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Evolutionary Operation–Factorial Design Technique for Optimization of Conversion of Mixed Agroproducts Into Gallic Acid

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Abstract

This article presents the optimization of gallic acid production using filamentous fungi from tannin-rich mixed substrates taking into account the interaction effects of six variable process parameters. The methodology adopted for optimization was the evolutionary operation (EVOP)–factorial design technique. This technique combines the factorial method for designing experiments with the EVOP methodology for analyzing the experimental results systematically and arriving at conclusions according to its decision-making procedure. Standard deviation and error limits based on 95% confidence were calculated according to the relationship given in the literature. It was found that the best combinations of the process parameters at the optimum levels were 30°C, 80% relative humidity, pH 5.0, 48-h incubation period, 3 mL of induced inoculum, and 35 g of mixed substrate, resulting in a gallic acid yield of 94.8% under modified solid-state fermentation.

Index Entries: Mixed substrates; modified solid-state fermentation; optimization; evolutionary operation–factorial design; filamentous fungi; gallic acid.

Introduction

There are many techniques of optimization, each having its own advantages and disadvantages. The probable interactions among the factors may be ignored by the single variable search technique, and thus the true optimum conditions may not be determined *(1)*. The basic theory of

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experimental design is based on changing more than one factor at a time, instead of changing only one factor *(2)*. To optimize the yield or production using the experimental design approach, both a design, and an optimization technique are required. The design specifies the different variants to be varied and tested in the experiment, the number of replicates, and the arrangements of the tests into homogeneous blocks.

Microbes have been reported to produce the industrially important enzyme tannase, more advantageously by solid-state fermentation (SSF) *(3)*. Tannase hydrolyzes tannins to gallic acid, which finds application in many fields including pharmaceutical, dye making, leather, and chemical industries *(3–6)*. Agroresidues and forest products serve well as substrates for production of microbial enzymes by SSF, with the substrate needing to be properly selected based on its cost, availability, and suitability for obtaining the desired product of fermentation *(7)*. In the present study, a coculture of the filamentous fungi *Rhizopus oryzae* and *Aspergillus foetidus* was used as inoculum for the bioconversion of tannin-rich mixed agroresidues to gallic acid by SSF. However, to overcome the harmful effects on microbial growth and activity owing to the heat produced during SSF, a modified solid-state fermentation (MSSF) was carried out. The GROWTEK bioreactor has been used for this purpose in which the solid substrate placed on its float comes into continuous contact with the liquid medium in the vessel *(5)*.

Microbial synthesis of enzymes by SSF and the product yield in turn are influenced by factors such as fermentation temperature, relative humidity, incubation period, pH, substrate quantity, and inoculum amount and moisture *(7)*. In the present study, the interaction effects of such influencing process parameters on gallic acid production by the coculture method using mixed agricultural residues by MSSF were evaluated and their optimum values determined by the evolutionary operation (EVOP)–factorial design technique.

The main advantage of EVOP methodology is its clear-cut decision-making procedure, which indicates the change in influencing variables toward the desired maxima or minima. Two other advantages of the EVOP technique, apart from its simplicity, are that the product quality is not deteriorated and the production of the existing plant is not interrupted *(8)*. The methodology is, however, constrained by its ability to represent physically a system having more than three independent variables at a time. To overcome this shortcoming, the EVOP-based factorial design technique has been proposed as a generalized approach for analysis of multivariable biologic systems, including SSF. This new methodology has the advantages of both EVOP and factorial design, thus providing a new and powerful tool for *n*-variable system optimization *(9)*. Therefore, our experiments were designed based on the factorial technique, and the results were analyzed by the EVOP procedure.

Optimization of Six Variables by EVOP-Based Factorial Technique

This section discusses the application of the EVOP–factorial design technique to maximize the response function of gallic acid production for optimization of six variable parameters simultaneously.

For studying six variable parameters, the total number of new experiments required to be conducted are 2^6, in addition to the 2 control (search-level) experiments. The parameters for these experiments are arranged in both lower level (–) and higher level (+) compared with the search-level region (0), which is assumed to be the initial optimum level. The parameters and the total number of experiments can be represented in a $[6 \times (2^6 + 2)]$ matrix, which can be divided into two blocks (blocks 1 and 2). There is one set of control experiment (search level) in each block. For the desired arrangement, each new experiment in block 1 should have an odd number of lower level (–) parameter(s) (e.g., 1, 3, and 5), and block 2 must have an even number of lower-level (–) parameters (e.g., 2, 4, and 6). The other parameters in the two blocks correspond to the higher level (+) in both the blocks. The overall responses of block 1 are distinguished from those of block 2 by the aforementioned arrangements. Whereas block 1 exhibits an overall lower-level (–) response, block 2 exhibits higher-level (+) responses compared with those of control experiments. The experimental design for "*n*" variable parameters is presented in Table 1.

The current investigation was conducted to study the effect of six parameters—temperature, relative humidity, pH, incubation period, inoculum amount, and substrate quantity—on gallic acid production by coculture method using mixed agricultural residues. These parameters are abbreviated Te, H, P, Ti, V, and W, respectively.

The level of each of the parameters in different experiments was decided in accordance with the earlier described procedure as presented in Table 1, which shows the distribution of the experiments between blocks 1 and 2. Experiments were conducted in duplicate in two cycles for minimizing the standard deviation (SD) and error limits, and responses for cycles 1 and 2 were recorded for conducting further analysis.

For a six-variable system, the effects of individual parameters are to be termed zero-order interactions, and the total number of such effects can be estimated as ${}^6c_1$ or 6. Therefore, the total number (*N*) of effects may be expressed as follows:

$$N = \sum_{i=1}^{6} 6\, C_i + \text{the change in mean effect}$$

Calculation of the effect can be based on the theory of fractional factorials *(10,11)*. All possible interaction levels for a six-variable system were derived by the multiplication of individual levels as shown in Table 1.

The effects of individual and interaction parameters are evaluated based on the average values of the two-cycle responses. Any individual or

Table 1
Experimental Design for "n" Variable Parameters

Experimental setup/ parameter	A1	A2	A3	A4	A5	–	–	–	$(A2)^{n-1}+1$	$(A2)^{n-1}+2$	$(A2)^{n-1}+3$	–	$(A2)^{n}+2$
P1	0	–	–	+	+				–	0	+		+
P2	0	–	+	–	+				–	0	–		+
P3	0	–	+	+	–				–	0	–		+
P4	0	–	–	–	–				+	0	–		+
P5	0	–	–	–	+				–	0	–		+
P6	0	–	–	–	–				–	0	–		+
–													
–													
Pn	0	–	–	–	+				+	0	–		+
Overall response	0	–	–	–	–			–	0	+	+		
	→	Block	1						←	→	Block	2	←
Experimental response	a1	a2	a3	a4	a5	–	–	–	$(a2)^{n-1}+1$	$(a2)^{n-1}+2$	$(a2)^{n-1}+3$	–	$(a2)^{n}+2$

interaction effect can be estimated from the generalized form as shown in Eq. 1:

$$E_1 = 1/n' \left[\sum_{i=1}^{n'} (\text{higher interaction level effects}) - \sum_{i=1}^{n'} (\text{lower interaction level effects}) \right] \quad (1)$$

The change in mean effects of all the experimental conditions can be expressed in generalized form as shown in Eq. 2:

$$E_2 = 1/2 \left\{ 1/m \left[\left(\sum_{i=2}^{m} a_i - ma_1 \right) \right] + 1/m \left[\left(\sum_{j=m+2}^{2m} a_j - ma_{m+1} \right) \right] \right\} \quad (2)$$

in which E_1 is the effect; E_2 is the change in mean effects; n' is the number of higher or lower interaction level effects; and m is the number of new experiments in block 1 or 2. Different effects and the change in mean effect for the six-variable systems were estimated using Eqs. 1 and 2 and Table 1.

The SD and error limits based on a 95% confidence level are estimated from the differences according to the relationship given in the literature *(12,13)* and are calculated as given here:

σ (SD) = $1/2(\sigma_1 + \sigma_2)$, in which $\sigma_1 = R_1 \times f$ and $\sigma_2 = R_2 \times f$

R_1 = (largest difference – smallest difference) in block 1

R_2 = (largest difference – smallest difference) in block 2

f (statistical constant) = 0.3 for number of cycles (n'') = 2 and number of new experiments per cycle (k) = 64

Error limits

For average: $\pm(2/\sqrt{n''}) \times \sigma$

For effects: $\pm(0.71 \times 2/\sqrt{n''}) \times \sigma$

For change in mean: $\pm(0.63 \times 2/\sqrt{n''}) \times \sigma$.

Decision-Making Procedure

After calculating the change in mean effects and error limits, it is necessary to examine whether any change in the control (search-level) experimental conditions will help improve the objective response and, if so, which should be the desired direction of change *(8)*. The magnitudes of the effects are compared with that of the error limits for this purpose. If all or any of the effects are larger than the error limits, a change in the experimental conditions might yield better results. The decision on the desired direction of change of a variable is taken as described in the literature *(9)*.

Materials and Methods

Organism

A newly isolated strain of *A. foetidus* (GMRB 013 MTCC 3557) and a strain of *R. oryzae* (RO IIT KGP RB-13, NRRL 21498) were used. Both fungal strains were isolated from the soil of the Indian Institute of Technology, Kharagpur campus, and were maintained routinely on 2% malt-extract agar slants.

Raw Material

Caesalpinia digyna pod cover powder and powdered fruits of *Terminalia chebula* were used as substrate for MSSF.

Preparation of Inoculum

Because tannase is an inducible enzyme, preinduced inoculum was used that was prepared using liquid modified Czapekdox medium in which 2% tannic acid was used as a sole carbon source. Induced inoculum was prepared with 2 mL of spore suspension and incubated at 30°C under shaking for 72 h.

Fermentation Process and Gallic Acid Extraction

MSSF was carried out in batch process in a GROWTEK bioreactor *(5)*. A weighed amount of powdered substrate was placed on the mesh of the bioreactor, below which a measured volume of modified Czapekdox medium was added. This was autoclaved at 121°C for 15 min and, after cooling, the appropriate volume of induced inoculum, comprising a coculture of the filamentous fungi, *A. foetidus* and *R. oryzae*, was added to the substrate. After incubation for the appropriate time, the fermented biomass and the medium in the bioreactor were mixed thoroughly and warmed. After cooling, gallic acid was extracted with organic solvent, which was then separated using a rotary vacuum evaporator.

Application of Methods for Evaluation of Combined Effects of Six Process Parameters for Maximizing Gallic Acid Production

To investigate the region of the optimum, in a multiple-factor system the most important aspect is the design of a suitable factorial technique *(14)* and the judicious selection of the design parameters. The EVOP–factorial design technique was applied to select the optimum levels of the six process parameters (as mentioned earlier). First, the control experimental conditions (A1 and A34) were selected based on the results of an earlier investigation (data not shown) on the effect of individual parameters on gallic acid production by MSSF using tannin-rich mixed substrates. Then, new experimental conditions were selected with lower and higher levels of parameters compared with the control or search level. The selection procedure illustrated in Table 1 was followed for determining the number of parameters present at lower and higher levels in each experiment. Fermentation was carried out and gallic acid extraction done as mentioned earlier with lower and higher levels of parameters, and all experiments were repeated for two cycles. Differences in gallic acid yield between cycles 1 and 2, and average gallic acid yield, were calculated for estimating the effects and error limits. The experimental conditions and results for the effects of the six variable process parameters are given in Table 2. The magnitudes of the effects, error limits, and change in mean effect were examined according to the decision-making procedure in order to arrive at the optimum.

Results and Discussion

The experimental conditions for the first set and the corresponding gallic acid yields are presented in Table 2. The change in mean effect, effects, and error limits for this set of results was calculated using the equations given earlier.

According to the decision-making procedure, after calculating the change in mean effects and error limits, an examination is necessary to determine whether any change in the control (search-level) experimental conditions will help to improve the objective function (i.e., response) and, if so, which should be the desired direction of change *(8)*. For this purpose, the magnitudes of the effects were compared with those of the change in mean effects and error limits. If the effects were larger than the error limits and/or the change in mean effect was small or positive, a change in the experimental conditions might yield better results *(9,15)*.

The experimental conditions in set 1 other than the control set did not show any improvement in the gallic acid yield. Analysis of the results demonstrates that the change in mean effect was negative and large (–25.14), which satisfies the basic requirement for determining the maximum gallic acid yield and is indicative of achieving the optimum condition. The effects and error limits (±7.21 for averages, ±5.14 for effects, and ±4.56 for change in mean) were also calculated. As per the decision-making rule *(9,15)*, the interaction effects are required to be less than the error limit, which was also more or less fulfilled.

However, to ensure that the condition at the search region (A1 and A34) of set 1 was the real optimum condition, a second set of experiments was conducted with the same control level as that in set 1 since there was no increase in gallic acid yield under any other experimental conditions. In this case, a new set of experiments (set 2) was designed keeping the same control point as that in set 1 but reducing the range of magnitudes. New search points were selected around the initial control with different magnitudes. The experimental conditions and results of set 2 experiments are presented in Table 3. It was observed that the control set gave maximum gallic acid yield. From the analysis of set 2 results, the change in mean effect was found to be negative and large (–28.06). Further, most of the effects

Table 2
Experimental Conditions and Results of Set 1

	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11
Parameter											
Te (°C)	30	25	25	25	25	25	25	25	25	25	25
H (%)	80	70	70	70	70	90	90	90	90	90	90
P	5	4	6	6	6	4	4	4	6	6	6
Ti (h)	48	72	24	72	72	24	72	72	24	24	72
V (mL)	3	4	4	2	4	4	2	4	2	4	2
W (g)	35	40	40	40	30	40	40	30	40	30	30
Yield (cycle 1)	94.8	77.1	68.5	74.3	74.9	68.8	53.4	77.4	60.8	73.2	70
Yield (cycle 2)	94.8	69.7	66.5	70.1	81.3	67	60.4	79.8	66.9	64.4	69.6
Difference	0	7.4	2	4.2	–6.4	1.8	–7	–2.4	–6	8.8	0.4
Average gallic acid yield (%)	94.8	73.4	67.5	72.2	78.1	67.9	56.9	78.6	63.8	68.8	69.8
	A12	A13	A14	A15	A16	A17	A18	A19	A20	A21	A22
Parameter											
Te (°C)	35	35	35	35	35	35	35	35	35	35	25
H (%)	70	70	70	70	70	70	90	90	90	90	90
P	4	4	4	6	6	6	4	4	4	6	6
Ti (h)	24	72	72	24	24	72	24	24	72	24	72
V (mL)	4	2	4	2	4	2	2	4	2	2	4
W (g)	40	40	30	40	30	30	40	30	30	30	40
Yield (cycle 1)	55.8	56	72.8	72.8	60.4	74.8	75.6	54.6	65.8	67.4	70.6
Yield (cycle 2)	61	61	74.2	66.8	56.4	70.8	79.2	46.2	59.4	69.8	76
Difference	–5.2	–5	–1.4	6	4	4	–3.6	8.4	6.4	–2.4	–5.4
Average gallic acid yield (%)	58.4	58.5	73.5	69.8	58.4	72.8	77.4	50.4	62.6	68.6	73.3

	A23	A24	A25	A26	A27	A28	A29	A30	A31	A32	A33
Parameter											
Te (°C)	35	35	35	35	35	35	25	25	25	25	25
H (%)	70	90	90	90	90	70	90	70	70	70	70
P	6	4	6	6	6	4	4	6	4	4	4
Ti (h)	72	72	24	72	72	24	24	24	72	24	24
V (mL)	4	4	4	2	4	2	2	2	2	4	2
W (g)	40	40	40	40	30	30	30	30	30	30	40
Yield (cycle 1)	73.8	60.8	82.3	75.6	62.3	75.8	68.9	73.5	55.5	57.8	67.2
Yield (cycle 2)	72	58.8	81.3	68	55.1	81.8	70.7	82.1	58.1	61.2	70.4
Difference	1.8	2	1	7.6	7.2	–6	–1.8	–8.6	–2.6	–3.4	–3.2
Average gallic acid yield (%)	72.9	59.8	81.8	71.8	58.7	78.8	69.8	77.8	56.8	59.5	68.8
	A34	A35	A36	A37	A38	A39	A40	A41	A42	A43	A44
Parameter											
Te (°C)	30	25	25	25	25	25	35	35	35	35	35
H (%)	80	70	90	90	90	90	70	70	70	70	90
P	5	6	4	6	6	6	4	6	6	6	4
Ti (h)	48	72	72	24	72	72	72	24	72	72	24
V (mL)	3	4	4	4	2	4	4	4	2	4	4
W (g)	35	40	40	40	40	30	40	40	40	30	40
Yield (cycle 1)	94.8	63.4	76.8	65.9	69.3	84.2	68.6	76.5	56.7	69.9	67.8
Yield (cycle 2)	94.8	72.2	82	60.9	70.3	76.4	64.8	80.7	62.9	65.7	71.2
Difference	0	–8.8	–5.2	5	–1	7.8	3.8	–4.2	–6.2	4.2	–3.4
Average gallic acid yield (%)	94.8	67.8	79.4	63.4	69.8	80.3	66.7	78.6	59.6	67.8	69.5

(continued)

Table 2
Experimental Conditions and Results of Set 1 *(continued)*

	A45	A46	A47	A48	A49	A50	A51	A52	A53	A54	A55
Parameter											
Te (°C)	35	35	35	35	35	35	35	35	35	35	25
H (%)	90	90	90	90	90	90	70	70	70	70	90
P	4	4	6	6	6	4	6	4	4	4	6
Ti (h)	72	72	24	24	72	24	24	72	24	24	24
V (mL)	2	4	2	4	2	2	2	2	4	2	2
W (g)	40	30	40	30	30	30	30	30	30	40	30
Yield (cycle 1)	66.6	73.9	76.5	56.7	74.9	87.6	77.8	69.6	66.4	83.6	66.5
Yield (cycle 2)	70.2	70.7	70.5	61.9	82.3	82.6	72.8	68	64.4	77	61.9
Difference	–3.6	3.2	6	–5.2	–7.4	5	5	1.6	2	6.6	4.6
Average gallic acid yield (%)	68.4	72.3	73.5	59.3	78.6	85.1	75.3	68.8	65.4	80.3	64.2
	A56	**A57**	**A58**	**A59**	**A60**	**A61**	**A62**	**A63**	**A64**	**A65**	**A66**
Parameter											
Te (°C)	25	25	25	25	25	25	25	25	25	25	35
H (%)	90	90	90	70	70	70	70	70	70	70	90
P	4	4	4	6	6	6	4	4	4	4	6
Ti (h)	72	24	24	72	24	24	72	72	24	24	72
V (mL)	2	4	2	2	4	2	4	2	4	2	4
W (g)	30	30	40	30	30	40	30	40	40	30	40
Yield (cycle 1)	73.4	80.7	74.5	66.8	82.7	69.7	77.1	75.2	55.6	57.7	77.8
Yield (cycle 2)	66.8	78.1	67.1	69.8	80.3	62.9	78.5	80.7	57	61.3	79.8
Difference	6.6	2.6	7.4	–3	2.4	6.8	–1.4	–5.5	–1.4	–3.6	–2
Average gallic acid yield (%)	70.1	79.4	70.8	68.3	81.5	66.3	77.8	77.95	56.3	59.5	78.8

Table 3
Experimental Conditions and Results of Set 2

	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11
Parameter											
Te (°C)	30	28	28	28	28	28	28	28	28	28	28
H (%)	80	78	78	78	78	82	82	82	82	82	82
P	5	4.5	5.5	5.5	5.5	4.5	4.5	4.5	5.5	5.5	5.5
Ti (h)	48	60	36	60	60	36	60	60	36	36	60
V (mL)	3	3.5	3.5	2.5	3.5	3.5	2.5	3.5	2.5	3.5	2.5
W (g)	35	38	38	38	32	38	38	32	38	32	32
Yield (cycle 1)	94.8	81.0	52.76	64.44	68.18	55.4	83.4	69.6	50.9	63.93	72.44
Yield (cycle 2)	94.8	78.2	54.6	56.44	69.2	49.4	80.6	67	50.7	61.93	76.1
Difference	0	2.8	–1.84	8	–1.02	6	2.8	2.6	0.2	2	–3.66
Average gallic acid yield (%)	94.8	79.6	53.68	60.44	68.69	52.4	82	68.3	50.8	62.93	74.27
	A12	A13	A14	A15	A16	A17	A18	A19	A20	A21	A22
Parameter											
Te (°C)	32	32	32	32	32	32	32	32	32	32	28
H (%)	78	78	78	78	78	78	82	82	82	82	82
P	4.5	4.5	4.5	5.5	5.5	5.5	4.5	4.5	4.5	5.5	5.5
Ti (h)	36	60	60	36	36	60	36	36	60	36	60
V (mL)	3.5	2.5	3.5	2.5	3.5	2.5	2.5	3.5	2.5	2.5	3.5
W (g)	38	38	32	38	32	32	38	32	32	32	38
Yield (cycle 1)	54.6	52.52	78.84	56	61.14	75.28	63.6	72.6	73.4	86.5	84.66
Yield (cycle 2)	56.3	60	76.8	58.2	59.2	76.4	62.4	74.4	74.2	84.6	86.3
Difference	–1.7	–7.48	2.04	–2.2	1.94	–1.12	1.2	–1.8	–0.8	1.9	–1.64
Average gallic acid yield (%)	55.45	56.26	77.82	57.1	60.17	75.84	63	73.5	73.8	85.55	85.48

(continued)

Table 3
Experimental Conditions and Results of Set 2 *(continued)*

	A23	A24	A25	A26	A27	A28	A29	A30	A31	A32	A33
Parameter											
Te (°C)	32	32	32	32	32	32	28	28	28	28	28
H (%)	78	82	82	82	82	78	82	78	78	78	78
P	5.5	4.5	5.5	5.5	5.5	4.5	4.5	5.5	4.5	4.5	4.5
Ti (h)	60	60	36	60	60	36	36	36	60	36	36
V (mL)	3.5	3.5	3.5	2.5	3.5	2.5	2.5	2.5	2.5	3.5	2.5
W (g)	38	38	38	38	32	32	32	32	32	32	38
Yield (cycle 1)	89.9	52.7	83.4	76.7	57.5	50.43	58.3	74.6	72.6	62.6	61.2
Yield (cycle 2)	87.4	54.4	80.6	78.2	58.6	52.4	56.3	76.2	74.36	68.32	64.8
Difference	2.5	–1.7	2.8	–1.5	–1.1	–1.97	2	–1.6	–1.76	–5.72	–3.6
Average gallic acid yield (%)	88.65	53.55	82	77.45	58.05	51.42	57.3	75.4	73.48	65.46	63

	A34	A35	A36	A37	A38	A39	A40	A41	A42	A43	A44
Parameter											
Te (°C)	30	28	28	28	28	28	32	32	32	32	32
H (%)	80	78	82	82	82	82	78	78	78	78	82
P	5	5.5	4.5	5.5	5.5	5.5	4.5	5.5	5.5	5.5	4.5
Ti (h)	48	60	60	36	60	60	60	36	60	60	36
V (mL)	3	3.5	3.5	3.5	2.5	3.5	3.5	3.5	2.5	3.5	3.5
W (g)	35	38	38	38	38	32	38	38	38	32	38
Yield (cycle 1)	94.8	86.3	78.53	55.95	58.9	58.24	51.9	54.17	56.07	58.03	54.05
Yield (cycle 2)	94.8	76.0	75.6	58.35	58.6	56.4	52.3	55.8	56.8	60.7	58.3
Difference	0	10.3	2.93	–2.4	0.3	1.84	–0.4	–1.63	–0.73	–2.67	–4.25
Average gallic acid yield (%)	94.8	81.15	77.07	57.15	58.75	57.32	52.1	54.99	56.44	59.37	56.18

	A45	A46	A47	A48	A49	A50	A51	A52	A53	A54	A55
Parameter											
Te (°C)	32	32	32	32	32	32	32	32	32	32	28
H (%)	82	82	82	82	82	82	78	78	78	78	82
P	4.5	4.5	5.5	5.5	5.5	4.5	5.5	4.5	4.5	4.5	5.5
Ti (h)	60	60	36	36	60	36	36	60	36	36	36
V (mL)	2.5	3.5	2.5	3.5	2.5	2.5	2.5	2.5	3.5	2.5	2.5
W (g)	38	32	38	32	32	32	32	32	32	38	32
Yield (cycle 1)	71.59	76.55	66.01	66.16	67.53	54.03	70.6	68.6	63.4	64	69.6
Yield (cycle 2)	74.8	78.8	63.5	68.2	64.6	58.2	72.4	66.8	64.8	66.2	70.2
Difference	–3.21	–2.25	2.51	–2.04	2.93	–4.17	–1.8	1.8	–1.4	–2.2	–0.6
Average gallic acid yield (%)	73.19	77.68	64.76	67.18	66.07	56.15	71.5	67.7	64.1	65.1	69.9

	A56	A57	A58	A59	A60	A61	A62	A63	A64	A65	A66
Parameter											
Te (°C)	28	28	28	28	28	28	28	28	28	28	32
H (%)	82	82	82	78	78	78	78	78	78	78	82
P	4.5	4.5	4.5	5.5	5.5	5.5	4.5	4.5	4.5	4.5	5.5
Ti (h)	60	36	36	60	36	36	60	60	36	36	60
V (mL)	2.5	3.5	2.5	2.5	3.5	2.5	3.5	2.5	3.5	2.5	3.5
W (g)	32	32	38	32	32	38	32	38	38	32	38
Yield (cycle 1)	61.85	66.3	63.4	64.33	64.11	61.31	69	66.26	63.2	66.96	74.6
Yield (cycle 2)	66.5	67.8	71.4	66.5	66.8	61.2	70.8	68.2	60.5	70.6	68.4
Difference	–4.65	–1.5	–8	–2.17	–2.69	0.11	–1.8	–1.94	2.7	–3.64	6.2
Average gallic acid yield (%)	64.17	67.05	67.4	65.42	65.46	61.25	69.9	67.23	61.85	68.78	71.5

were also less than the error limits (±7.17 for averages, ±5.07 for effects, and ±4.53 for change in mean), and the interaction effects of the variables were also mostly less than the error limit. Thus, in accordance with the decision-making rule, the optimum levels of the process parameters were attained, which gives the highest yield of gallic acid under such conditions.

Conclusion

The optimum levels of the process parameters for obtaining a maximum gallic acid yield under MSSF using tannin-rich mixed substrates determined by the EVOP–factorial technique were 30°C, 80% relative humidity, 48-h incubation period, pH 5.0, 3 mL of inoculum, and 35 g of substrate quantity.

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