131.35 ± 16.13^{a}
Na_2HPO_4	8.76 ± 0.26^{b}	295.14 ± 10.63^{d}	11.73 ± 0.92^{d}	96.49 ± 14.87^{c}
K_2HPO_4	9.25 ± 0.77^{a}	357.30 ± 15.21^{b}	12.83 ± 0.84^{b}	114.59 ± 16.50^{b}
$NH_4H_2PO_4$	6.55 ± 0.39^{c}	187.57 ± 16.64^{e}	13.21 ± 0.27^{a}	72.97 ± 10.97^{e}
$(NH_4)_2HPO_4$	5.94 ± 0.82^{d}	189.19 ± 24.08^{e}	11.93 ± 0.73^{d}	87.03 ± 13.66^{d}

TXD105 strain grown in 50 mL M1D medium (pH 6.0) inoculated with 2 mL inoculum size of 5-day-old culture and incubated at 120 rpm and 25 °C for 10 days. TER995 strain grown in 50 mL FBM medium (pH 6.0) inoculated with 4 mL inoculum size of 7-day-old culture and incubated at 120 rpm and 25 °C for 14 days. Calculated mean is for triplicate measurements from two independent experiments ± SD, $^{a–k}$ means with different superscripts in the same column for each source (C, N, and P) are considered statistically different (LSD test, $P \leq 0.05$).

Minneapolis, MN, USA) was used for designing the experimental trials. The obtained results were analyzed by the first-order model assumption to calculate the coefficient value of each factor. Medium constituents with high positive coefficient value (*t* value) have a major impact on response and a *p* value of variables lower than 0.05 indicates a significant effect. These constituents were selected for further concentration optimization.

2.7. Response surface methodology (RSM)

For both fungal strains, magnesium sulphate, sodium acetate, and phenylalanine are the relevant factors in PB design (with high *t* value and low *P* value). These factors were optimized by RSM using a three–coded level Box–Behnken (BB) design constructed by the Design–Expert software (Box and Behnken, 1960). For each fungal strain, 17 trials were formulated using BB design (Table 5) for the three independent factors [A: magnesium sulphate (0.2, 0.6 and 1.0 g L^{-1}), B: sodium acetate (0.5, 1.5 and 2.5 g L^{-1}) and C: phenylalanine (0.025, 0.075 and 0.125 g L^{-1})]. Broth media were prepared, sterilized, cooled and then separately inoculated under the optimum culture conditions mentioned previously.

The obtained data on paclitaxel production from RSM program was analyzed by ANOVA (analysis of variance) and behavior of the design was illustrated by the following equation:

$$Y = \beta_0 + \Sigma\beta_i x_i + \Sigma\beta_{ij} x_i x_j + \Sigma\beta_{ii} {x_i}^2$$

where *Y* is the predicted response, β_o the offset term, β_i the linear offset, β_{ii} the squared offset, β_{ij} the interaction effect and χ_i is the dimensionless coded value of χ_j. The statistical significance of the above model equation was determined by Fisher's F–test value and the proportion of variance explained by the model was given by the multiple coefficients of determination, R^2 value. 2D contour and 3D response surface plots were generated by the statistical Design–Expert software on the basis of the response analysis to explain the interaction between the significant factors.

2.8. UV irradiation

The freshly collected spore suspensions (10^6 spores/mL) of the two fungal strains were separately exposed to UV-light radiation (254 nm, 70 μW/cm^2) carried out in a "Dispensing–Cabinet" for different periods of time (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 min) at a distance of 10 cm from the UV source. To avoid photo–reactivation, the UV-exposed spore suspensions were stored in dark overnight at 4 °C.

2.9. ^{60}Co gamma irradiation

Spore suspensions (10^6 spores/mL) of the two fungal strains were irradiated separately by ^{60}Co gamma rays at different doses as follows: 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00, 2.25 and 2.50 KGy. The irradiated spore suspensions were kept in darkness overnight at 4 °C to avoid photo–reactivation.

2.9.1. Gamma source specifications

The process of irradiation was carried out at the Nuclear Research Center, Atomic Energy Authority, Cairo, Egypt. The facility used was ^{60}Co gamma chamber, MC20, Russia. The dose rate of the facility was 1.27 KGy/h at the time of the experiment.

Table 2
Media components and their two levels used in PB design.

M1D broth components				FBM broth components			
Symbol	Composition	Lower level (g L^{-1})	Higher level (g L^{-1})	Symbol	Composition	Lower level (g L^{-1})	Higher level (g L^{-1})
A	$ZnSO_4$	0.0025	0.0108	A	$ZnSO_4$	0.001	0.004
B	$MnSO_4.H_2O$	0.5	2	B	$FeCl_3.6H_2O$	0.002	0.008
C	$Ca(NO_3)_2.4H_2O$	0.00065	0.0026	C	$Cu(NO_3)_2$	0.001	0.004
D	$FeCl_3.6H_2O$	0.0027	0.01	D	L-tyrosine	0.005	0.02
E	Sodium benzoate	0.05	0.2	E	Vitamin B1	0.05	0.2
F	$MgSO_4.6H_2O$	0.2	0.8	F	$MgSO_4.6H_2O$	0.2	0.8
G	NaOAc	0.1	0.4	G	NaOAc	0.5	2
H	Phenylalanine	0.017	0.068	H	Phenylalanine	0.017	0.068

2.10. Effect of irradiation on the production of paclitaxel

The irradiated spore suspensions (UV and gamma) of the TXD105 strain, were tested separately for their paclitaxel producing-ability using the optimized M1D broth (MM1D). In the case of TER995 strain, the irradiated spore suspensions (UV and gamma) were tested separately for their production ability of paclitaxel using the optimized FBM broth (MFBM). Broth media were prepared, sterilized, cooled and then separately inoculated under the optimum culture conditions mentioned previously.

2.11. Analytical methods

2.11.1. Determination of dry biomass

At the end of the incubation period, the obtained biomass of both strains (TXD105 and TER995) cultures were separately quantified by filtering the fungal cultures through pre-weighted filter papers (Whatman no.1). The cells were oven dried to a constant weight at 70 °C before estimating dry biomass.

2.11.2. Extraction of paclitaxel

Paclitaxel was extracted from the culture filtrate of the two strains using a reported method (Strobel et al., 1996). To avoid fatty acid contamination in the paclitaxel extraction process, Na_2CO_3 (0.025%, w/v) was added to the filtrate. Paclitaxel was extracted with an equal volume of methylene chloride and then filtered over anhydrous sodium sulfate. The solvent was removed by evaporation under reduced pressure at 35 °C using rotary evaporator (IKA, RV10, Germany). The dried concentrated extract was dissolved in methanol (absolute) and used for qualitative and quantitative analysis.

Table 4
Statistical analysis of medium components using PB design for paclitaxel production by *A. fumigatus* TXD105 and *A. tenuissima* TER995.

Component	Effect		Contribution (%)		Prob > *F*	
	TXD105	TER995	TXD105	TER995	TXD105	TER995
A	10.43	21.58	0.79	10.71	0.4639	0.0346
B	14.67	9.52	1.56	2.09	0.3236	0.2023
C	−58.01	4.66	24.32	0.50	0.0187	0.4845
D	21.44	16.05	3.32	5.93	0.1836	0.0713
E	7.22	0.75	0.38	0.013	0.6029	0.9062
F	60.86	36.67	26.77	30.95	0.0164	0.0082
G	60.51	37.93	26.46	33.11	0.0166	0.0075
H	42.50	24.97	13.05	14.34	0.0420	0.0236
Model	–	–	–	–	0.0381	0.0229

For TXD105 strain: Factor A $ZnSO_4$, Factor B $MnSO_4.H_2O$, Factor C $Ca(NO_3)_2.4H_2O$, Factor D $FeCl_3.6H_2O$, Factor E Sodium benzoate, Factor F magnesium sulphate, Factor G sodium acetate, Factor H Phenylalanine.
For TER995 strain: Factor A $ZnSO_4$, Factor B $FeCl_3.6H_2O$, Factor C $Cu(NO_3)_2$, Factor D L-tyrosine, Factor E Vitamin B1, Factor F magnesium sulphate, Factor G sodium acetate, Factor H Phenylalanine.

2.11.3. Qualitative and quantitative analysis of paclitaxel

Thin layer chromatography (TLC, 0.25 mm GF-254 silica thin layer plate, Loba Chemie Pvt. Ltd., Mumbai, India) was used for separation of samples from extracts of the two fungal strains, together with the paclitaxel standard (Sigma, St. Louis, MO, USA). TLC plates were developed using the running solvent consisted of chloroform: methanol (7:1, v/v) (Strobel et al., 1996). The developed TLC plates were air-dried and sprayed with a vanillin-H_2SO_4 reagent then incubated at 110 °C for 15 min. The reagent was composed of 2 g of vanillin dissolved in a mixture of 100 mL methanol and 1 mL H_2SO_4. Paclitaxel shows dark

Table 3
PB experimental design matrix for screening factors affecting paclitaxel production (μg L^{-1} culture filtrate) by *A. fumigatus* TXD105 and *A. tenuissima* TER995.

Run	Factors								Paclitaxel conc. (μg L^{-1})	
	A	B	C	D	E	F	G	H	*A. fumigatus* TXD105	*A. tenuissima* TER995
1	1	1	−1	1	1	1	−1	−1	404.36 ± 11.24	111.43 ± 9.15
2	−1	1	1	−1	1	1	1	−1	383.82 ± 10.73	106.76 ± 8.65
3	1	−1	1	1	−1	1	1	1	432.83 ± 10.82	158.72 ± 7.62
4	−1	1	−1	1	1	−1	1	1	420.84 ± 9.54	104.39 ± 10.32
5	−1	−1	1	−1	1	1	−1	1	321.72 ± 10.63	92.21 ± 8.49
6	−1	−1	−1	1	−1	1	1	−1	415.42 ± 7.42	116.17 ± 11.63
7	1	−1	−1	−1	1	−1	1	1	413.87 ± 12.65	112.44 ± 5.92
8	1	1	−1	−1	−1	1	−1	1	406.43 ± 11.93	108.27 ± 7.45
9	1	1	1	−1	−1	−1	1	−1	296.75 ± 10.35	98.85 ± 8.52
10	−1	1	1	1	−1	−1	−1	1	313.82 ± 11.56	82.41 ± 3.95
11	1	−1	1	1	1	−1	−1	−1	259.04 ± 9.05	58.56 ± 7.82
12	−1	−1	−1	−1	−1	−1	−1	−1	295.11 ± 11.63	16.87 ± 3.17

TXD105 strain grown in 50 mL M1D medium (pH 6.0) inoculated with 2 mL inoculum size of 5-day-old culture and incubated at 120 rpm and 25 °C for 10 days. TER995 strain grown in 50 mL FBM medium (pH 6.0) inoculated with 4 mL inoculum size of 7-day-old culture and incubated at 120 rpm and 25 °C for 14 days. Calculated means is for triplicate measurements from two independent experiments.

Table 5
BB experimental design matrix representing the response of paclitaxel production (μg L^{-1} culture filtrate) by *A. fumigatus* TXD105 and *A. tenuissima* TER995.

Run	Factor A		Factor B		Factor C		Paclitaxel conc. (μg L^{-1})			
	Magnesium sulphate (g L^{-1})		Sodium acetate (g L^{-1})		Phenylalanine (g L^{-1})		*A. fumigatus* TXD105		*A. tenuissima* TER995	
	Actual	Coded	Actual	Coded	Actual	Coded	Actual	Predicted	Actual	Predicted
1	0.2	(−1)	0.5	(−1)	0.075	(0)	387.54 ± 12.36	391.92	111.54 ± 11.08	112.47
2	1	(1)	0.5	(−1)	0.075	(0)	416.66 ± 11.58	416.65	115.85 ± 9.48	114.53
3	0.2	(−1)	2.5	(1)	0.075	(0)	414.82 ± 8.65	414.83	114.49 ± 7.39	115.81
4	1	(1)	2.5	(1)	0.075	(0)	380.25 ± 10.52	375.87	153.11 ± 10.63	152.18
5	0.2	(−1)	1.5	(0)	0.025	(−1)	418.09 ± 6.37	414.11	118.27 ± 12.77	118.36
6	1	(1)	1.5	(0)	0.025	(−1)	412.63 ± 11.63	413.04	138.71 ± 10.53	141.05
7	0.2	(−1)	1.5	(0)	0.125	(1)	399.42 ± 10.28	399.02	108.34 ± 9.63	106.00
8	1	(1)	1.5	(0)	0.125	(1)	381.87 ± 7.35	385.86	121.83 ± 10.92	121.74
9	0.6	(0)	0.5	(−1)	0.025	(−1)	407.91 ± 12.52	407.51	132.31 ± 7.93	131.29
10	0.6	(0)	2.5	(1)	0.025	(−1)	395.79 ± 10.37	399.77	157.79 ± 12.44	156.38
11	0.6	(0)	0.5	(−1)	0.125	(1)	391.55 ± 4.73	387.57	118.62 ± 10.36	120.04
12	0.6	(0)	2.5	(1)	0.125	(1)	377.04 ± 13.72	377.44	134.94 ± 8.96	135.96
13	0.6	(0)	1.5	(0)	0.075	(0)	428.32 ± 11.47	433.17	156.77 ± 10.43	157.16
14	0.6	(0)	1.5	(0)	0.075	(0)	433.49 ± 10.55	433.17	155.75 ± 7.64	157.16
15	0.6	(0)	1.5	(0)	0.075	(0)	436.17 ± 9.42	433.17	159.66 ± 10.55	157.16
16	0.6	(0)	1.5	(0)	0.075	(0)	432.13 ± 13.81	433.17	155.87 ± 12.19	157.16
17	0.6	(0)	1.5	(0)	0.075	(0)	434.28 ± 12.44	433.17	157.77 ± 10.04	157.16

TXD105 strain grown in 50 mL M1D medium (pH 6.0) inoculated with 2 mL inoculum size of 5-day-old culture and incubated at 120 rpm and 25 °C for 10 days. TER995 strain grown in 50 mL FBM medium (pH 6.0) inoculated with 4 mL inoculum size of 7-day-old culture and incubated at 120 rpm and 25 °C for 14 days. Calculated means is for triplicate measurements from two independent experiments.

blue spots on TLC plates (Cardellina, 1991). The spots corresponding to the paclitaxel standard (Rf = 0.67) were carefully collected by scrapping off the silica gel and dissolved in methanol. Quantitative analysis of paclitaxel was performed using UV-spectroscopic analysis performed (JENWAY-305 spectrophotometer, UK). Paclitaxel concentration was estimated after recording the absorption at 273 nm against a standard curve (Gangadevi and Muthumary, 2008; Zhang et al., 2009).

The identity of paclitaxel from TXD105 and TER995 cultures was further confirmed by HPLC analysis (EZChrom Elite Client/Server, Agilent, USA). The HPLC analysis column specifications are 4.5 × 250 nm, 2.5 μm of Hypersil BDS-C18 at 24 °C. The mobile phase consisted of methanol: water at a ratio of 65:35 (v:v) and was used at a flow rate of 1.0 mL min^{-1}. The UV detector was set at a wavelength of 228 nm and an injection volume of 20 μL was used (Gangadevi and Muthumary, 2008; Deng et al., 2009). The data of HPLC analysis of the sample extracted (either from TXD105 or TER995 strain) were identical with those of the authentic sample of paclitaxel analyzed under the same conditions (Fig. S1).

2.12. Statistical analyses

Statistical significance was performed using the ANOVA test (SPSS software version 22, IBM Corp., NY) and by the least significant difference (LSD) test at 0.05 level. The obtained results were expressed as the mean ± standard deviation (SD).

3. Results

3.1. Optimization of major medium constituents

3.1.1. Carbon sources

Sucrose for the TXD105 strain and glucose for the TER995 strain were found the most favorable substrates among the tested carbon sources where significant differences ($P \leq 0.05$) in paclitaxel concentrations were obtained (Table 1). For the TXD105 strain, the maximum concentration of paclitaxel recorded was 317.84 ± 19.03 μg L^{-1} in sucrose amended M1D broth, however, the combination of carbon sources in the original medium (sucrose, glucose, and fructose) recorded 307.03 ± 07.39 μg L^{-1}. Moreover, fructose followed by glucose was good carbon sources for paclitaxel production where comparable paclitaxel levels were achieved (314.59 ± 23.24 μg L^{-1} and 312.43 ± 12.48 μg L^{-1}). Regarding the TER995 strain, the best carbon source that supported the maximum paclitaxel production was glucose (124.05 ± 13.24 μg L^{-1}). Sucrose followed by fructose was good carbon sources for paclitaxel production where the production level reached was 120.54 ± 19.39 μg L^{-1} and 118.65 ± 12.48 μg L^{-1}, respectively. For both fungal strains, glucose was the best carbon source that supported maximum cell growth (10.33 ± 0.29 g L^{-1} for TXD105 and 13.41 ± 0.67 g L^{-1} for TER995 strain).

Fungal growth and paclitaxel production increased with increased concentrations of either sucrose or glucose till a maximum value was attained after which a decreased levels of paclitaxel of both the two fungal strains were obtained. In the case of TXD105 strain, maximum growth (10.62 ± 0.53 g L^{-1}) was attained on using M1D broth amended with sucrose as a sole carbon source at a concentration of 40 g L^{-1}. However, the maximum paclitaxel production (321.62 ± 26.36 μg L^{-1}) was achieved at a concentration of 20 g sucrose L^{-1}. Comparable concentrations of paclitaxel (315.68 ± 23.38 μg L^{-1}) were also obtained on using 10 g sucrose L^{-1}. Regarding the TER995 strain, the maximum growth (13.14 ± 0.84 g L^{-1}) was obtained on using FBM amended with glucose as a sole carbon source at a concentration of 100 g L^{-1}. However, the maximum paclitaxel production (124.78 ± 17.51 μg L^{-1}) was recorded at a concentration of 80 g glucose L^{-1} (Fig. 1a–b).

3.1.2. Nitrogen sources

Data recorded in Table 1 revealed that dry biomass and paclitaxel levels by both strains were greatly influenced by the type of nitrogen source employed. The maximum fungal growth of both strains was obtained on using casein (10.30 ± 0.52 g L^{-1} for TXD105 strain and 13.83 ± 0.55 g L^{-1} for TER995 strain). However, maximum paclitaxel production (327.57 ± 17.43 μg L^{-1} and 131.27 ± 12.98 μg L^{-1}, for TXD105 and TER995 strains; respectively) was achieved on using ammonium nitrate for the first strain and peptone for the second strain. For both fungal strains, urea proved to be unsatisfactory nitrogen source for both growth and paclitaxel production where the lowest values were recorded.

The results in Fig. 1c–d revealed that any increase in either ammonium nitrate or peptone concentrations was accompanied by an

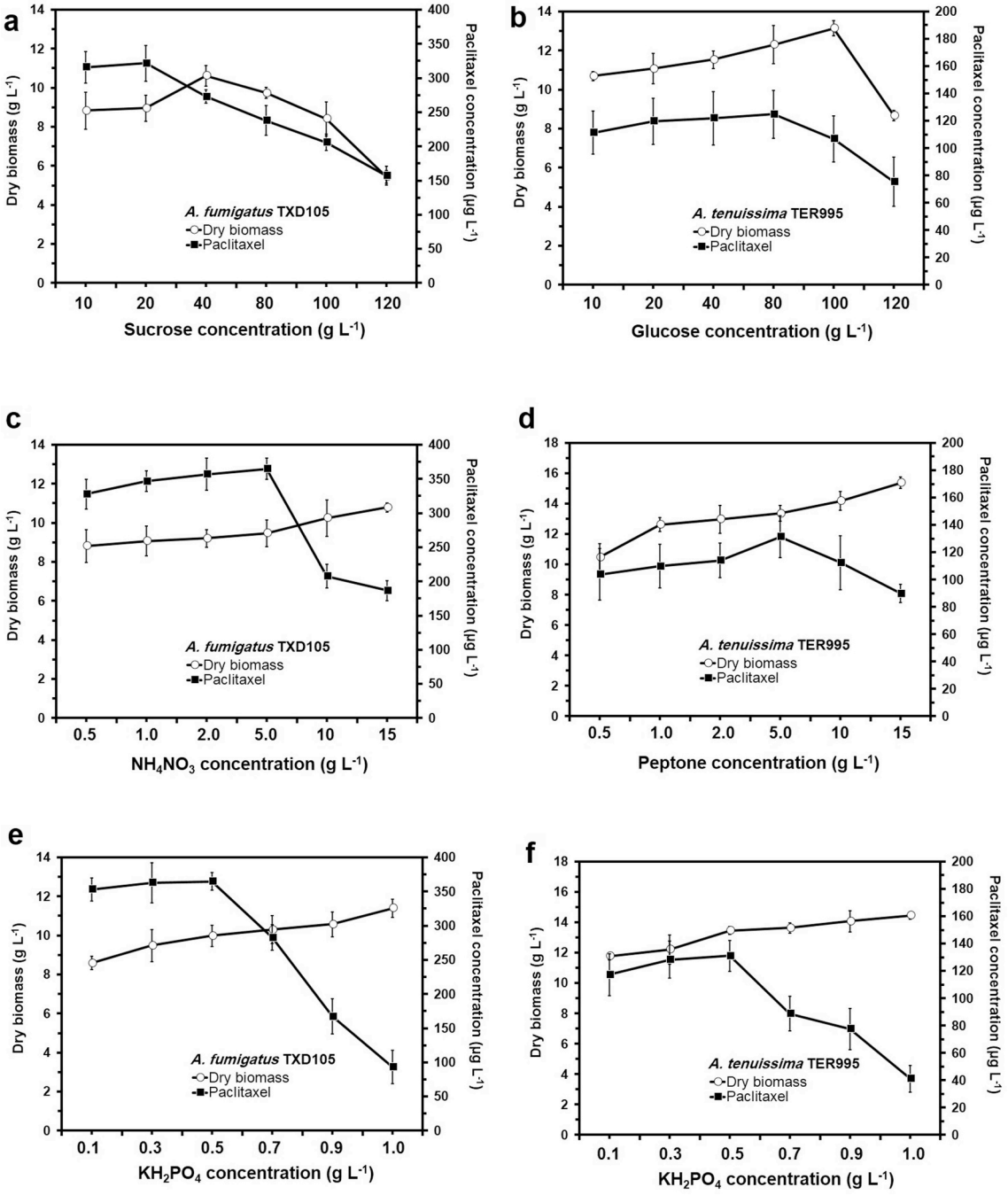

Fig. 1. Effect of different concentrations of sucrose, NH_4NO_3 and KH_2PO_4 on growth (g L^{-1}) and paclitaxel production (µg L^{-1} culture filtrate) by *A. fumigatus* TXD105 **(a, c, e)** and effect of different concentrations of glucose, peptone and KH_2PO_4 on growth (g L^{-1}) and paclitaxel production (µg L^{-1} culture filtrate) by *A. tenuissima* TER995 **(b, d, f)**. In **(a), (c)** and **(e)**, TXD105 cultures were carried out in 50 mL M1D medium inoculated with 2 mL inoculum size of 5-day-old culture and incubated at 120 rev min^{-1} and 25 °C for 10 days. In **(b)**, **(d)** and **(f)**, TER995 cultures were carried out in 50 mL FBM medium inoculated with 4 mL inoculum size of 7-day-old culture and incubated at 120 rev min^{-1} and 25 °C for 14 days. All data are shown as the mean ± SD of triplicate measurements from two independent experiments.

increase in the fungal growth of both strains reaching maximum (10.79 ± 0.23 g L^{-1} and 15.37 ± 0.38 g L^{-1} for TXD105 and TER995 strains, respectively) at 15 g L^{-1}. The obtained results also indicated that the average values of paclitaxel concentration were increased gradually with the increase of nitrogen substrate concentration reaching maximum (364.49 ± 15.47 µg L^{-1} by TXD105 strain, and 131.35 ± 15.41 µg L^{-1} by TER995 strain) at 5.0 g L^{-1}, after which a decline in the values of paclitaxel production was obtained.

3.1.3. Phosphorus sources

Potassium dihydrogen phosphate as a sole phosphorus source was the most effective for both growth and production of paclitaxel by the two strains where the maximum values were obtained (9.47 ± 0.91 g L^{-1} biomass and 364.86 ± 27.43 µg L^{-1} paclitaxel for TXD105 strain and 13.47 ± 0.36 g L^{-1} biomass and 131.35 ± 16.13 µg L^{-1} paclitaxel for TER995 strain). On using dipotassium hydrogen phosphate, satisfactory paclitaxel concentrations by both fungal strains were obtained (357.30 ± 15.21 µg L^{-1} for the first

strain and 114.59 ± 16.50 μg L^{-1} by the second strain). However, on using ammonium dihydrogen phosphate as a phosphorus source, the lowest paclitaxel concentrations were obtained (Table 1).

The results in Fig. 1e–f revealed that the concentration of potassium dihydrogen phosphate in the basal broth media (either M1D or FBM) greatly affected both growth and production of paclitaxel by both fungal strains. The average values of fungal growth of both strains were increased gradually with the increase of potassium dihydrogen phosphate concentration reaching maximum (11.39 ± 0.46 g L^{-1} by TXD105 strain, and 14.45 ± 0.24 g L^{-1} by TER995 strain) at 1.0 g L^{-1}. Regarding paclitaxel production by both strains, the productivities of the two strains were increased gradually with the increase of potassium dihydrogen concentration reaching maximum (364.86 ± 12.67 μg L^{-1} by TXD105 strain, and 131.08 ± 11.24 μg L^{-1} by TER995 strain) at 0.5 g L^{-1}, after which a decline was obtained at 0.7–1.0 g L^{-1}.

3.2. Plackett–Burman (PB) design

The matrices developed by the PB design and the obtained results (paclitaxel production by the two fungal strains) are presented in Table 3. Values of "Prob > *F*" less than 0.05 indicate model terms are significant. From the regression analysis, values of "Model Prob > *F*" were 0.0381 and 0.0229 for TXD105 and TER995 strains, respectively; which indicated that the used two models for each fungal strain are significant (Table 4).

Data presented in Table 4 indicated that the most three relevant factors (magnesium sulphate, sodium acetate, and phenylalanine) that had a high contribution to paclitaxel production by both strains. The three selected factors were subjected to response surface methodology for further determining their optimum concentrations.

3.3. Response surface methodology (RSM) optimization

Table 5 presents the Box–Behnken (BB) design matrices for the factors, given in both coded and actual values, plus the predicted and experimental paclitaxel concentration results of the two fungal strains. The obtained results for each fungal strain were analyzed by linear multiple regression using the Design-Expert (Version 9.0; Stat-Ease, Inc.) and the following equations were obtained:

$$\text{Paclitaxel}_{TXD105} = +433.17 - 3.56A - 4.47B - 10.57C - 15.92AB - 3.02AC - 0.60BC - 11.71A^2 - 21.64B^2 - 18.46C^2$$

$$\text{Paclitaxel}_{TER995} = +157.16 + 9.61A + 10.25B - 7.92C + 8.58AB - 1.74AC - 2.29BC - 23.77A^2 - 9.64B^2 - 11.60C^2$$

where A, B, and C are the symbols of the concentration of magnesium sulphate, sodium acetate, and phenylalanine, respectively. The significance of the fit of the second-order polynomial for the concentration of paclitaxel production by the two fungal strains was assessed by carrying out analysis of variance (ANOVA) as shown in Table 6.

Regarding TXD105 strain, the model terms are significant because values of "Prob > F" are less than 0.05. A model very low probability value [(Prob > F) less than 0.0001] imply that the model shows a significant fit to the experimental data. From the regression model, the model terms B, C, A^2, B^2, C^2, and AB were significant with a probability of 95%. The terms A, AC and BC had no significant effect on the production process of paclitaxel. The coefficient of determination (R^2) of the model was 0.9789, which indicated that the model adequately represented the relationship between the tested factors and response (paclitaxel production). The coefficient of variation (C.V.) obtained was 1.10%. The Coefficient of Variation (C.V) indicates the degree of precision with which the treatments were carried out. The Lack of Fit Prob > *F*-value of 0.1332 implies that the lack of fit is insignificant (Table 6).

In the case of TER995 strain (Table 6), the model which showed very low probability value [(Prob > F) less than 0.0001] imply that the model shows significant fit to the experimental data. From the regression model, the model terms A, B, C, A^2, B^2, C^2, and AB were significant with a probability of 95%. The terms AC and BC had no significant effect on the production process of paclitaxel. The coefficient of determination (R^2) of the model was 0.9946, which indicated that the model adequately represented the relationship between the tested factors and response (paclitaxel production). The coefficient of variation (C.V.) obtained was 1.59%. The Lack of Fit Prob > *F*-value of 0.1689 implies that the lack of fit is insignificant.

In order to detect the optimum concentrations, 2D contour and 3D response surface plots (graphical representations of regression equation) were generated during data analysis (Figs. 2 and 3). The study of these plots indicated that maximum paclitaxel production by the two fungal strains was obtained when the concentration of magnesium sulphate, sodium acetate and phenylalanine were 0.6, 1.5 and 0.075 g L^{-1}, respectively.

To evaluate the performance of the optimum micronutrients concentrations on the production of paclitaxel, each fungal strain was cultivated in its corresponding optimized medium. In the case of TXD105 strain, the predicted paclitaxel concentration at the optimum levels was 433.17 μg L^{-1} while the actual experimental concentration was 435.43 μg L^{-1}. Regarding TER995 strain, the predicted and actual paclitaxel concentrations at the optimum levels were 157.16 μg L^{-1} and 163.75 μg L^{-1}, respectively. The use of RSM leaded in increases in the paclitaxel production TXD105 and TER995 strains by 16.25 and 19.86%, respectively. The results from two independent experiments were coincident with the estimated value, thereby confirming the high accuracy of the model used for each fungal strain.

3.4. Effect of UV irradiation on the growth and paclitaxel production

Data presented in Fig. 4a–b showed that the dry biomass and paclitaxel production of both strains were significantly influenced by UV-light irradiation. The reduced effect of UV irradiation on cell growth of both strains was found to increase by increasing time of exposure. The dry biomass of TXD105 and TER995 strains after 40 min of UV-exposure was reduced significantly ($P \leq 0.05$) by 83.76 and 76.33% of their respective controls (non-irradiated). After 50 min of UV-exposure, no fungal growth of the first strain was observed, however, the dry biomass of the second strain was significantly ($P \leq 0.05$) reduced by 92.51%.

The production of paclitaxel by TXD105 and TER995 strain increased by increasing the time period of irradiation, and maximum paclitaxel concentrations (504.56 ± 14.02 and 195.64 ± 12.49 μg L^{-1}, respectively) were significantly ($P \leq 0.05$) obtained after 30 min of UV-exposure. Increasing the exposure time more than 30 min, a decline was observed on increasing the time period (Fig. 4a–b). The obtained paclitaxel concentrations by TXD105 and TER995 strain irradiated for 30 min were 1.16 and 1.20 fold, respectively of their controls.

3.5. Effect of ^{60}Co gamma irradiation on the growth and paclitaxel production

Fig. 4c–d showed that the effect of gamma irradiation on the fungal growth of both strains was found to be dose-related. At higher doses of irradiation (1.50 and 1.75 KGy), the maximum reduction in the dry biomass of both fungal strains was recoded. At 1.50 KGy, the dry biomass of the TXD105 strain was reduced by 81.75%, as compared with control (non-irradiated). Regarding TER995 strain, the recorded dry biomass was reduced by 89.87% of that at control culture.

The most effective dose of gamma irradiation on paclitaxel production was 0.75 KGy, and significant differences ($P \leq 0.05$) in paclitaxel concentrations by the two strains were obtained at this dose. Maximum paclitaxel concentrations recorded were 495.31 ± 16.31 μg L^{-1} and 195.41 ± 15.35 μg L^{-1}, by TXD105 and

Table 6
Analysis of variance (ANOVA) for paclitaxel production by *A. fumigatus* TXD105 and *A. tenuissima* TER995.

Source	*A. fumigatus* TXD105					*A. tenuissima* TER995				
	SS	d.f.	MS	*F*-value	Prob > *F*	SS	d.f.	MS	*F*-value	Prob > *F*
Model	6622.63	9	735.85	36.16	< 0.0001	6056.21	9	672.91	144.04	< 0.0001
A	101.25	1	101.25	4.97	0.0609	738.43	1	738.43	158.07	< 0.0001
B	159.85	1	159.85	7.85	0.0264	840.71	1	840.71	179.96	< 0.0001
C	893.38	1	893.38	43.90	0.0003	501.65	1	501.65	107.38	< 0.0001
AB	1014.10	1	1014.10	49.83	0.0002	294.29	1	294.29	63.00	< 0.0001
AC	36.54	1	36.54	1.80	0.2221	12.08	1	12.08	2.58	0.1519
BC	1.43	1	1.43	0.070	0.7987	20.98	1	20.98	4.49	0.0718
A^2	577.49	1	577.49	28.38	0.0011	2379.40	1	2379.4	509.33	< 0.0001
B^2	1971.97	1	1971.97	96.90	< 0.0001	391.65	1	391.65	83.84	< 0.0001
C^2	1434.24	1	1434.24	70.47	< 0.0001	567.01	1	567.01	121.37	< 0.0001
Lack of Fit	102.46	3	34.15	3.42	0.1332	22.28	3	7.43	2.85	0.1689
	S.D.	4.51	R^2	0.9789		S.D.	2.16	R^2	0.9946	
	Mean	408.79	Adjusted R^2	0.9519		Mean	135.98	Adjusted R^2	0.9877	
	C.V. %	1.10	Predicted R^2	0.7484		C.V. %	1.59	Predicted R^2	0.9388	
	PRESS	1701.83	Adequate Precision	16.562		PRESS	372.69	Adequate Precision	30.865	

SS sum square, d.f. degree of freedom, MS mean square, S.D. standard deviation, C.V. coefficient of variation, R^2 coefficient of determination.

TER995 strain, respectively. On increasing the radiation dose in the range of 1.50–1.75 KGy, the lowest paclitaxel production was then gradually obtained. No fungal growth was observed by the two fungal strains at doses of 1.75 and 2.00 KGy, respectively.

4. Discussion

Paclitaxel is a mitotic inhibitor that has been used in chemotherapy for many forms of cancers since the 1970s (Zhang et al., 2009). In this study, the effect of nutritional requirements represented by carbon, nitrogen, and phosphorus sources as well as micronutrient on paclitaxel production was investigated. Testing different carbon sources showed that maximum paclitaxel production by the TXD105 strain was attained on using sucrose (2%, w/v) as a sole carbon source. In partial agreement with our results, Sonaimuthu et al. (2010) reported that among the tested carbon sources, sucrose (1%, w/v) supported both maximum growth and paclitaxel production of *Pestalotiopsis oxyanthi* SVJM060. However, Garyali et al. (2014) used sucrose at a concentration of 8% (w/v) for maximum paclitaxel production by *Fusarium redolens*. Regarding the TER995 strain, glucose (8%, w/v) was the optimum carbon source for the maximum paclitaxel production process. In concurrence with these results, Xu et al. (2006) showed that the highest paclitaxel yield was obtained on using medium amended with glucose (8%, w/v) as a sole carbon source. However, Deng et al. (2009) used glucose at a concentration of 4% (w/v) for maximum paclitaxel production. Also, Dai and Tao (2008) used glucose for maximum paclitaxel production by *Fusarium mairei* UH23. Meanwhile, Rui et al. (2011) reported that maltose (1%, w/v) was the best carbon source for maximum paclitaxel production process among the tested carbon sources utilized by the endophytic strain XC1-07.

Testing the effect of different nitrogen sources on the production of paclitaxel by the two fungal strains showed that maximum paclitaxel production was attained on using ammonium nitrate for the TXD105 strain and peptone for the TER995 strain both at a concentration of 0.5% (w/v). In literature, different concentrations of ammonium nitrate were used for the maximum paclitaxel production process. Xu et al. (2006) used 0.5%, w/v, Garyali et al. (2014) used 0.8%, w/v and Rui et al. (2011) used 0.9%, w/v. Meanwhile, Deng et al. (2009) used a combination of peptone, yeast extract and ammonium sulphate for maximum paclitaxel production by *Fusarium solani* Tax-3. Sonaimuthu et al. (2010) showed that among the tested nitrogen sources ammonium tartarate (1%, w/v) supported both maximum growth and paclitaxel production by *Pestalotiopsis oxyanthi* SVJM060.

The present study showed that maximum paclitaxel production by both fungal strains was recorded on using potassium dihydrogen phosphate as a phosphorus source at a concentration of 0.5 g L^{-1}. In accordance with these results, Xu et al. (2006) showed that maximum paclitaxel by *F. maire* Y1117 was obtained on using medium amended with 0.5 g potassium dihydrogen phosphate L^{-1} as a sole phosphorus source. Other reports used the same phosphorus source for paclitaxel production but at higher concentrations. Deng et al. (2009) used 2 g L^{-1} potassium dihydrogen phosphate for maximum paclitaxel production by *Fusarium solani* Tax-3. However, some reports used sodium dihydrogen phosphate at a concentration of 20 mg L^{-1} for paclitaxel production by *Pestalotiopsis microspora* and *Epicoccum nigrum* (Li et al., 1996; Somjaipeng et al., 2015). The superiority of specific carbon, nitrogen and phosphorus sources over other sources in the production of paclitaxel and/or fungal growth could be attributed to the nature of the fungal strain. Medium components are intrinsically linked to the nature of the microbial strain. This is because no one medium works best for all the strains being tested (Kennedy and Krouse, 1999).

In this study, optimization of media compositions was performed by using response surface methodology (RSM). The obtained results showed that maximum paclitaxel production by the two fungal strains was obtained separately when the concentration of magnesium sulphate, sodium acetate and phenylalanine were 0.6, 1.5 and 0.075 g L^{-1}, respectively. The obtained results further showed that the use of RSM has developed increases in the paclitaxel production process of the TXD105 and TER995 strains by 16.25% and 19.86%, respectively, when compared with their unoptimized control cultures. In partial agreement with these results, Xu et al. (2006) reported that sodium acetate, ammonium nitrate, and magnesium sulphate were the most important components in the cultivation medium which were optimized by RSM. These authors found that the optimal concentrations of these components were 2.02, 7.84 and 0.68 g L^{-1}, respectively. The authors further concluded that using RSM has led to enhancement in the production of paclitaxel which was 31% higher than the basal media. Also, Garyali et al. (2014) reported that ammonium nitrate (6.25 g L^{-1}), magnesium sulphate (0.63 g L^{-1}) and sodium acetate (1.25 g L^{-1}), were the significant constituents for achieving maximum paclitaxel production after applying RSM optimization. Their study revealed that optimization of culture components intensified the production of paclitaxel from 66 to 198 µg L^{-1}, which is three fold higher than that produced in the original medium. Zhao et al. (2011) used orthogonal design to optimize the fermentation medium of paclitaxel produced by the fungus *Nodulisporum sylviforme* and they found that modified S-7 medium supplemented with serine (10 mg L^{-1}), salicylic acid (80 mg L^{-1}), silver nitrate (8.5 mg L^{-1}) and ammonium acetate

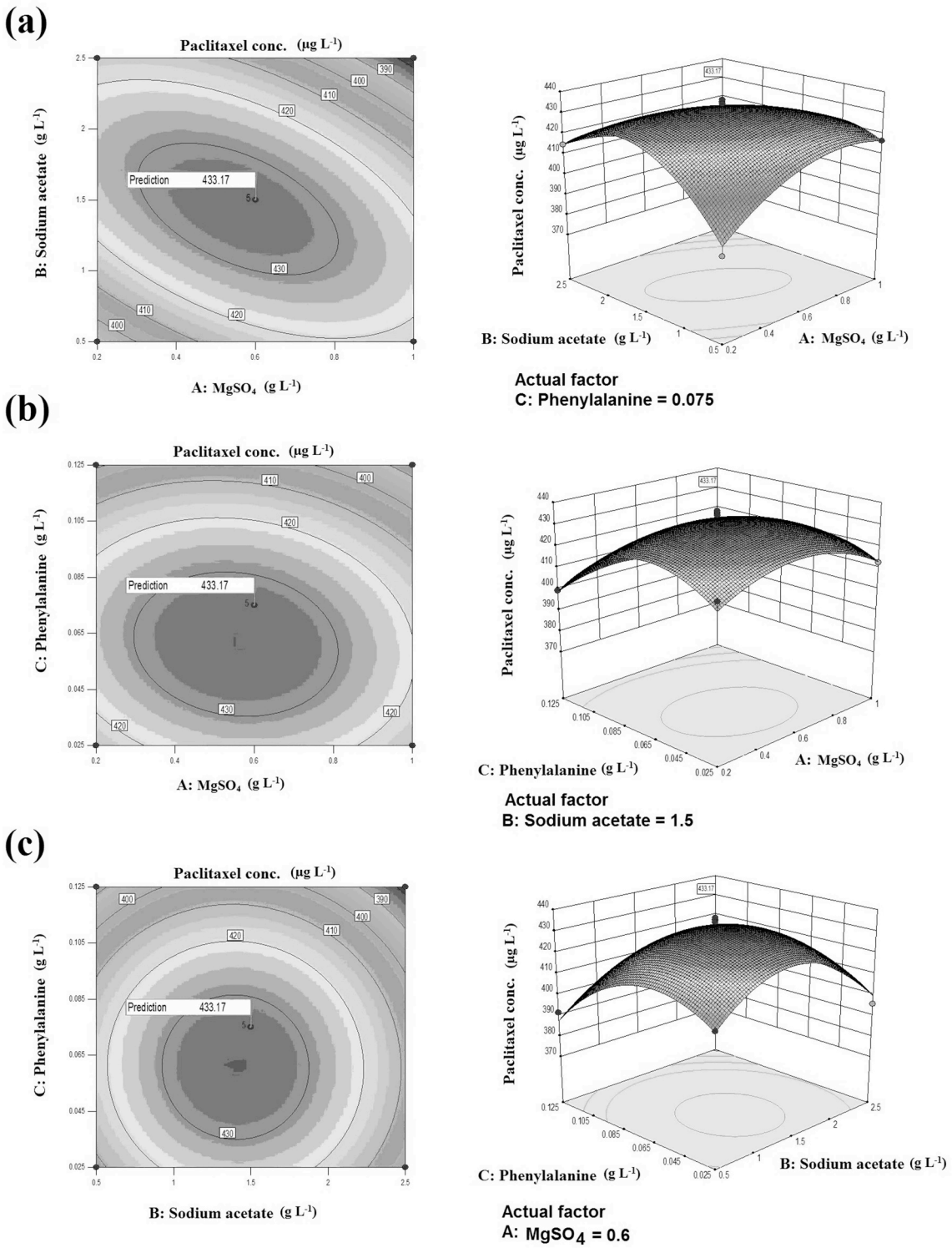

Fig. 2. 2D contour and 3D surface plots showing the effect of different factors on paclitaxel production by *A. fumigatus* TXD105. **(a)** Effect of sodium acetate and magnesium sulphate on paclitaxel production. **(b)** Effect of magnesium sulphate and phenylalanine on paclitaxel production. **(c)** Effect of sodium acetate and phenylalanine on paclitaxel production.

(20 mg L^{-1}) has increased the yield of paclitaxel by 115% of the yield obtained with the original medium. Additionally, Rui et al. (2011) used orthogonal design to optimize the fermentation medium for maximum paclitaxel production by XC1-07 strain. They showed that the yield of paclitaxel increased from 780 to 1124.34 µg L^{-1} after using the optimal medium containing ammonium nitrate, maltose and magnesium sulfate at concentrations of 10, 90 1.0 g L^{-1}, respectively.

In the present study, results of determining the spore viability of two experimental strains following exposure to UV and gamma rays showed clearly that the TER995 strain was more resistant than the TXD105 strain to UV and gamma radiation where higher lethal doses were recorded by the first strain. Regarding gamma irradiation, the lethal dose of the TXD105 strain was 1.75 KGy, however, it was 2.00 KGy for the TER995 strain. In the case of UV light irradiation, the lethal doses of the

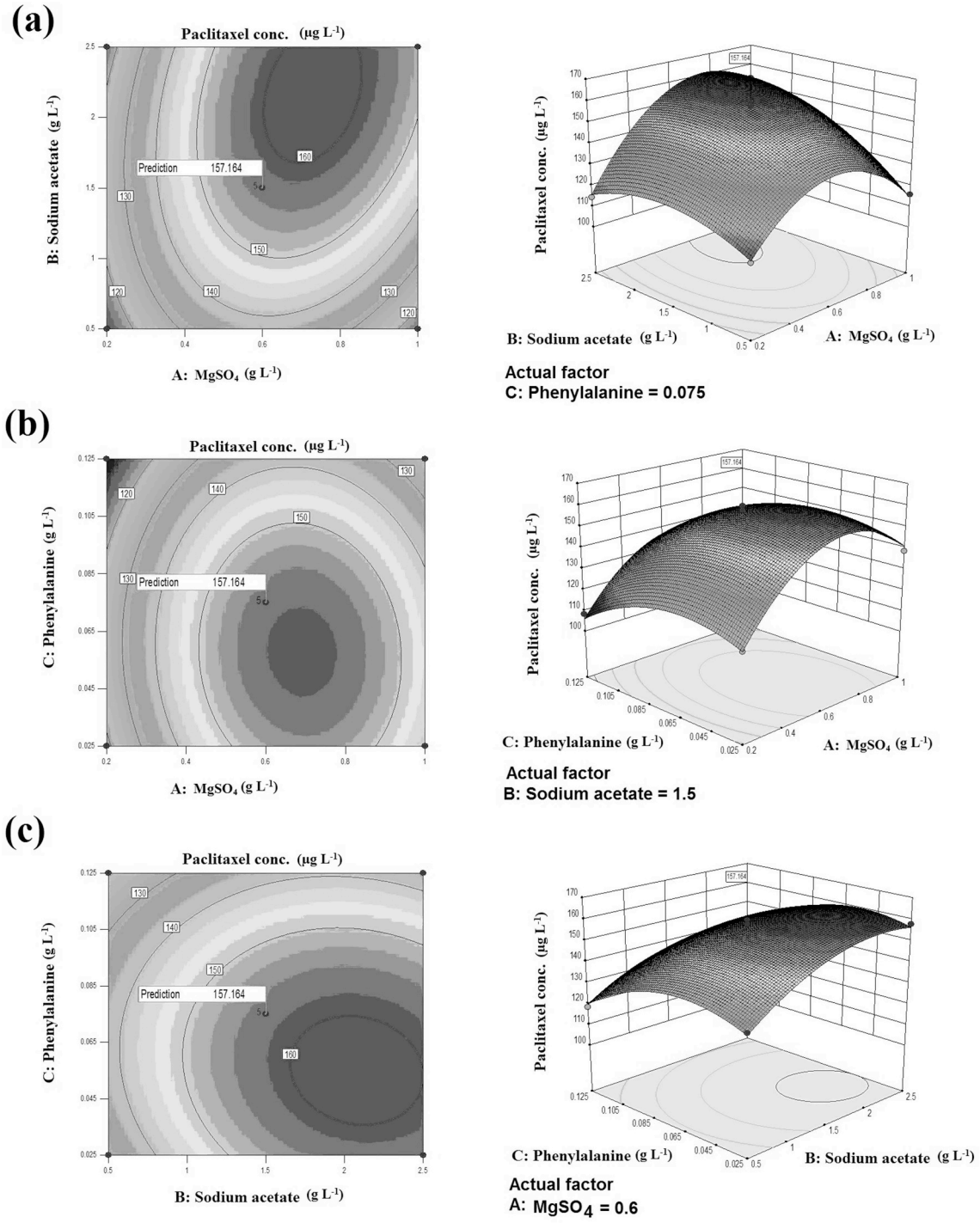

Fig. 3. 2D contour and 3D surface plots showing the effect of different factors on paclitaxel production by *A. tenuissima* TER995. **(a)** Effect of sodium acetate and magnesium sulphate on paclitaxel production. **(b)** Effect of magnesium sulphate and phenylalanine on paclitaxel production. **(c)** Effect of sodium acetate and phenylalanine on paclitaxel production.

two strains were 50 and 60 min, respectively. In this respect, Dadachova and Casadevall (2008) attributed this resistance property to the presence of melanin pigments. Melanin pigments are complex polymers with a variety of properties that can be made enzymatically from relatively simple precursors. A remarkable feature of melanins is their ability to absorb all types of electromagnetic radiation that endows them with the capacity for both energy transduction and shielding (Dadachova and Casadevall, 2008).

The obtained results showed that maximum paclitaxel concentration was attained after 30 min of UV-exposure. The paclitaxel production by TXD105 and the TER995 strain was 1.16 fold and 1.20 fold of their control cultures. Regarding the effect of gamma radiation on paclitaxel production, 0.75 KGy was the optimum dose for maximum paclitaxel concentrations either from TXD105 or TER995 strain. In the same connection, Zhao et al. (2004) used a series of mutagenic agents including UV and gamma radiation for improvement of *Nodulisporium sylviforme* HQD33 strain for higher paclitaxel production (from 125.7 to 314.07 µg L^{-1}). Moreover, the same gamma radiation dose (0.75 KGy) was used in the production enhancement of mycophenolic acid by two *Penicillium roqueforti* strains (Ismaiel et al., 2014, 2015). Zhao et al.

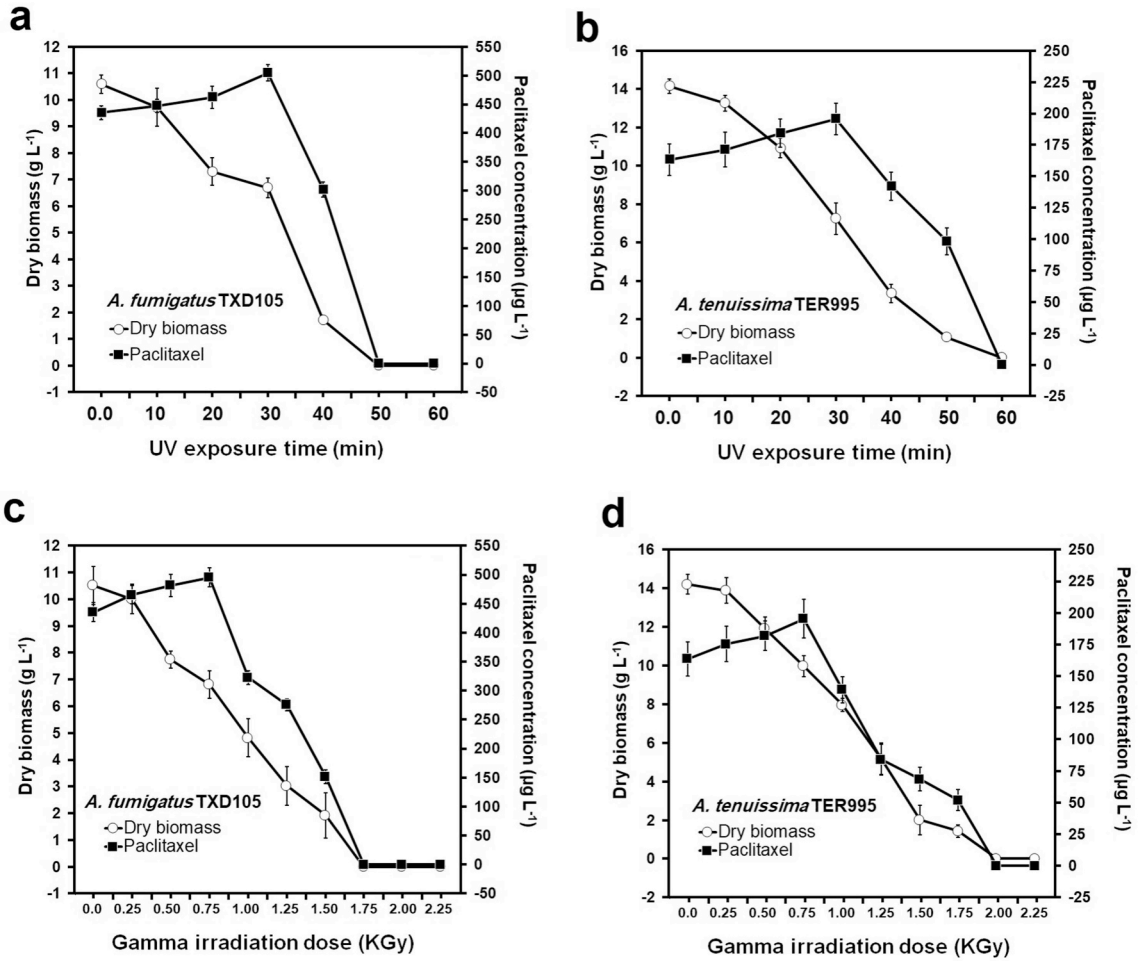

Fig. 4. Effect of UV light irradiation (254 nm, a distance 10 cm for different periods of time) on growth (g L^{-1}) and paclitaxel production (µg L^{-1} culture filtrate) by *A. fumigatus* TXD105 **(a)** and *A. tenuissima* TER995 **(b)**. Effect of different gamma-irradiation doses (KGy) on growth (g L^{-1}) and paclitaxel production (µg L^{-1} culture filtrate) by *A. fumigatus* TXD105 **(c)** and *A. tenuissima* TER995 **(d)**. TXD105 cultures were carried out in 50 mL MM1D medium inoculated with 2 mL inoculum size of 5-day-old culture and incubated at 120 rev min^{-1} and 25 °C for 10 days. TER995 cultures were carried out in 50 mL MFBM medium inoculated with 4 mL inoculum size of 7-day-old culture and incubated at 120 rev min^{-1} and 25 °C for 14 days. All data are shown as the mean ± SD of triplicate measurements from two independent experiments.

(2008a) reported enhanced production of paclitaxel by UV radiation and chemical mutagenesis of HD1-3 strain. They succeeded to obtain a high-yielding strain whose production was 1.41 times than that of the original strain. Xu et al. (2006) succeeded in the improvement of *Fusarium mairie* strain (paclitaxel-producing fungus) and optimization of the medium constituents. The authors reported an increase in the paclitaxel yield from 20 to 225.2 µg L^{-1}. Zhao et al. (2008b) have applied the fundamental principles of genome shuffling in the breeding of the paclitaxel-producing fungus *Nodulisporium sylviforme*. They found that three hereditarily stable strains showed high paclitaxel production. One of the three strains *Nodulisporium sylviforme* F4-26 produced a higher yield of paclitaxel (516.37 µg L^{-1}) than the original (356.80 µg L^{-1}).

5. Conclusion

Optimization of media compositions was performed by using response surface methodology. Maximum paclitaxel production by the two fungal strains was obtained when the concentration of magnesium sulphate, sodium acetate and phenylalanine were 0.6, 1.5 and 0.075 g L^{-1}, respectively. The use of response surface methodology has developed increases in the paclitaxel production process of the two strains by 16.25% and 19.86%, respectively, when compared with their unoptimized control cultures. In order to maximize their paclitaxel productivity, the two fungal cultures were subjected to different doses of UV and gamma radiation. Maximum paclitaxel concentration was attained after 30 min of UV-exposure. The paclitaxel production by the two strain was 1.16 fold and 1.20 fold of their control cultures. Regarding the effect of gamma radiation on paclitaxel production, 0.75 KGy was the optimum dose for maximum paclitaxel concentrations from both strain. Therefore, *A. fumigatus* TXD105 and *A. tenuissima* TER995 appear to be promising and attractive sources for developing a cost-effective paclitaxel fermentation process. Further research work is in progress to improve the fungal strains using radiation mutagenesis at the favorable doses. Moreover, the application of immobilization technique to scale up the process of paclitaxel production is used.

Competing interests

The authors declare that they have no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.01.034.

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