Research Article **Microbiology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

APPLICATION OF PLACKETT-BURMAN DESIGN TO EVALUATE MEDIA COMPONENTS AFFECTING ANTIOXIDANT ACTIVITY OF AN ENDOPHYTIC FUNGI *PENICILLIUM* SP. ISOLATED FROM BARK OF *AEGLE MARMELOS*

ROBIN SHARMA*[1], R. RASHMI[2] AND B. S. VIJAYA KUMAR[1]

[1]Department of Biosciences, Sri Sathya Sai Institute of Higher Learning, Prasanthi Nilayam Campus, Puttaparthi, Anantapur Dist., Andhra Pradesh, India.
[2]Microbiology Division, Defence Food Research Laboratory, Mysore, Karnataka, India.

ABSTRACT

Antioxidants are molecules that scavenge or neutralize free radicals. Free radicals are generated in human body during normal physiological processes and have implications on various human diseases. Endophytic fungi from medicinal plants are gaining tremendous impetus as potential source of antioxidant compounds. In this studyPlackett-Burman design was used to evaluate the media components affecting antioxidant activity of an endophytic fungus *Penicillium* sp. isolated from bark of *Aegle marmelos*. Antioxidant activity was assayed by different procedures and was compared for correlation (if any) with total phenolic content. The screening results from the Plackett-Burman design revealed sucrose and $NaNO_3$ to be the most significant in influencing antioxidant activity.This study therefore, highlights the determination of the media components, using the Plackett-Burman design, affecting the antioxidant activity of an endophytic fungus *Penicillium* sp. and also paves the way for future studies on optimization of the media components for enhancement of antioxidant activity of this endophyte.

KEY WORDS: ***Aegle marmelos*, Endophytic, *Penicillium* sp., Antioxidant, Plackett-Burman design**

ROBIN SHARMA
Department of Biosciences, Sri Sathya Sai Institute of Higher Learning,
Prasanthi Nilayam Campus, Puttaparthi, Anantapur Dist., Andhra Pradesh, India.

INTRODUCTION

Physiological and nutritional conditions greatly influence the growth as well as the activities of microorganisms [1]. Hence, choice of the best source of nutrients, such as carbon, nitrogen, minerals, trace metals, buffering agents, growth promoters and inducer/precursor forms one of the most critical stage in optimization of anyfermentation process. The conventional one-factor-at-a-time approach of optimization is tedious due to involvement of multivariate parameters where one faces an immense task of screening alarge number of constituents under each of the secategories that dictates the requirement of conducting alarge number of experiments that involves copious time and resources[2].In addition it ignores the interaction effect of the different parameters and is often incapable of providing the true optimum conditions. The optimization process generally includes the initial screening of important factors, followed by optimization of these selected factors by different techniques[1]. The Plackett-Burman design is a well-known and widely used statistical technique for screening and selection of most significant culture varibles[3, 4]. Therefore, in the present study we screened various media components for their role in antioxidant activity of endophytic fungal isolate *Penicillium* sp. by various assay procedures that include, DPPH assay, reducing power assay, FRAP assay and NO ion scavenging assay, using Plackett-Burman design. An effort was also made to find out the correlation (if any) between antioxidant activity and total phenolic content.

MATERIALS AND METHODS

Isolation and identification

An endophyte, *Penicillium* sp.isolated from bark of *Aegle marmelos*, growing in Vengalammacheruvu forest area of Puttaparthi Mandal (14º8´N,77º50´E), Anantapur Dist., Andhra Pradesh, India was identified based on rDNA sequencing of internal transcriber sequence (ITS) region in our previous study (NCBI GenBank accession no. KC560012). The culture was also deposited at the National Fungal Culture Collection of India (NFCCI), Agharkar Research Institute, Pune, India with NFCCI accession no. 3004.

Preparation of growth media and inoculation

The endophyte *Penicillium* sp.was grown in a 250-mL conical flask containing100.0 mL Czapek Dox's broth (containing defined proportion of various components). Few discs (8.0 mm) of fungal mycelia obtained from 7 days grown culture was inoculated unto the potato dextrose agar (PDA) plates. The growth was carried out under stationary conditions at 25ºC. After incubation for ten days, the culture broth was filtered through Whatman filter paper 1 (pore size: 11.0 µm) and the filtrate so obtained was analyzed for various antioxidant potential and also examined for total phenolic content.

Design of experiment using Plackett-Burman statistical method

In this study we anticipated to identify various ingredient of the medium that have significant effect on antioxidant potential of endophytic fungus *Penicillium* sp.using Plackett-Burman statistical experimental design. This design tests only two levels of each medium component and is a preliminary optimization tool for the rapid evaluation of the effects of various medium components[5]. However, it cannot provide the optimal quantity of each component required in the medium. Moreover, the design is orthogonal in nature, implying that the effect of each variable worked out is pure in nature and not confounded with interaction among variables[6]. Therefore, the screening of important parameters effecting antioxidant potential was studied using Plackett-Burman design as described in Chandra & Arora [1]. Five components of Czapek Dox's medium (Sucrose, $NaNO_3$, K_2HPO_4, KCl, and $MgSO_4$) were examined (Table 1). A total of 14 tests were designed including 12 combinations and 2 repetitions at central point with varying concentration of each factor. As indicated in Table 2 each row represents one trial and each column a single variable (medium component) respectively. The -1 and +1 element represent the lower and upper levels of each variable present

within each trial. Difference between the average of the + and – responses were considered for the determination of effect of each factor. The difference between the lower and the upper level of each factor was statistically significant or not was ascertained using student's t-test and the associated *p*-value. Effect of the medium constituent was calculated using the following equation as described in Naveen *et al.,*[7].

$E(x)_1 = 2 (\sum H_i - \sum L_i)/N$

Where, N = number of experiments (12 in this case), $\sum H_i$ = sum of the yields of the experiments where the level of i^{th} constituent is high, $\sum L_i$ = sum of the yields of the experiments where the level of the i^{th} constituent is low.

Variables		Levels		
		Low (-)	Central Point (g/100.0 mL)	High (+) (g/100.0 mL)
X_1	Sucrose	0	2.500	5.000
X_2	$NaNO_3$	0	0.175	0.350
X_3	K_2HPO_4	0	0.090	0.180
X_4	$MgSO_4$	0	0.045	0.090
X_5	KCl	0	0.045	0.090

Table 1
Medium components for screening using Plackett-Burman design

ANTIOXIDANT ASSAYS

DPPH radical scavenging activity

2.5 mL of the filtrate was mixed with methanolic solution containing 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals (0.1 mM, 0.5 mL). The solution was stirred and allowed to react (in dark) for 30 minutes. By measuring the absorbance at 517.0 nm using Spectramax M5 the reduction in the DPPH radical concentration was determined. DPPH solution without the extract was taken as control and methanol was taken as blank [8]. The percentage of DPPH scavenged was calculated using the equation:

% Scavenged = $[(A_c-A_s)/A_c] \times 100$

where, A_c is the absorbance of control, and A_s is the absorbance of solution containing sample extracts. 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (TROLOX) was used as standard. All experiments were carried out in triplicate.

Reducing power assay

Reducing power was measured by the method of Oyaizu [9] as described in Babu & Rao [8]. 2.5 mL of the filtrate was mixed with sodium phosphate buffer (0.2 M, pH 6.5, 2.5 mL) and potassium ferricyanide $[K_3Fe(CN)_6]$ (1%, 2.5 mL). The mixture was then incubated at 50ºC for twenty minutes. To this mixture trichloroacetic acid (10% w/v, 2.5 mL) was added and centrifuged at 3000 rpm for ten minutes. To the supernatant layer (2.5 mL), 2.5 mL of deionised water and ferric chloride (0.1%, 0.5 mL) were added, and the absorbance was measured at 700.0 nm (Spectramax M5). Higher absorbance indicated better reducing power. TROLOX was used as standard. All experiments were carried out in triplicate.

Ferric reducing antioxidant power (FRAP) assay

In Ferric reducing antioxidant power (FRAP) method, the complex formed when ferric tripyridyl triazine (Fe^{3+}-TPTZ) complex is reduced to the ferrous (Fe^{2+}) ion was determined using Spectramax M5. The oxidant in the FRAP assay was prepared by mixing TPTZ (10.0 mM in 40.0 mM HCl, 2.5 mL), acetate buffer (0.3 M pH 3.6, 25.0 mL), and 2.5 mL of $FeCl_3.6H_2O$ (20.0 mM). To 3600.0 µL of freshly prepared FRAP reagent, 360.0 µL of water and 120.0 µL of endophytic fungal filtrate was added. The mixture was then incubated at 37ºC for thirty minutes. The absorbance was measured at 595.0 nm. Higher absorbance indicated better ferric reducing ability of the extracts. Butylated hydroxytoluene (BHT) was used as standard [10]. All experiments were carried out in triplicate.

Nitric oxide ion scavenging assay

Nitric oxide was generated from sodium nitroprusside and measured by the Griess reaction as described in Panda *et al.,*[11]. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions which can be estimated by use of the Greiss reagent.. Sodium nitroprusside (5.0 mM) solution in phosphate-buffered saline (PBS) was mixed with 6.0 mL of fungal filtrate and incubated at 25°C for 180 minutes. A control experiment without test compound, but with the equivalent amount of double distilled H_2O was conducted in an identical manner. At intervals, samples (0.5 mL) of the incubation solution were removed and diluted with 0.5 mL of Griess reagent(1% sulphanilamide, 2% H_3P0_4 and 0.1% naphthylethylenediamine dihydrochloride). The absorbance of the chromaphore formed during diazotization of nitrite with sulphanilamide and subsequent coupling with naphthylethylenediamine was read at 546.0 nm using Spectramax M5 and referred to the absorbance of standard solutions of potassium nitrite treated in the same way with Griess reagent. The percentage of NO ion scavenged was calculated using the equation: % Scavenged = $[(A_c-A_s)/A_c] \times 100$ Where A_c is the absorbance of control, and A_s is the absorbance of solution containing sample extracts.

Determination of total phenolic content

Folin-Ciocalteu reagent [12] was used for determining the total phenolic content. To the mixture containing 1.5 mL distilled water and 0.5 mL Folin-Ciocalteu's reagent, 1.0 mL sample was added. After 1 minute, 1.0 mL 20% sodium carbonate solution was added. The final mixture was stirred and incubated at 25ºC for 2 hours in the dark. The absorbance of the mixture was measured at 760.0 nm using Spectramax M5. Using gallic acid (1.0-20.0 µg/mL) as standard, a standard graph was plotted, giving an equation as: Absorbance = 0.0305 gallic acid (µg/mL) + 0.0188 (R^2 = 0.9972) (Fig. 1). All tests were carried out in triplicate and the results were expressed as gallic acid equivalents (mg GAE/100.0 mL culture broth).

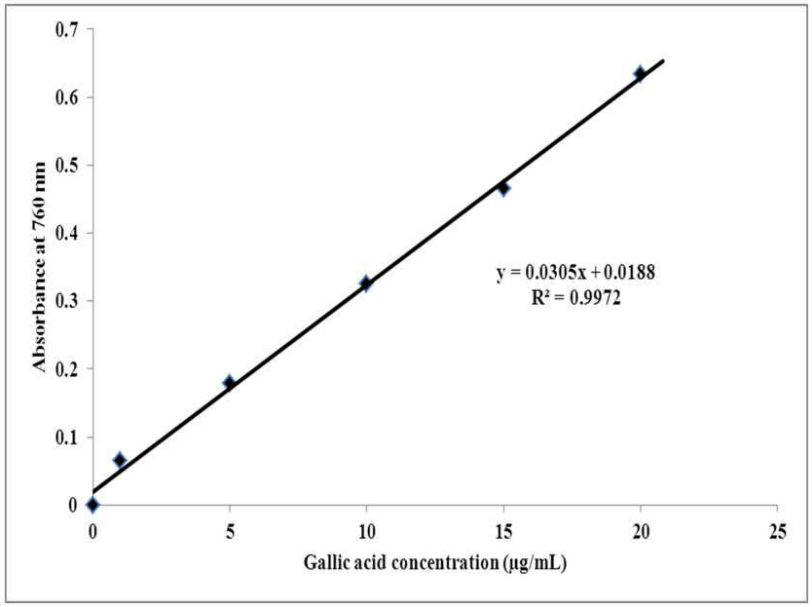

Figure 1
Folin-Ciocalteu Gallic acid standard graph at λ = 760 nm

RESULTS AND DISCUSSION

Endophytic fungi are reported as producers of important secondary metabolites with diverse biological activities. Recently they have also been identified to produce secondary metabolites that possess antioxidant activity [13]. Fungi allow easy manupulations and are easy to grow [1]. Nutritional and fermentation conditions play an important role and have direct influence on physiology and synthesis of secondary metabolites by microorganisms. Therefore, optimization of these parameters forms a very crucial step for the enhancement of yield. Lately many optimization processes have relied on statistical experimental designs [5, 14, 15] for ease of analysis. Antioxidant potential of *Penicillium* sp.assayed by different procedures and extracellularly produced total phenolic content varied significantly with the 14-run of different combinations of the media components (Table 2). The maximum antioxidant potential along with high TPC was observed in run order six and thirteen. The results were subjected to student's *t*-test which revealed sucrose to have statistically significant effect on antioxidant activities that include, DPPH scavenging activity, reducing power activity, NO ion scavenging activityandtotalphenoliccontentwith *p*-value≤0.05. On the other hand $NaNO_3$ had statistically significant effect on the antioxidant activities, that include, DPPHscavengingactivity,reducing poweractivity,FRAPactivityandtotalphenolicco ntentwith*p*-value≤ 0.1. Thus, we see that ofthefivevariablessucrose and $NaNO_3$ play the most critical role for antioxidant activity (Table 3 to7). We also performed a conventional testing of hypothesis procedure, where our null hypothesis (H_0) was that the antioxidant activity measured by various assay remains the same either in presence (+1) or in absence (-1) of different media components for different antioxidant assays and total phenolic content and the alternative hypothesis (H_1) was that the antioxidant activity by various assay is greater in presence (+1) of a media component than the antioxidant activity in the absence (-1)of a media component. The corresponding test-statistic is denoted by t_{Stat} and it was compared with the critical value t_{Crit} tabulated from the t-table with the right degrees of freedom. The level of significance (α) considered was 0.05.When $t_{Stat} < t_{Crit}$ then we consider the acceptance of null hypothesis (H_0). On the contrary, when $t_{Stat} > t_{Crit}$ the null hypothesis (H_0) is rejected. The analysis confirmed that for sucrose $t_{Stat} > t_{Crit}$ for DPPH assay, NO ion scavenging assay and total phenolic content (Table 3, 6 &7), therefore the rejection of the null hypothesis that sucrose has no effect on various antioxidant activities. This is in concordance with the inference from *p*-values as discussed above. The findings of this study complement the results of various previous reports[1, 16, 17] thereby, endorsing the importance of sucrose as carbon source and $NaNO_3$ as nitrogen source to regulate microbial growth and secondary metabolite production. However, this does not undermine the role of K_2HPO_4, KCl, and $MgSO_4$ as components of the media. It is well known that magnesium and potassium are necessary for all the fungi for a variety of regulatory functions and in the control of biosynthesis of plethora of secondary metabolites[1].

Run	Variables (%)					Antioxidant activity (% activity)				
	Sucrose	$NaNO_3$	K_2HPO_4	$MgSO_4$	KCl	DPPH assay	Reducing power assay	FRAP assay	NO scavenging assay	TPC mg/mL
1	1	-1	1	-1	-1	10.9000±02.8000	0.0970±0.0140	0.4920±0.0150	09.6500±7.1100	02.2300±0.0010
2	1	1	-1	1	-1	11.4400±00.7200	0.1540±0.0030	0.4970±0.0040	30.5700±4.0100	05.7600±0.0020
3	-1	1	1	-1	1	-	0.1100±0.0080	0.4720±0.0070	01.3700±0.0000	01.1300±0.0010
4	1	-1	1	1	-1	11.9400±03.8100	0.1160±0.0060	0.4850±0.0130	04.1300±2.3700	01.8300±0.0005
5	1	1	-1	1	1	37.0600±16.2500	0.1450±0.0090	0.5230±0.0090	17.0000±4.8100	05.4000±0.0010
6	1	1	1	-1	1	22.5100±01.1500	0.2100±0.0090	0.5910±0.0020	37.2300±4.9300	12.2000±0.0020
7	-1	1	1	1	-1	13.3100±04.1000	0.1180±0.0080	0.5490±0.0030	-	00.7600±0.0010
8	-1	-1	1	1	1	08.2700±01.4300	0.1160±0.0110	0.5490±0.0410	02.7500±3.5500	01.0300±0.0005
9	-1	-1	-1	1	1	04.3200±00.0000	0.1340±0.0090	0.4190±0.0090	33.1000±7.7700	01.4300±0.0000
10	1	-1	-1	-1	1	09.6000±03.4000	0.1440±0.0070	0.4800±0.0040	33.7900±2.4600	03.1600±0.0010
11	-1	1	-1	-1	-1	10.0700±03.2100	0.1160±0.0240	0.5030±0.0020	10.1100±2.7600	00.8600±0.0000
12	-1	-1	-1	-1	-1	-	-	-	-	-
13	0	0	0	0	0	19.3700±02.9000	0.4210±0.0110	0.6830±0.0050	16.5500±1.3600	26.3000±0.0050
14	0	0	0	0	0	12.6200±04.1400	0.1610±0.0070	0.5290±0.0010	58.6100±2.0500	05.1000±0.0010

Table 2
Plackett-Burman design variables with different antioxidant potential as response. Each value is expressed as mean ± standard deviation (n=3)

Factors	Components	Effect	S.E.	*t*-value	*p*-value	t_{Stat}	t_{Crit}
X1	Sucrose	11.25	1.6744	2.2785	0.0283	**2.2785**	1.8945
X2	$NaNO_3$	08.26	1.6744	1.4959	0.0926	1.4959	1.9431
X3	K_2HPO_4	-00.93	1.6744	-0.1525	0.4412	-0.1524	1.8595
X4	$MgSO_4$	05.54	1.6744	0.9515	0.1830	0.9515	1.8331
X5	KCl	04.02	1.6744	0.6751	0.2623	0.6751	1.9431

Table 3
Effect estimates for DPPH scavenging activity from the result of Plackett-Burman design

Factors	Components	Effect	S.E.	*t*-value	*p*-value	t_{Stat}	t_{Crit}
X1	Sucrose	0.045	0.0080	1.7765	0.0546	1.7765	1.8331
X2	$NaNO_3$	0.041	0.0080	1.5624	0.0763	1.5624	1.8331
X3	K_2HPO_4	0.012	0.0080	0.4251	0.3403	0.4251	1.8331
X4	$MgSO_4$	0.018	0.0080	0.6148	0.2806	0.6148	1.9431
X5	KCl	0.043	0.0080	1.6590	0.0657	1.6590	1.8331

Table 4
Effect estimates for Reducing Power activity from the result of Plackett-Burman design

Factors	Components	Effect	S.E.	*t*-value	*p*-value	t_{Stat}	t_{Crit}
X1	Sucrose	0.096	0.0254	1.1014	0.1604	1.1014	2.0150
X2	$NaNO_3$	0.118	0.0254	1.4026	0.1098	1.4026	2.0150
X3	K_2HPO_4	0.119	0.0254	1.4168	0.1031	1.4168	1.9431
X4	$MgSO_4$	0.081	0.0254	0.9094	0.1990	0.9094	1.9431
X5	KCl	0.085	0.0254	0.9586	0.1873	0.9586	1.9431

Table 5
Effect estimates for FRAP activity from the result of Plackett-Burman design

Factors	Components	Effect	S.E.	*t*-value	*p*-value	t_{Stat}	t_{Crit}
X1	Sucrose	14.17	2.4504	1.8425	0.0476	**1.8425**	1.8124
X2	$NaNO_3$	02.14	2.4504	0.2414	0.4070	0.2414	1.8124
X3	K_2HPO_4	-11.57	2.4504	-1.4260	0.0921	-1.4260	1.8124
X4	$MgSO_4$	-00.78	2.4504	-0.0861	0.4665	-0.0861	1.8124
X5	KCl	11.80	2.4504	1.4593	0.0892	1.4593	1.8331

Table 6
Effect estimates for NO scavenging activity from the result of Plackett-Burman design

Factors	Components	Effect	S.E.	*t*-value	*p*-value	t_{Stat}	t_{Crit}
X1	Sucrose	4.22	0.5695	2.6777	0.0219	**2.6777**	2.0150
X2	$NaNO_3$	2.74	0.5695	1.4571	0.0976	1.4571	1.9431
X3	K_2HPO_4	0.43	0.5695	0.2074	0.4204	0.2074	1.8595
X4	$MgSO_4$	-0.56	0.5695	-0.2724	0.3965	-0.2724	1.8945
X5	KCl	2.15	0.5695	0.3144	0.3809	0.3114	1.8124

Table 7
Effect estimates for Total Phenolic content from the result of Plackett-Burman design

Correlation between antioxidant activity and TPC

The results obtained indicate *Penicillium* sp.to be a potent antioxidant producer having broad spectrum activity against various free radicals. Chandra & Arora [1] demonstrated a strong linear correlation between total phenolic content and antioxidant activity.

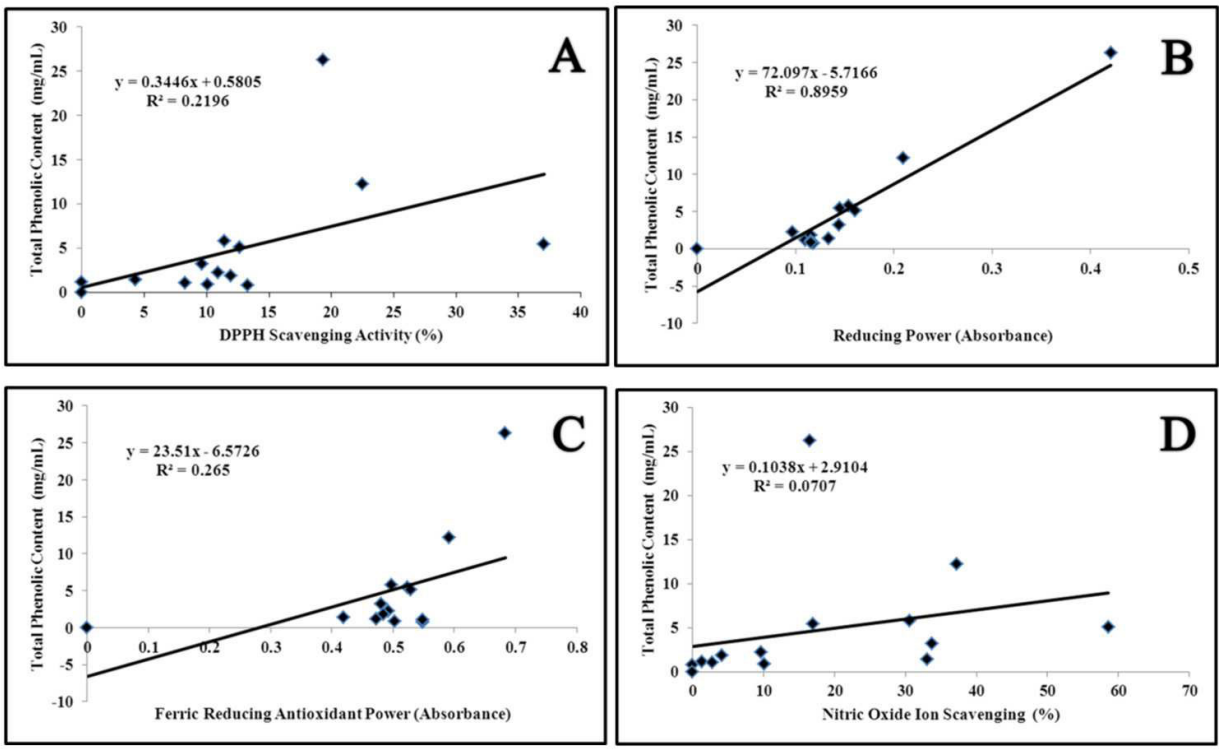

Figure 2

(A) Correlation between TPC and DPPH scavenging activity (B) Correlation between TPC and RP assay (C) Correlation between TPC and FRAP assay (D) Correlation between

TPC and NO ion scavenging assay

In this study too, the total phenolic content of *Penicillium* sp.correlated with the antioxidant activity, but in a lesser proportion. The extract of *Penicillium* sp.showed moderate activity against DPPH radical by neutralizing the free radical character of purple colour DPPH, either by transfer of electron or hydrogen atom, to yellow- colored diamagnetic molecule revealing hydrogen donating property of the extract [18]. The highest scavenging percentage was 37.06±16.25 noted for run number five (Table 2). The correlation analysis to find the measure of association between the phenolic contents and DPPH radical scavenging property revealed a coefficient of R^2 = 0.2196 (Figure 2A) which was much lesser as compared to the correlation reported in previous studies [19]. However, a high positive correlation of R^2 = 0.8959 (Figure 2B) was found between reducing power assay and total phenolic content. Reducing power proves the potential of the phenolic compounds in the extracts to act as reductones that inhibit lipid peroxidation by donating a hydrogen atom thereby terminating the free radical chain reaction. Moreover, this reducing potential may be due to the dihydroxy or monohydroxy substitution in the aromatic rings that possess potent hydrogen-donating ability [20]. The maximum reducing potential was reported for run number six with the value of 0.210±0.009 (Table 2). Results of FRAP assay revealed a coefficient of R^2 = 0.265 (Figure 2C) which was much lesser as compared to the correlation reported in previous study by Chandra & Arora [1]. The reducing property may be attributed to the breaking of free radical chain by donating a hydrogen atom [21]. The highest FRAP activity of 0.683±0.005 was reported for the run order thirteen (Table 2). The study revealed a moderate nitric oxide ion scavenging activity of *Penicillium* sp. with the highest value of 58.61±2.05% in the run order fourteen (Table 2). However, there was no correlation reported between the NO ion scavenging and total phenolic content in this study. The results revealed a very low coefficient with R^2 = 0.0707 (Figure 2D). NO ion is an effective reactive radical that acts as an important oxidative biological signalling molecule in a large variety of biological

processes. However, overproduction of reactive oxygen species is called nitrosative stress, that results to nitrosative stress which leads to nitrosylation reactions that can alter the structure of proteins and so inhibit their normal function and act as a potent-oxidizing agent that can cause DNA fragmentation and lipid peroxidation[1]. Thus from the results of correlation we get an overall idea that phenolic compounds have diverse mode of action. They are exhibiting antioxidant activity by either donating hydrogen or reduction and less through scavenging or inhibition ability. This variation in correlation coefficient also highlight that a single assay is not sufficient to evaluate the total antioxidant activity [22]. However, the antioxidant activity observed in these cases can be attributed to the presence of other phytochemicals and also the synergistic effects among compounds that may contribute to the total antioxidant capacity [23].

CONCLUSION

Endophytic fungi are known as potential producers of various secondary metabolites one of them are the antioxidant molecules. Even though the endophytes have been reported as antioxidant producers, there are no reports till date on the use of statistical tools for screening of media components with the aim to optimize growth conditions for enhancement of activity. In this study we highlighted the usefulness of Plackett-Burman design in determining the media components affecting antioxidant activity of an endophytic *Penicillium* sp. This study emphatically identified two components of the Czapex Dox media, sucrose and $NaNO_3$ as important player in antioxidant activity. However, the study demands further work on determining the optimum value of factors under investigation and also the prediction of responses under optimized conditions.

ACKNOWLEDGEMENT

Authors dedicate this study with gratitude to the founder Chancellor, Sri Sathya Sai Institute of Higher Learning, Bhagawan Sri Sathya Sai Baba. Also, we gratefully acknowledge Dr. Debarshi Dey, Biostatistician, Novartis Healthcare Pvt. Ltd., Hyderabad, Andhra Pradesh, India for rendering help in analyzing and interpreting the findings.

REFERENCES

1. Chandra P and Arora DS, Optimization of antioxidant potential of *Penicillium granulatum* Bainier by statistical approaches. ISRN Microbiol,2012, (2012).
2. Srinivas M, Chand N and Losane B, Use of Plackett-Burman design for rapid screening of several nitrogen sources, growth/product promoters, minerals and enzyme inducers for the production of alpha-galactosidase by *Aspergillus niger* MRSS 234 in solid state fermentation system.Bioprocess Eng,10(3):139-144, (1994).
3. Kothekar D, and Dasgupta Debjani, Evaluation of in vitro conditions influencing Phosphatidylinositol-specific Phospholipase C production by *Staphylococcus aureus* ATCC 9144. Int J Pharm Bio Sci,4(1):59-69, (2013).
4. Rangabhashiyam S, Consequence of copper ions on thermal stability of glucoamylase from *Aspergillus niger*. Int J Pharm Bio Sci,2(2):365-371, (2011).
5. Siva Kiran R, Konduri R, Rao G and Madhu G, Statistical optimization of endo-polygalacturonase production by overproducing mutants of *Aspergillus niger* in solid-state fermentation. J Biochem Technol,2(2):154-157, (2010).
6. Naveena B, Altaf M, Bhadriah K and Reddy G, Selection of medium components by Plackett–Burman design for production of l (+) lactic acid by *Lactobacillus amylophilus* GV6 in SSF using wheat bran.Bioresour Technol,96(4): 485-490, (2005).
7. Naveen D, Shubha D, Majumder C and Mondal P, Response surface optimization of critical medium

components for the production of lactic acid by *Rhizopus arrhizus*. ResJ Chem Sci,2(3):1-6, (2012).
8. Babu DR and Rao GN, Antioxidant properties and electrochemical behaviour of cultivated commercial Indian edible mushrooms.J Food Sci Technol,50(2):301-308, (2011).
9. Oyaizu M, Studies on products of the browning reaction. Antioxidative activities of browning reaction products prepared from glucosamine. Jpn J Nutr,44(6):307-315, (1986).
10. Benzie I and Strain J, The ferric reducing ability of plasma (FRAP) as a measure of "Antioxidant Power". The FRAP assay. Anal Biochem, 239(1):70–76, (1996).
11. Panda B, Mohanta S, Manna A and Si S, In vitro antioxidant activity on the aerial parts of *Cocculus hirsutus* Diels. J Adv Pharm Res, 2(1):18-23, (2011).
12. Djeridane A, Yousfi M, Nadjemi B, Boutassouna D, Stocker P and Vidal N, Antioxidant activity of some Algerian medicinal plants extracts containing phenolic compounds. Food Chem,97(4):654-660, (2006).
13. Jalgaonwala RE, Mohite BV and Mahajan RT, A review: natural products from plant associated endophytic fungi. J Microbiol Biotechnol Res, 1(2):21-32, (2011).
14. Deshmukh DV and Puranik PR, Application of Plackett-Burman design to evaluate media components affecting antibacterial activity of alkaliphilic cyanobacteria isolated from Lonar Lake. Turk J of Biochem,35(2):112-118 (2010).
15. Gouda M and Elbahloul Y, Statistical optimization and partial characterization of amylases produced by Halotolerant *Penicillium* sp. World J Agric Sci, 4(3):359-368, (2008).
16. Arora DS and Chandra P, Assay of antioxidant potential of two *Aspergillus* isolates by different methods under various physio-chemical conditions.Braz J Microbiol, 41(3):765-777, (2010).
17. Arora DS and Chandra P, In vitro antioxidant potential of some soil fungi: screening of functional compounds and their purification from *Penicillium citrinum*. Appl Biochem Biotechnol, 165(2):639-651, (2011).
18. Shon MY, Kim TH and Sung NJ, Antioxidants and free radical scavenging activity of *Phellinus baumii* (*Phellinus* of Hymenochaetaceae) extracts. Food Chem, 82(4):593-597, (2003).
19. Liu X, Dong M, Chen X, Jiang M, Lv X and Yan G, Antioxidant activity and phenolics of an endophytic *Xylaria* sp. from *Ginkgo biloba*. Food Chem, 105(2):548-554, (2007).
20. Zhao GR, Xiang ZJ, Ye TX, Yuan YJ and Guo ZX, Antioxidant activities of *Salvia miltiorrhiza* and *Panax notoginseng*. Food Chem, 99(4):767-774, (2006).
21. Othman A, Ismail A, Abdul Ghani N and Adenan I, Antioxidant capacity and phenolic content of cocoa beans. Food Chem,100(4):1523-1530, (2007).
22. Arora DS, Chandra P and Kaur GJ, Optimization and assay of antioxidant potential of two *Penicillium* spp. by different procedures. Curr Biotechnol, 1(2012):2-10, (2012).
23. Kamiloglu Ö, Ercisli S, Senguel M, Toplu C and Serçe S, Total phenolics and antioxidant activity of jujube (*Zizyphus jujube* Mill.) genotypes selected from Turkey. Afr J Biotechnol, 8(2):303-307, (2009).