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Optimization of Growth Medium using a Statistical Approach for the Production of Plant Gallic Acid from a Newly Isolated *Aspergillus tubingenesis* NJA-1

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The present study is to optimize and produce gallic acid of plant tannin under solid state fermentation of different process parameters like temperature, pH of the medium, agitation speed (RPM), volume of Innoculum (ml), moisture, fermentation time, tannic acid, ammonium nitrate, sucrose, substrate influencing gallic acid production from plant tannin were carried out by Minitab ™ 15 software. A systematic study with RSM based on FFD and CCD at 95% confidence level was used to study the interactions among the different variables of fermentation process to maximize the gallic acid production. Under the optimized cultivation conditions *A. tubingenesis* synthesized 94.34% was produced at 35°C temperature, pH 5.5, 96hrs fermentation period, 300mM ammonium nitrate, 600mg sucrose, 916.291mM tannic acid from plant tannin under solid state fermentation. Model validations at optimum operating conditions showed excellent agreement between the experimental results and the predicted responses. Thus the present fungal species to be efficient for gallic acid production. The present study demonstrates the pomegranate peels as a significant substrate statistical optimization with major parameters for gallic acid production can be highly applicable for industrial scale further.

Key words: Full factorial design, Central composite design, *Aspergillus tubingenesis*, Pomegranate.

Tannins are a large class of complex phenolic compounds, comprising hydrolysable, condensed and complex tannins. Hydrolysable tannins are constituted of several organic acids, such as gallic and ellagic. Gallic acid (3,4,5 –tri hydroxyl benzoic acid), a type of phenolic acid and organic substance and occurring in many plants either as a free molecule or as part of hydrolysable tannins is a useful product with significant applications. It is one of the most significant organic acid plays important role for synthesis of propyl gallate[25,8] and preparation of trimethoprim[9] mainly in food and pharmaceutical industry. It is utilized in the production of trimethoxy benzaldehyde, which is used in ink industry, dye industry photographic developer, in testing free mineral acids, dihydroxy acetone and alkaloids . In pharmaceutical industry 3, 4, 5 trimethoxy benzaldehyde is converted to trimethoprim, a broad spectrum antibiotic. A combination of trimethoprim and sulphonamide is effective against many otherwise resistant species of bacteria. Though technological advances have introduced a number of antibiotics in markets, trimethoprim is still very significant. In combination with sulphonamides, it is highly effective against many drug resistant species of bacteria. Of the total requirement of gallic acid of 8000 tons per

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year, 75 % of it is used in production of trimethoprim. Gallic acid can also be used as a raw material for manufacturing an intermediate for anti oxidants, preservatives like propyl gallate. It is very important to have an economical indigenous technology for its commercial production. It has huge demand in India though it is an imported item .Gallic acid has anti-fungal and anti-viral properties. Gallic acid acts as an antioxidant and helps to protect human cells against oxidative damage. Gallic acid was found to show cytotoxicity against cancer cells, without harming healthy cells. Gallic acid is used as a remote astringent in cases of internal haemorrhage. Gallic acid is also used to treat albuminuria and diabetes. Some ointments to treat psoriasis and external haemorrhoids contain gallic acid.A different fungal[2,14,13,15,16,24] strains have been utilized for tannase and gallic acid production[16]. Recovered through fermentation by using *Aspergillus* 30% of gallic acid of tara pod powder. Vermeire and Vandamme[24] (1990) was studied gallic acid production by *Aspergillus* sp from gallotannin.

Gallic acid production from myrabolon and terripod using the immobilized cells of *Rhizopus oryzae*[13,14] reported gallic acid production from tannins of myrabolon and teripod powder through MSSF by two fungal strains *R.oryzae* and *A.foetidus*. Therefore it has been found that it has been found that production of gallic acid through fermentation is possible provided that plenty of raw material (tannin) and desired organism for bioconversion are in hand. Gallic acid production by solid-state fermentation (SSF) is more advantageous over submerged or liquid surface fermentation[12,1]. Conventionally gallic acid is produced by acid hydrolysis of tannins but this process releases a large amount of toxic effluent that causes environmental hazards. Gallic acid production through the fermentation of tannic acid using suitable tannase producing microorganism is preferred today. In the present investigation for the first time gallic acid production by *Aspergillus tubingenesis* NJA-1 has been carried out under solid state fermentation of locally available raw plant tannin. Agro-residues and forest products serve well for being used as the substrate for production of microbial enzymes by SSF.

In the present study tannin-rich agro-residues comprising of powder of pomegranate peels were used for carrying out SSF. In the present study attempts were made to enhance gallic acid production from the culture of *Aspergillus tubingenesis* NJA-1 by SSF.

Response surface methodology is a collection of statistical and mathematical techniques useful for developing, improving and optimizing the design process. RSM stems from science discipline in which physical experiments are performed to study the unknown relation between a set of variables and the system output, or response for which only a few experiment values are acquired[18]. In the 'conventional design' approach, a design is improved by evaluating its 'response' and making design changes based on experience or intuition. This approach does not always lead to the desired result, that of a 'best' design, since the design objectives are often in conflict. It is therefore not always clear how to change the design to achieve the best compromise of these objectives[23]. The improvement procedure that incorporates design criteria into a mathematical framework is referred to as design optimization. Some of the statistical software's used for the optimization purpose are statgraphics, Minitab, statgraphics plus, doe, software, statistica etc.

Response Surface Methodology (RSM) is becoming very famous and precised approach for process optimization by using much mathematical software like Minitab, statistical etc which are used for the experimental design, determination for the model fit, optimization and analysis of variance i.e. ANOVA etc [5]. Full factorial design (FFD), and Plackket - Burman design are used to find significant factors for 2-15 factors and 2-47 factors respectively in a process[19-21]. R^2 value in ANOVA suggests us that model is a good fit or not, 95% confidence level indicates that 0.95 probability is there for the model to be fit for the data in the experimental domain. The higher F value of individual responses than F critfit also confirms adequacy of model. The lack of fit measures the failure of the model to represent data in the experimental domain at points which are not included in the regression[17]. However, based on high R^2 values and F-values, the model can be considered as a good fit when R^2 value is much high i.e. more than 99 and lack of fit can be ignored even it is higher and then based on R^2 and F value the model can be considered as good fit. The P

values indicates the significance of the regression terms for responses if ($P \leq 0.05$) at 95% significant level[7]. The Minitab 15 software is used generally to calculate coefficients for the actual functional relationships for predicting responses also student's *t*-ratio were determined for all the variables in its linear term square and multiplication of two or more variables terms, T value greater than T_{crit} irrespective of their sign indicates the significance of terms[5,7].

Factors which affects plant gallic acid production by *Aspergillus tubingenesis NJA-1* in Czepakdox media are temperature, pH of media, moisture, fermentation time period, inoculums volume (CFU*10^7), substrate weight[3], agitation rate (rpm), tannic acid concentration, ammonium nitrate concentrate, and sucrose level[22].

MATERIALS AND METHODS

Microorganism

Screening of fungal organism for gallic acid production was carried out and the potential organism was identified as *Aspergillus tubingenesis NJA-1* by 18s r RNA sequencing analysis. The strain of A.*tubingenesis NJA-1* used in this study was isolated earlier in the laboratory from the soil sample of paddy fields plant debris after harvesting in Palakkad (Kerala) India and was identified as *A.tubingenesis*. In the lab it was routinely maintained on czapekdox agar at 4ÚC. slants were sub-cultured routinely at an interval of 4–5 weeks.

Raw Material or substrate

Pomegranate peels collected from the local supermarket nearby Vellore and dried at a temperature of 60°C for 24hrs in an oven to remove extra free moisture. The dried peels were then powdered in a grinder.

Inoculum preparation

10ml of sterile distilled water was taken in 50ml conical flask. The mycelia of the slant were scraped off in 2ml of distilled water. The resulting spore suspension was mixed to obtain a uniform suspension .This suspension was then was then added to distilled water to give 10ml of spore suspension for spore dilution. 50ml medium is transferred to each of 250ml conical flask and then sterilized. These flasks were inoculated aseptically with 2ml of spore suspension prepared from the culture slants. These flask were kept in a rotator shaker 190rpm at 37°C. After 3days incubation the fungal mycelia was washed thoroughly with distilled water for subsequent studies this inoculum was used by centrifuging at 5000rpm for 15 minutes and wet mass is used as inoculums.

Preparation of pre induced inoculums

The spores were collected using Tween 80 (0.01 %). Czapekdox medium was prepared using the 2% tannic acid[6] as the sole carbon source (pH=5.0). *Aspergillus tubingenesis* spores were inoculated in this medium at a concentration of $2x10^7$ spores/mL). This induced inoculum was used for subsequent fermentations.

Fermentation cultural conditions

The substrate powdered cinnamon sticks and aqueous mixture containing sodium nitrate 2.5g/L, Dipotassium hydrogen phosphate 1g/L, Magnesium sulphate 0.5g/L, Potassium chloride 0.5g/L , Ferrous sulphate 0.01g/L respectively were placed in a 250ml Erlenmeyer flask .After sterilization the medium was inoculated with induced inoculums 2×10^7 spore of fungal propagules of *A.tubingenesis* and kept it for incubation for bioconversion of natural tannin to gallic acid under different process conditions ,as per the experimental design.

Gallic acid extraction[11]

Identification of significant factors using FFD

Full Factorial Design is used to determine the variables that significantly affect the process. Two levels ten factor design with two blocks was adopted in the study. The input variables considered to be important during the extraction process were temperature, pH of media, moisture, fermentation time period, inoculums volume (CFU2x107), substrate weight[3] agitation rate (rpm), tannic acid concentration, ammonium nitrate concentrate, and sucrose level[22]. In coded terms the lowest, medium and the highest levels of five variables were –1, 0 and +1, respectively. A complete FFD with coded and uncoded values are shown in table 1. Data were analyzed using MINITAB-15™ software to find the interaction between the variables and their responses. The experiment evaluates the influence of the independent variables and its possible interactions on the response i.e. gallic acid production.

Optimization of significant factors by CCD

A central composite design (CCD) with

four variables (significant factors obtained from FFD), two base blocks and 30 base runs was used to study the response pattern and to determine the optimum combination of the variables. CCD combines vertices of the hypercube whose co-ordinates are given by a 2^n factorial design and two star points (outsider points) to provide for the estimation of curvature of the model[10]. The variables optimized were tannic acid concentration in mM (X_1), ammonium nitrate concentration in mM (X_2), sucrose weight in mg (X_3) and temperature in °C (X_4) each at three levels: –1, 0, and 1 (Table 2). The relation between the coded values and actual values are described by the following eq. [9]:

$$x_i = \frac{Xi - Xo}{Xh - Xo} \quad \ldots (1)$$

where, xi = coded value of the variable, Xi = the actual value of the variable, Xo = the actual value of Xi at the centre point, Xh = the actual value of Xi at high level. A set of 13 experiments including 5 repeats at centre point were carried out.

Production of gallic acid in mg/l was taken as the response. The quadratic equation (equation 2) of the variables was used[9]. Where Y = predicted response, β_o = a constant, β_i = linear coefficient, $\Sigma\beta_{ii}$ = squared coefficient, and β_{ij}= interaction coefficient. This was used to build surfaces for variables. Minitab 15.0 was used to analyze the results and to generate response plots.

$$Y = \beta_o + \Sigma\beta_i x_i + \Sigma\beta_{ii} x_i^2 + \Sigma\beta_{ij} x_i x_j \quad \ldots (2)$$

A complete CCD coded and un-coded value table shown in table 3. An analysis of variance (ANOVA) was conducted to determine the significant effects of process variables on each response. Optimum conditions for medium for gallic acid production from *Aspergillus* were determined to obtain maximum gallic acid concentration during the fermentation. Quadratic model equations obtained in this study (coefficients from) were utilized for each response in order to determine optimum conditions.

The statistical analysis of the model was performed in the form of analysis of variance (ANOVA) using Minitab 15 software. This analysis also included the Fisher's F-test (overall model significance), determination coefficient R^2 which measures the adequacy of fit for the regression model. It also includes the Student's t-value for the estimated coefficients, t value grater than T_{crit} irrespective of their sign indicates the significance of terms. For each variable, the quadratic models were represented as 3D surface plots[17]. The optimal conditions were also done by drawing optimization plots i.e. Response Optimizer (goal: maximize, target: 2.9 mg/l, lower: 2.6 mg/l) as well as overlaid contour plot (maximize, lower: 2.8, upper: 2.9) by using Minitab15 software. The optimized conditions obtained from response optimizer and overlaid contour plot were performed experimentally, the predicted value given by software and experimental values are compared for validation.

Statistical analysis

All statistical analysis was performed using experimental results and expressed as mean of parallel duplicates ANOVA correlation were performed. $P < 0.05$ was considered statistically significant.

RESULTS

The experimental conditions of different process parameters for such as temperature, pH of the medium, agitation speed (RPM) , volume of Innoculum (ml), moisture, fermentation time, tannic acid, ammonium nitrate, sucrose, substrate influencing gallic acid production from plant tannin were carried out by Minitab ™ 15 software .

Identification of significant factors using FFD

In this experiment, FFD was used to screen the main factors of the gallic acid production by *Aspergillus tubingenesis* through fermentation process. The 21 experiments of design matrix and the measured response as gallic acid production are shown in Table 1. Out of these ten factors, four were found to be significant (Tannic acid concentration, ammonium nitrate concentration, weight of sucrose and temperature) as shown in half normal plot (Fig 1). From ANOVA table (table 4) we can conclude that experimental design (FFD) for gallic acid production is fit for the model since R^2 is 97.61% and lack of fit is 1, so we can consider significant factors shown in half normal plot (Fig 1).

Optimization of significant factors using CCD

Tannic acid concentration, ammonium nitrate concentration, sucrose weight and

Table 1. Complete FFD table with coded[a], uncoded values and respective response (gallic acid production)

Run	Factors										Response
Number	Tannic Acid (mM)	Ammonium Nitrate (mg)	Sucrose (mg)	Substrate (mg)	Temperature (°C)	pH of media	Agitation rate (rpm)	Inoculum volume (CFUx 10^7)	Moisture (XXX)	Fermentation Time (Hr)	Gallic Acid (mg/l)
1	(+1) 1200	(-1) 100	(-1) 200	(-1) 15	(+1) 65	(-1) 3	(+1) 220	(+1) 7	(-1) 40	(-1) 24	1.6235
2	(+1) 1200	(+1) 500	(-1) 200	(-1) 15	(-1) 30	(+1)10	(+1) 220	(-1) 2	(+1) 80	(+1) 96	0.47
3	(-1) 200	(+1) 100	(+1) 1000	(-1) 15	(+1) 65	(+1) 10	(+1) 220	(-1) 2	(-1) 40	(+1) 96	1.09065
4	(-1) 200	(+1) 500	(+1) 1000	(-1) 15	(-1) 30	(-1)3	(+1) 220	(+1) 7	(+1) 80	(-1) 24	0.85248
5	(+1) 1200	(-1) 100	(-1) 200	(+1) 25	(+1) 65	(+1)10	(-1) 100	(-1) 2	(+1) 80	(-1) 24	1.5
6	(+1) 1200	(+1) 500	(-1) 200	(+1) 25	(-1) 30	(-1)3	(-1) 100	(+1) 7	(-1) 40	(+1) 96	0.47
7	(-1) 200	(-1) 100	(+1) 1000	(+1) 25	(+1) 65	(-1)3	(-1) 100	(+1) 7	(+1) 80	(+1) 96	1.09065
8	(-1) 200	(+1) 500	(+1) 1000	(+1) 25	(-1) 30	(+1)10	(-1) 100	(-1) 2	(-1) 40	(-1) 24	0.85248
9	(0) 700	(0) 300	(0) 600	(0) 20	(0) 47.5	(0) 6.5	(0) 160	(0) 4.5	(0) 60	(0) 60	0.63862
10	(0) 700	(0) 300	(0) 600	(0) 20	(0) 47.5	(0) 6.5	(0) 160	(0) 4.5	(0) 60	(0) 60	0.7
11	(-1) 200	(-1) 100	(-1) 200	(-1) 15	(-1) 30	(-1) 3	(-1) 100	(-1) 2	(+1) 80	(+1) 96	1.09065
12	(-1) 200	(+1) 500	(-1) 200	(-1) 15	(+1) 65	(+1)10	(-1) 100	(+1) 7	(-1) 40	(-1) 24	0.85248
13	(+1) 1200	(-1) 100	(+1)1000	(-1) 15	(-1) 30	(+1)10	(-1) 100	(+1) 7	(+1) 80	(-1) 24	1.565
14	(+1) 1200	(+1) 500	(+1)1000	(-1) 15	(+1) 65	(-1) 3	(-1) 100	(-1) 2	(-1) 40	(+1) 96	1.47409
15	(-1) 200	(-1) 100	(-1)200	(+1) 25	(-1) 30	(+1)10	(+1) 220	(+1) 7	(-1) 40	(+1) 96	1.09065
16	(-1) 200	(+1) 500	(-1)200	(+1) 25	(+1) 65	(-1) 3	(+1) 220	(-1) 2	(+1) 80	(-1) 24	0.867
17	(+1) 1200	(-1) 100	(+1)1000	(+1) 25	(-1) 30	(-1) 3	(+1) 220	(-1) 2	(-1) 40	(-1) 24	1.6235
18	(+1) 1200	(+1) 500	(+1)1000	(+1) 25	(+1) 65	(+1)10	(+1) 220	(+1) 7	(+1) 80	(+1) 96	1.47409
19	(0) 700	(0) 300	(0) 600	(0) 20	(0) 47.5	(0) 6.5	(0) 160	(0) 4.5	(0) 60	(0) 60	0.638
20	(0) 700	(0) 300	(0) 600	(0) 20	(0) 47.5	(0) 6.5	(0) 160	(0) 4.5	(0) 60	(0) 60	0.63862

[a]The numbers in parentheses are coded value for FFD.

temperature for fermentation were optimized with RSM approach by using CCD. Response i.e. gallic acid concentration in mg/l determines efficiency of media. A complete CCD table with coded, uncoded values and responses are shown in table 3. The ANOVA of CCD (table 5) indicates the high values of R^2 and F-value for response as 98.61% and 5.68 respectively with $F_{crit\ (0.05,\ 14,\ 14)}$ 2.483.

Table 2. Variables with their - α, + α, maximum (+1) and minimum (-1) values

Factors	-α	-1	0	1	+α
Tannic acid (mM)	-300[a]	200	700	1200	1700
Ammonium nitrate (mM)	-100 [a]	100	300	500	700
Sucrose (mg)	-200 [a]	200	600	1000	1400
Temperature (°C)	12.5	30	47.5	65	82.5

α = 2

[a] Negative values of these are not possible so consider them as zero.

Table 3. Complete CCD table with coded[b], uncoded values and respective responses

S. No.	Tannic Acid (mM)(X_1)	Ammonium Nitrate (mM)(X_2)	Sucrose (mg)(X_3)	Temperature (°C)(X_4)	gallic acid (mg/l)
1	(-1) 200	(-1) 100	(-1) 200	(-1) 30	0.488
2	(+1) 1200	(-1) 100	(-1) 200	(-1) 30	2.01
3	(-1) 200	(+1) 500	(-1) 200	(-1) 30	2.445
4	(+1) 1200	(+1) 500	(-1) 200	(-1) 30	2.508
5	(-1) 200	(-1) 100	(+1) 1000	(-1) 30	2.47
6	(+1) 1200	(-1) 100	(+1) 1000	(-1) 30	2.038
7	(-1) 200	(+1) 500	(+1) 1000	(-1) 30	1.922
8	(+1) 1200	(+1) 500	(+1) 1000	(-1) 30	2.579
9	(-1) 200	(-1) 100	(-1) 200	(+1) 65	0.569
10	(+1) 1200	(-1) 100	(-1) 200	(+1) 65	0.488
11	(-1) 200	(+1) 500	(-1) 200	(+1) 65	0.4322
12	(+1) 1200	(+1) 500	(-1) 200	(+1) 65	0.522
13	(-1) 200	(-1) 100	(+1) 1000	(+1) 65	0.488
14	(+1) 1200	(-1) 100	(+1) 1000	(+1) 65	0.558
15	(-1) 200	(+1) 500	(+1) 1000	(+1) 65	0.465
16	(+1) 1200	(+1) 500	(+1) 1000	(+1) 65	0.456
17	(0) 700	(0) 300	(0) 600	(0) 47.5	2.537
18	(0) 700	(0) 300	(0) 600	(0) 47.5	2.918
19	(0) 700	(0) 300	(0) 600	(0) 47.5	2.572
20	(0) 700	(0) 300	(0) 600	(0) 47.5	2.831
21	(-α) -300 [a]	(0) 300	(0) 600	(0) 47.5	0.0927
22	(+α) 1700	(0) 300	(0) 600	(0) 47.5	3.098
23	(0) 700	(-α) -100 [a]	(0) 600	(0) 47.5	0.654
24	(0) 700	(+α) 700	(0) 600	(0) 47.5	1.573
25	(0) 700	300	(-α) -200[a]	(0) 47.5	0.453
26	(0) 700	300	(+α) 1400	(0) 47.5	1.009
27	(0) 700	300	(0) 600	(-α) 12.5	1.209
28	(0) 700	300	(0) 600	(+α) 82.5	0.321
29	(0) 700	300	(0) 600	(0) 47.5	2.084
30	(0) 700	300	(0) 600	(0) 47.5	2.47

[a] Negative values of these are not possible so consider them as zero.

[b]The numbers in parentheses are coded value for FFD.

Table 4. Analysis of Variance (ANOVA) for gallic acid production in FFD

Source	DF	SeqSS	AdjMS	F	P
Main effect	10	1.71965	0.171965	7.36	0.064
2-way interaction	4	0.23503	0.058757	2.52	0.237
Curvature	1	0.70806	0.708057	30.31	0.012
Residual error	3	0.07008	0.023359		
Lack of Fit	1	0.06819	0.068194	72.39	0.014
Pure error	2	0.00188	0.000942		
Total	19	2.93798			
R sq	97.61%				
Lack of fit	1				

Table 5. Regression coefficients and respective t-values for coagulation time

Term	Coefficient	T-value	P-value
Constant	2.47503	11.099	0.000[a]
Tannic acid (mM)	0.39257	3.813	0.430[c]
Ammonium nitrate (mM)	0.23067	1.909	0.077[b]
Sucrose (mg)	0.18648	1.544	0.145[c]
Temperature (°C)	-0.59408	-5.219	0.000[a]
Tannic acid (mM)* Tannic acid (mM)	0.13699	1.301	0.214[c]
Ammonium nitrate (mM)* Ammonium nitrate (mM)	-0.41973	-3.302	0.005[a]
Sucrose (mg)* Sucrose (mg)	-0.53861	-4.237	0.001[a]
Temperature (°C)* Temperature (°C)	-0.42490	-4.034	0.001[a]
Tannic acid (mM)* Ammonium nitrate (mM)	-0.01739	-0.125	0.903[c]
Tannic acid (mM)* Sucrose (mg)	-0.08174	-0.586	0.567[c]
Tannic acid (mM)* Temperature (°C)	-0.10876	-0.780	0.448[c]
Ammonium nitrate (mM)* Sucrose (mg)	-0.15526	-1.114	0.284[c]
Ammonium nitrate (mM)* Temperature (°C)	-0.16724	-1.200	0.250[c]
Sucrose (mg)* Temperature (°C)	-0.10014	-0.718	0.484[c]

[a] significant at 99% confidence level $Tcrit_{(0.05,4)}$ = 2.776
[b] significant at 95% confidence level
[c] significant at 90% confidence level

Table 5. The analysis of variance (ANOVA) table for the CCD for gallic acid as response

Source	DF	SeqSS	AdjMS	F	P
Regression	14	24.7366	1.76690	5.68	0.001a
Linear	4	9.4496	2.63932	8.49	0.001a
Square	4	13.9923	3.49809	11.25	0.000a
Interaction	6	1.2946	0.21577	0.69	0.659c
Residual Error	14	4.3541	0.31100		
Lack-of-Fit	10	4.1728	0.41728	9.21	0.023b
Pure Error	4	0.1813	0.04532		
Total	29	29.0921			
R square	85.03%				
Model F value	5.68				
Lack of Fit F value	9.21				

a) Significant at 99% confidence level
b) Significant at 95% confidence level
Fcrit(0.05,14,14) = 2.483
c)significant at 95% confidence level

Table 6. Optimized conditions with respective predicted responses from overlaid contour plot and response optimizer plot

Run	Optimized condition				Predicted	Experimental
	Tannic acid (mM)	Ammonium nitrate (mM)	Sucrose (mg)	Temperature (°C)	Response Gallic acid (mg/l)	Response
1 [a]	1046.0838	324.4991	622. 2222	37. 2996	2.8907	2.901
2 [b]	1274.81	363. 213	600	47.5	2.80715	2.885
3 [b]	1303.74	300	633.062	47.1	2.80911	2.893
4 [b]	916.921	300	600	35.3870	2.81346	2.975

[a] Global solution obtained by response optimizer with desirability 0.96897
[b] solution obtained by overlaid contour plot

ANOVA table also indicates the significant lack of fit for model which is 9.21. For gallic acid formation the variable (tannic acid (mM) and temperature(°C)) as well as the interactive terms of ammonium nitrate (mM)* ammonium nitrate (mM), sucrose (mg)* sucrose (mg) and temperature (°C)* temperature (°C) are significant since their T value is greater than T_{crit} (table 5). Based on the T_{crit} value and T values quadratic model equations obtained for Coagulation time is given in equation (Equation 3).

$$Y = 2.47503 + 0.39257 * X1 - 0.59408 * X4 - 0.41973 * X2 * X2 - 0.53861 * X3 * X3 - 0.42490 * X4 * X4 \quad ...(3)$$

where, Y is response i.e. gallic acid concentration (mg/l), X1, X2, X3 and X4 are factors i.e. tannic acid concentration (mM), ammonium nitrate concentration (mM), sucrose weight (mg) and temperature (°C) respectively.

Response surface plots were generated for these terms to study the interactive effect among variables on gallic acid production (Figure 2). From the surface plot it was observed that the response i.e. gallic acid (mg/l) decreases with increase of temperature while increases with increase in tannic acid concentration, ammonium nitrate concentration and sucrose level also observed that among all the four factors temperature and tannic acid concentration are having high impact compared to other two factors. Gallic acid concentration was found to be maximum (3.098 mg/l) at highest value of tannic acid and mid value of other factors according to experimental design (tannic acid= 1700mM, Ammonium nitrate

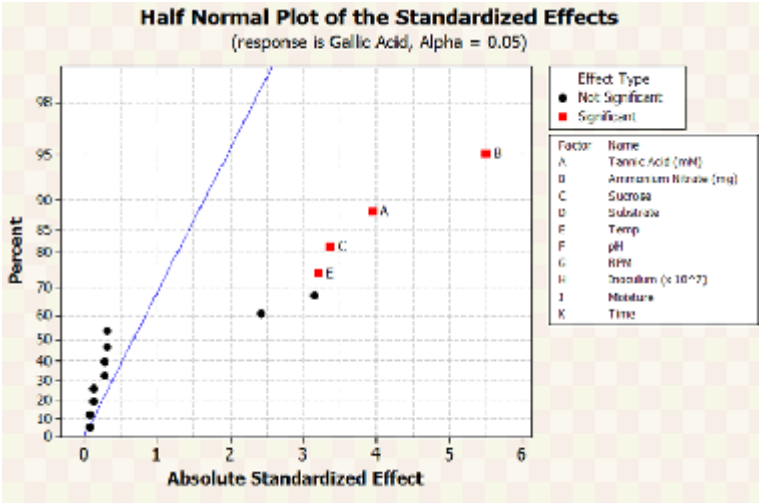

Fig. 1. Half normal plot for gallic acid production, factor A (Tannic acid concentration), B (ammonium nitrate concentration), C (sucrose weight) and E (temperature) are significant

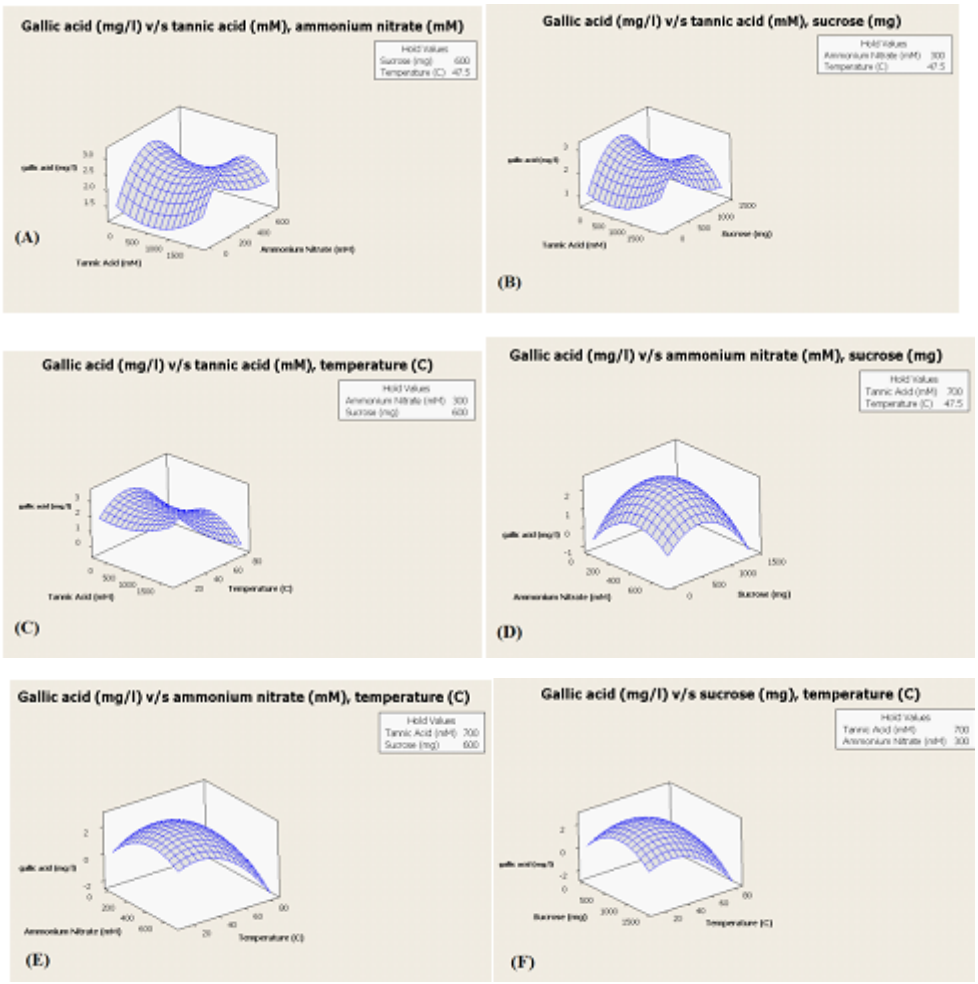

Fig. 2. Response surface plots for gallic acid (mg/l) v/s (A) Tannic acid (mM), ammonium nitrate (mM) (B) Tannic acid (mM), sucrose (mg) (C) Tannic acid (mM), temperature (°C) (D) Ammonium nitrate (mM), sucrose (mg) (E) ammonium nitrate (mM), temperature (°C) (F) Sucrose (mg),temperature (°C)

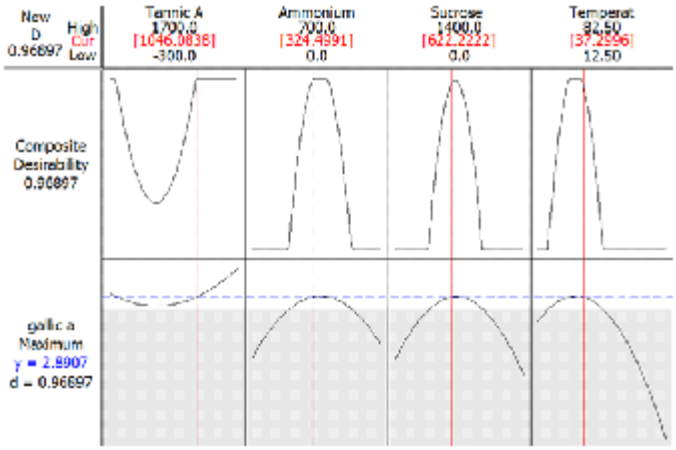

Fig. 3. Optimization Plot for factors and responses in gallic acid production from *Aspergillus* sp

=300mM, sucrose=600mg, temperature=47.5°C; coded value +±, 0, 0, 0).

By applying the method of desirability function i.e. response optimizer (Figure 3) and graphic optimization i.e. overlaid contour plot (Figure 4) the optimized conditions were determined as tabulated in table 6 which also shows the predicted value for coagulation time at this optimized and was validated experimentally, which validate the predictability of the model.

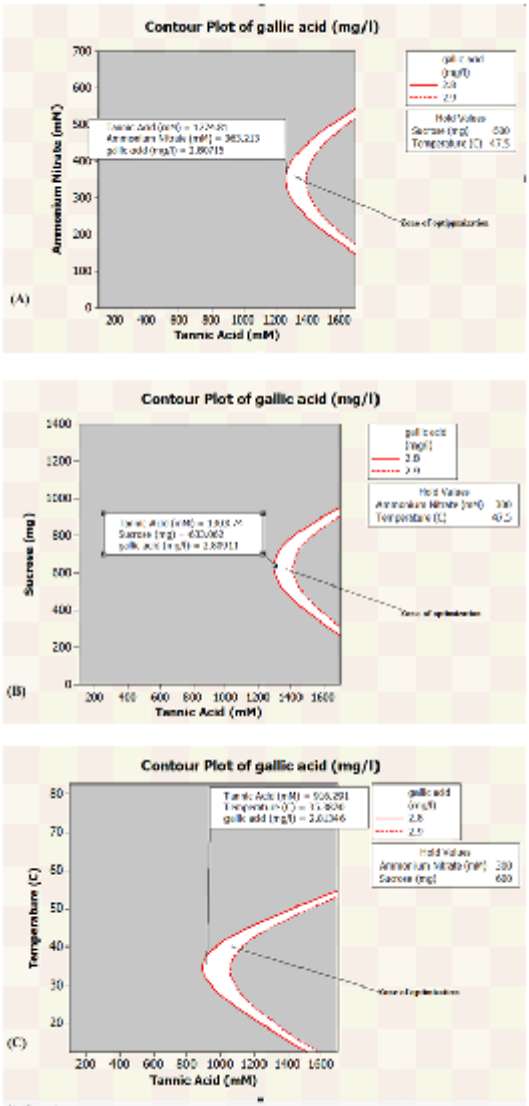

Fig. 4. Overlaid contour plot showing the zone of optimization (white zone) of response as a function of (A) tannic acid (mM) and ammonium nitrate (mM) (B) tannic acid(mM) and sucrose(mg) (C) tannic acid(mM) and temperature (°C)

DISCUSSION

The main objective of this research is to detect the significant factors responsible for gallic acid production by *Aspergillus tubingenesis* NJA-1 from fermentation process with FFD experimental design and then by keeping other factors at mid value according to FFD optimization of significant factors by response surface method (RSM) using CCD experimental design. This process helps to make more precised decision for any process.From the above findings it can be concluded that extract *Aspergillus tubingenesis NJA-1* is showing good response as gallic acid formation in optimized media which can be used in food and pharmaceutical industry. The decrease in gallic acid production with increase in temperature can be attributed by the fact that high temperature might inactivates the culture and increase can be attributed by the fact that tannic acid, ammonium nitrate and sucrose are the source of nutrition for bacteria.

CONCLUSION

In the present study, a simple and reproducible method for the production of gallic acid in herbal formulation method is developed. The advantage of the method lies in the simplicity. The validated parameters indicate that the developed method is quick, high selectivity and economic. Hence the developed method is more suitable for the production of gallic acid in multi-component herbal formulation. This is for the first time gallic acid production has been made from the raw tannin of pomegranate peels through fermentation by *Aspergillus tubingenesis* NJA-1. Plenty of such cost effective raw material ensured the possibility for exploitation of this organism in large scale production of gallic acid .

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