Kinetics and antimicrobial activity of gallic acid by novel bacterial co-culture system using Taguchi's method and submerged fermentation

Subbalaxmi Selvaraj
Manipal Institute of Technology
Julia Moraes Amaral
Universidade Estadual Paulista
VYTLA RAMACHANDRA MURTY (murty.vytla@manipal.edu)
Manipal University: Manipal Academy of Higher Education

Research

Keywords: Bacterial co-culture, Gallic acid, Taguchi design, Kinetics, Antimicrobial activity

Posted Date: April 26th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-445597/v1

Kinetics and antimicrobial activity of gallic acid by novel bacterial co-culture system using Taguchi's method and submerged fermentation

Subbalaxmi Selvaraj[1], Julia Moraes Amaral[2], Vytla Ramachandra Murty[1]*

[1]Department of Biotechnology, Manipal Institute of Technology, Manipal Academy of Higher Education, Manipal-576104, India.

, Brazil.

*Corresponding author:

Vytla Ramachandra Murty

Professor

Department of Biotechnology

Manipal Institute of Technology

Manipal-576104, India.

E-mail: murty.vytla@manipal.edu

Fax No.: 91-820-2571071

Phone: +91-9448529691

Abstract

In this present study, a tannase positive *Bacillus gottheilii* M2S2 and *Bacillus cereus* M1GT were co-cultivated for the production of gallic acid by using tannic acid as the sole carbon source through submerged fermentation. Optimizing various factors is important for the improvement of coculture metabolite production. Taguchi orthogonal array (OA) of Design of experimental (DOE) methodology was used to estimate the influence and significance of five factors (tannic acid concentration, glucose concentration, agitation speed, and inoculum size) on the gallic acid production in shake flask. L16 OA with five factors in four levels was considered with an experimental matrix of 16 trials. Among all the factors, Agitation speed contributed the highest for gallic acid production (28.28%), followed by glucose concentration (21.59%), inoculum size (19.6%), tannic acid concentration (19.54%), and pH (11.09%). Validation experiments were executed at the found optimized conditions which resulted in a 6.36-fold increase in gallic acid yield compared to the unoptimized condition. Further, the kinetics of growth, tannic acid degradation, and gallic acid yield were evaluated at the optimized conditions. The kinetic parameters $Y_{x/s}$, $Y_{p/s}$, and $Y_{p/x}$ were determined as 0.292 mg of cells/mg of tannic acid, 22.2 µg of gallic acid/mg of tannic acid, and 70.76 µg of gallic acid/mg of cells with a growth rate of 0.273 h^{-1} after 24 h of fermentation. Finally, antimicrobial activity of the product gallic acid was investigated against food-borne pathogenic *E. coli*, *and Serriatia marcescens, Bacillus* sp. and *S. aureus* using the agar disc diffusion technique. Gallic acid showed bacteriostatic against *E. coli, S. aureus, and Serriatia marcescens* with a zone of inhibition of 2 cm, 1.6 cm, and 1.3 cm respectively. Thus the cost-effective bioproduct gallic acid proved to be potentially effective to control food poisoning diseases and preserve foodstuff.

Keywords: Bacterial co-culture, Gallic acid, Taguchi design, Kinetics, Antimicrobial activity.

Background

Gallic acid (3,4,5-trihydroxy benzoic acid) is an organic compound, was discovered by Carl Wilhelm Scheele in 1878, and is a chemical constituent of tannic acid molecules found in plants and fruits [1, 2]. Gallic acid has wide applications in the production of an antibacterial drug called trimethoprim (pharmaceutical industry); in the synthesis of pyrogallol, inks, dihydroxyacetone, alkaloids, and photographic developer (chemical industry); and in the production of propyl gallate, an antioxidant, and gallate esters, as preservatives (food industry) [3, 4]. It is also known to exhibit several biological and pharmacological properties such as antibacterial, antiallergic, antioxidant, antimutagenic, anti-inflammatory, neuroprotective, and anticarcinogenic activities [3, 5-7]. The global yearly demand for gallic acid is about 8000 tonnes, 75% of it is used in the production of trimethoprim [2, 8] and the natural existence is limited.

In India, gallic acid is manufactured by extracting tannic acid from imported tara powder and acid hydrolyzing it with sulphuric acid at high temperatures to obtain gallic acid which is crystallized to obtain the final product trimethoprim (an antibacterial agent). The manufacture of trimethoprim involves three stages, viz;

$$Tara\ powder \xrightarrow{Stage\ I} Gallic\ acid$$

$$Gallic\ acid \xrightarrow{Stage\ II} 3,4,5-Trimethoxy\ benzaldehyde$$

$$3,4,5-Trimethoxy\ benzaldehyde \xrightarrow{Stage\ III} Trimethoprim$$

While most of the manufacturers of trimethoprim are having the technology of Stage III, stages I and II are not very much established. Indian companies have, by and large, been successful in achieving good efficiencies. Even though the production process is somewhat simple and their production units have been stabilized, the important raw material tara powder is expensive as it is presently imported and also has irregular supply demanding for an alternate source. The

major constituent of tara powder is tannic acid of about 40%. Conventionally the compound 3,4,5 trimethoxybenzaldehyde is produced by chemical methods, but these processes generate not only the environment but also processing problems like low purity, high cost, and low yield. Therefore, the production of substrate gallic acid for the synthesis of drug intermediates stands unsuitable. On the other hand, microbial tannase can be used to hydrolysis tannic acid into gallic acid which is a simpler method, requires fewer stages, and is a non-polluting process [2, 8, 9]. The tannase is an inducible enzyme secreted by microbes, plants, and animals, upon hydrolysis of tannic acid, liberates 9 parts of gallic acid and 1 part of glucose. Among them, microbial tannase mainly from fungi is well reported [10-13], still, the documents on tannic acid hydrolysis are scarce. Predominantly *Aspergilli* have been used for the hydrolysis of tannic acid to yield gallic acid [14, 10, 8, 6, 4], among bacteria Enterobacter sp. [15], *Bacillus subtilis* AM1[1], *Lactobacillus plantarum* CIR1 [1], *Klebsiella pneumonia* [16], *Corynebacterium* sp. [16] have been reported to produce gallic acid.

The gallic acid and tannase can be produced by both submerged fermentation (SmF) and solid state fermentation (SSF) [17]. However, submerged fermentation is the most preferred choice for bacterial metabolite production than SSF as they require high moisture content for their growth and metabolism, doubling time is short, purification of the product is easier, and control of the fermentation is simpler [17]. Upon the discovery of an enzyme tannase in 1867, a great deal of research was carried out for its products mainly from fungus, as tannins were considered bacteriostatic. The discovery of bacterial tannase was reported in 1983 [18] and the strong focus on the production of bacterial tannase and gallic acid was seen from 1990 onwards [13, 19-21]. In a natural habitat, fermentation is generally carried out by mixed cultures instead of pure culture. The mixed culture may show interactions among themselves and may provide internal regulation of growth and product formation [22]. These interactions of the microbial population also depend on the environmental factors and create

two types of association, synergistic (proliferates the microorganisms and increases the metabolite or enzyme production) and antagonistic (inhibits the growth of other microorganisms) [23]. Bacterial co-culturing is a unique method in which two or more different bacterial cultures develop on the same medium with some degree of interaction between them. In this study, those bacterial co-cultures with a positive association with each other can be used to improve the production of metabolite gallic acid and enzyme tannase rather than bacterial monoculture. There are quite a few articles reported where co-culturing of two cultures was found to enhance enzyme production like alpha-amylase [23, 24]. However, to the best of the authors' knowledge, no reports have been published on the production of tannase or gallic acid by the bacterial co-culturing method.

In this communication, we made attempts to enhance gallic acid production by co-culturing *Bacillus gottheilii* M2S2 and *Bacillus cereus* M1GT through SmF. In this study, Taguchi orthogonal array of DOE technique was used to study the effects of process parameters, includes tannic acid concentration, glucose concentration, agitation speed, inoculum size, and initial pH on the production of gallic acid, and to define the most optimal levels of these parameters to attain the higher yield of gallic acid. Besides, to understand the design of fermentation processes utilizing the co-culturing method, the kinetic parameters specific growth rate (μ), maximum biomass concentration (X_m), biomass yield coefficient based on substrate consumption ($Y_{x/s}$), product yield coefficient based on substrate consumption ($Y_{p/s}$), and product yield coefficient based on biomass ($Y_{p/x}$) were evaluated at the optimized process conditions. Finally, the product gallic acid produced was used to test the antimicrobial activity against the bacterial strains causing food poisoning diseases.

Material and methods

Microorganism and bacterial co-culture cell suspension preparation

Gallic acid-producing strains of *Bacillus gottheilii* M2S2 and *Bacillus cereus* M1GT were previously isolated from tannery effluent soil and gastrointestinal tract of Goat respectively by adopting liquid enrichment and spread plate methods as described elsewhere [25]. The ribosomal RNA gene sequence of newly isolated *Bacillus gottheilii* M2S2 and *Bacillus cereus* M1GT was deposited at Gen Bank bearing an accession ID as KU866380 and KX033490 respectively.

A one loop-full of strain *Bacillus gottheilii* M2S2 or *Bacillus cereus* M1GT was grown in 10 mL of sterilized nutrient broth for 20 h at 180 rpm and 32°C. Then 1 mL of the respective strain was further grown in 50 mL sterilized nutrient broth at the same condition. Further to obtain co-culture suspension, the individual suspensions of *Bacillus gottheilii* M2S2 and *Bacillus cereus* M1GT were mixed in equal proportions of volume ratio of 1:1 as these strains exhibited approximately equal proportions of cell quantity based on the viable cell count experiment [26].

Co-culture fermentation

To run the coculture submerged fermentation, 2 mL of 20 h old *Bacillus gottheilii* M2S2 inoculum and 2 mL of 20 h old *Bacillus cereus* M1GT inoculum each containing $4x10^{11}$ CFU/mL were aseptically mixed into 250 mL Erlenmeyer flask containing 100 mL of production media composed of 1% (w/v) of tannic acid (as carbon source and tannase inducer), 0.5% (w/v) of NH_4NO_3 (as nitrogen source), and supplemented with 0.05% (w/v) of K_2HPO_4, 0.05% (w/v) of KH_2PO_4, 0.05% (w/v) of $MgSO_4.7H_2O$, 0.05% (w/v) of NaCl; adjusted the pH to 5.0. The Erlenmeyer flasks were incubated in a rotary shaker at 32°C and 180 rpm for 24 h. Experiments were carried out in triplicates and after fermentation, the gallic acid content, tannic acid content, biomass, and pH were determined.

Optimization of gallic acid production by using Taguchi's L_{16} – orthogonal array design

Based on our preliminary studies using co-culture of *Bacillus gottheilii* M2S2 and *Bacillus cereus* M1GT, process parameters such as tannic acid concentration, glucose concentration, agitation speed, initial pH, and inoculum size were found to be the most influential parameters for gallic acid production. Since the variations in these parameters can alter the course of an experiment which in-turn the "response parameter" i.e. gallic acid concentration can be increased thereby helping to set the experimental parameters while designing an experiment.

To study the effect of both chemical and physical parameters on gallic acid production by bacterial co-culture and to enhance its concentration for maximum gallic acid production an ordered L_{16} Orthogonal array of experiments was used. The above-mentioned five parameters were evaluated and optimized at four different levels designated by 1, 2, 3, and 4 as shown in Table 1 with 16 experimental runs as depicted in Table 2. Concerning the optimization of process conditions, each column in Table 2 would represent specific process parameters, and each row would depict experimental runs with different combinations of parameters. The main aim of the optimization of process conditions was to enhance gallic acid production. For this, Taguchi's statistical method was used to find the optimal conditions which are based on the signal-to-noise ratio (S/N) function. This method eliminates the experimental variations improving the overall outcome of the experiment. This is done by setting the 'signal-to-noise' ratio as *"larger the better"* [27, 28]. The main aim of this statistical method is to find the optimal experimental conditions to enhance the yield of gallic acid by using the aforementioned option. Here, the gallic acid concentration is measured as a response, and the formula for S/N ratio is given as:

$$\frac{S}{N} = -10\, log\left[\frac{i}{N}\sum_{N=1}^{N}\left(\frac{1}{{X_i}^2}\right)\right] \qquad \text{(i)}$$

Where, S/N =signal-to-noise ratio; N = number of experimental runs; and X_i,= gallic acid concentration of respective runs. The optimal values of each parameter are those at the largest S/N ratio.

The design matrix, its analysis, and the process of experimental runs were evaluated using statistical software MINITAB 17 (Trial version). The evaluated data was taken to rank the most influential parameters on the yield of gallic acid and to find the best fermentation environments for co-culture of *Bacillus gottheilii* M2S2 and *Bacillus cereus* M1GT to produce the highest gallic acid.

To validate Taguchi's L_{16} orthogonal array design, the experiments were carried out in triplicates at determining optimized process conditions.

---------- **Table 1 and Table 2** ----------

Kinetic studies of gallic acid production at optimized process conditions

The kinetics of gallic acid production from co-culture of *Bacillus gottheilii* M2S2 and *Bacillus cereus* M1GT was evaluated in optimized conditions, which was found based on the Taguchi's L_{16} Orthogonal array approach as described below. The composition of fermentation media and cultural conditions are as follows: tannic acid 1 %(w/v), agitation speed 120 rpm, and inoculum size 10 %(v/v); whereas other components were maintained at 0.5 %(w/v) of NH_4NO_3, 0.05 %(w/v) of K_2HPO_4, 0.05 %(w/v) of KH_2PO_4, 0.05 %(w/v) of $MgSO_4.7H_2O$, and 0.05 %(w/v) of NaCl; at optimum pH 6.0. Then, for every 4 h of fermentation, tannic acid content, gallic acid, biomass concentration, total protein, and glucose were determined. Experiments were carried out independently in triplicates.

To better understand the bacterial co-culture of gallic acid production, different kinetic parameters i.e. specific growth rate (μ), biomass yield coefficient based on substrate utilization (Y_{xs}), product yield coefficient based on biomass (Y_{px}), product yield coefficient based on

substrate utilization (Y_{ps}), the specific rate of substrate utilization (q_s), and specific rate of product formation (q_p) were estimated based on the methods described by Doran [29]. The following are the set of equations used to estimate the fermentation kinetic parameters:

$$\mu = \frac{1}{X}\frac{dX}{dt} \quad (1)$$

$$Y_{xs} = \frac{dX/dt}{dS/dt} \quad (2)$$

$$q_s = \frac{1}{X}dS/dt \quad (3)$$

$$Y_{ps} = \frac{dP/dt}{ds/dt} \quad (4)$$

$$Y_{px} = \frac{dP/dt}{dX/dt} \quad (5)$$

$$q_p = \frac{1}{X}dP/dt \quad (6)$$

In the above equations, μ, X, S, P, and t are specific growth rate, biomass concentration, substrate (tannic acid) concentration, product (gallic acid) concentration, and the fermentation time respectively. All these fermentation kinetic parameters were studied in the batch process mode at optimized environmental conditions.

Analytical methods

After submerged fermentation, the total fermentation broth was subjected to centrifugation (10,000 rpm, 15 min, and 4°C). The cell-free extract was utilized for analysis of gallic acid, tannic acid, and final pH; whereas wet residue (pellet) was used to determine dry cell weight.

The product gallic acid was estimated spectrophotometrically using rhodanine as a coloring agent and methyl gallate as substrate [30]. The amount of gallic acid released was correlated with the gallic acid standard curve, which was attained by measuring the absorbance of different concentrations of standard gallic acid solutions ranging from 0 to 100 nmol.

Further, the protein precipitation method of Ann-Hagerman and Larry-Butler [31] was adopted to quantify the tannic acid content in the cell-free extract.

Finally, the dry cell weight method was used to determine the biomass concentration; the pellet obtained after centrifugation of fermentation broth was dried at 80°C in a hot air oven until it reaches constant weight. The biomass concentration was calculated as defined below:

$$Biomass\ concentration\ (g/L) = \frac{(weight\ of\ centrifuge\ tube\ with\ pellet) - (weight\ of\ empty\ centrifuge)}{Volume\ taken\ for\ centrifugation} \quad \text{(ii)}$$

Antibacterial activity of the gallic acid

Bacterial strains

The effectiveness of antibacterial activity of gallic acid produced by co-culture of *Bacillus gottheilii* M2S2 and *Bacillus cereus* M1GT under SmF was estimated using two strains of Gram-positive (*Bacillus* species and *Staphylococcus*) and two strains of Gram-negative (*Escherichia coli and Serratia marcescens*) bacteria causing food poisoning disease. The above-mentioned strains for antibacterial check are procured from the culture collection of the Institute of Microbial Technology, Chandigarh, India.

Inoculum preparations

The above-mentioned bacterial strains were grown overnight in Nutrient agar slants at 32°C. Further, the growth of bacterial strains was harvested and diluted using sterile saline water to obtain a viable cell count of 10^6 CFU/mL and its absorbance was adjusted at 600 nm using a spectrophotometer.

Antibacterial activity of gallic acid produced

The diffusion assay method was adopted to estimate the antimicrobial activity of the gallic acid produced under SmF by co-culture fermentation of *Bacillus gottheilii* M2S2 and *Bacillus cereus* M1GT. The gallic acid extract was steam-sterilized at 121°C and 15 psi for 15 min. The overnight grown bacterial suspension of 0.1 mL was spread plated on petri dishes containing 20 mL solidified nutrient agar medium. Further with the sterilized cork borer holes were punched on nutrient agar plates. Then, the gallic acid solution of 0.5 mL was poured into the well and the plates were kept in the incubator for 24 h at 37°C. The exhibition of clear zones was recorded by Vernier caliper and considered as the presence of antibacterial activity.

Results and discussion

Microbial production of metabolites mainly depends on the physical and chemical environment of the microorganism employed. Initially, the production of gallic acid was studied by co-culture fermentation of *Bacillus gottheilii* M2S2 and *Bacillus cereus* M1GT at 32ºC, 180 rpm and for 48 h in production medium (% w/v) comprising tannic acid, 1; NH_4NO_3, 0.5; K_2HPO_4, 0.05; KH_2PO4, 0.05; $MgSO4.\ 7H_2O$, 0.05; and NaCl, 0.05; with pH of 5.0. The maximum gallic acid concentration of 22.76 µg/mL was exhibited at 24 h of fermentation. The product gallic acid was found to be growth associated and also similar results have been observed in *Lactobacillus plantarum* [32] and *Bacillus sphericus* [33]. Based on the preliminary studies, the parameters such as tannic acid (carbon source), glucose (inducer), inoculum size, initial pH, and agitation speed were found to be most critical for the production of gallic acid by co-culture fermentation of *Bacillus gottheilii* M2S2 and *Bacillus cereus* M1GT.

Yet, the interaction effects of these parameters on the gallic acid yield were not possible with traditional methods of optimization, which has proven to exhibit a significant effect in the

regulation of metabolism [25]. Hence, optimization of fermentation media was examined for gallic acid yield with five critical parameters at their selected levels as shown in Table 1.

Regression Analysis

The co-culturing of *Bacillus gottheilii* M2S2 and *Bacillus cereus* M1GT was adopted in this study and the strains have shown a good yield of gallic acid in the initial study completed. As the bacterial co-culture production of gallic acid was not reported, hence this study aimed to check the prospect of the bacterial cultures producing a high amount of gallic acid on a laboratory scale. The process parameters such as tannic acid concentration, glucose concentration, agitation speed, initial pH, and inoculum size were considered in the development of mathematical models. Table 2 illustrates the final gallic acid concentration at different levels of fermentation media compositions. The Taguchi L_{16} design matrix showed significant differences in the gallic acid yield (Table 2). Among all 16 experimental trials, the maximum and minimum gallic acid concentration was achieved in Run 5 (143.52 µg/mL: Tannic acid, 1 %w/v; Glucose, 0 % w/v; Agitation speed, 120 rpm; pH, 5 and inoculum size, 10 % v/v) and Run 16 (19.79 µg/mL: Tannic acid, 2 % w/v; Glucose, 0.4 %w/v; Agitation speed, 100 rpm; pH, 6 and inoculum size, 6 % v/v) respectively at 32°C and 24 h of fermentation. The differences in the concentrations of gallic acid with a change in the media composition indicate that the production of fermentation products is the main function of media composition. This statistical method of optimization showed a significant increase in the gallic acid yield from 22.76 to 143.52 µg/mL when compared to the traditional method of media optimization.

The S/N ratio in Taguchi design is generally used to eliminate the experimental variations caused due to uncontrollable parameters. Table 2 depicts the results of the L_{16} orthogonal array for the production of gallic acid, and the S/N ratio (larger is better). It is an

important parameter in Taguchi's L_{16} Orthogonal array design to identify the optimal conditions for the process. The values of the S/N ratio tell which combination of parameters has the maximum effect on the response i.e. gallic acid concentration. The upper value of the S/N ratio indicates that those constituents in composition have the maximum effect on the gallic acid yield. In this study, based on the main effects of each parameter the order of parameters ranked as agitation speed > glucose concentration > inoculum size > tannic acid concentration > initial pH, indicating that agitation speed had the highest effect and initial pH exhibited the least effect on the gallic acid yield by co-culture of *Bacillus gottheilii* M2S2 and *Bacillus cereus* M1GT. The main effect plots of individual parameters are generated by plotting the response average against each parameter level using the software MINITAB (Figure 1). These plots explain how an individual parameter affects the response i.e. gallic acid yield. The main effect of a parameter can be negligible or zero if the line is horizontal to the X-axis. If the line makes the larger deviation in verticle location from the horizontal X-axis, then the main effect can be maximum. In this study, it is noticed that tannic acid at level 2 (1 %w/v), glucose at level 1 (0 %w/v), agitation speed at level 2 (120 rpm), initial pH at level 4 (7.0), and inoculum size at level 3 (8 %v/v) exhibited the maximum main effect on the gallic acid yield (Fig. 1). These levels and their values indicate the optimal process conditions for the growth and metabolism of *Bacillus gottheilii* M2S2 and *Bacillus cereus* M1GT. The optimal process conditions can also be predicted from the response table (Table 2).

----------**Figure 1**----------

Table 3 depicts the response table for the S/N ratio for each parameter and each parameter is ranked based on the delta, which generally relates to the relative degree of the effects of different parameters. A parameter with the highest delta is ranked 1. A parameter with a high S/N ratio indicates the better performance of that parameter for the production of gallic acid with the least error of measurement The graphical representation of the S/N ratio is

provided in the supplementary material (Additional Figure 1). Agitation speed exhibits the maximum contribution and is ranked 1 for delta followed by inoculum size and concentration of glucose as shown in Table 3. The mean in the Taguchi design represents the average response of gallic acid yield for each combination of parameters and levels. The maximum percent of this contributes greatly to enhance gallic acid yield. Based on the mean response as shown in Additional Table 1, agitation speed gave maximum influence followed by glucose content, inoculum size, tannic acid concentration, and initial pH which is similar to the responses shown in Table 3.

---------- **Table 3**----------

During the analysis of the effect of each parameter with their levels, the observation was noticed that the highest average effect was seen at the agitation speed of level 2. Subsequently, the next highest was glucose concentration, inoculum size, tannic acid concentration, and initial medium pH at levels 1, 3, 2 & 4 respectively (Fig.1 and Additional Table 2). The effect of the parameter at the individual level is defined as the difference between the average value at a high and low level of each parameter. Larger the difference, the stronger the significance. The positive or negative signs of values of the effect of different parameters at each level will determine whether the contribution towards gallic acid production has increased or decreased (Additional Table 2). It was found that the relative influence of process parameters on gallic acid is shown below for the fermentation period of 24 h at 32°C.

Agitation speed > Glucose concentration > Inoculum size > Tannic acid concentration > Initial pH.

Impact of interaction effects of parameters

The MINITAB 17.0 software generated the interaction effects and was examined individually to better understand the production of gallic acid by co-culture of *Bacillus gottheilii* M2S2 and

Bacillus cereus M1GT. The severity index (SI) helps to understand the interaction of two parameters on gallic acid yield at defined levels. Severity Index measures the angle between the two straight interaction lines in an interaction plot. SI also indicates the strength of the presence of interaction. The angle between the interaction lines can vary between 0 to 90 degrees and is expressed on a scale of 0 to 100 as the SI (Table 5). The results of predicted interactions shown in Table 5 are in good agreement with the interaction plots shown in the supplementary material (Additional Fig. 2A, 2B, and 2C).

Based on the statistical results represented in Table 5, it can be noticed that the interaction of initial pH and glucose concentration of the media showed the highest effect of 96.81 %. But, it is also intrusive to note that the initial pH is having the least percent factor of 11.09 % (Table 4) which showed maximum interaction with the parameter glucose concentration (21.59 %). Among all the selected parameters, the interaction between glucose concentration and agitation speed exhibited the least severity index of 23.46%. Hence from the interaction studies, it can be established that the effect of individual parameters on gallic acid is different and also in the grouping is independent of the effect of the individual parameter. This result suggests that the insignificant parameters at their levels can be very much significant when interacting with other parameters to enhance the gallic acid yield.

----------Table 4 and 5----------

Analysis of variance

The results of Taguchi's design matrix were analyzed and evaluated the contribution of each parameter towards the yield of gallic acid by Analysis of variance (ANOVA). Further, the quality of the experimental results was given by F-ratio. This is a test statistic used for more than a few independent parameters. The test statistics can be calculated as follows:

$$Test\ statistic = \frac{system\ variance}{unsystematic\ variance} \quad \text{(iii)}$$

If the sum of squares (SS) measures the variance, then the test statistic can be shown as

$$Test\ statistic = \frac{SS_{Model}}{SS_{Residual}} \quad \text{(iv)}$$

And the total variance is calculated as, $SS_{Total} = SS_{Model} + SS_{Residual}$

Where SS_{Model}, expected variance; and $SS_{Residual}$, random variance.

F ratio and a Mean sum of squares (MS) can be calculated using the formula shown below:

$$\text{F ratio} = \frac{\text{Model mean sum of squares } (MS_{Model})}{\text{Model mean sum of squares } (MS_{Residual})} \quad \text{(v)}$$

$$\text{Sum of squares (SS)} = \frac{\text{Sum of squares (SS)}}{\text{Degrees of freedom (df)}} \quad \text{(vi)}$$

ANOVA was carried out to evaluate the variation in gallic acid production caused due to each parameter and also to estimate the optimal level value of a parameter for the maximum product formation. The ANOVA exhibited the values of the model sum of squares, mean squares, and Variance is 15988, 3198, and 1066 respectively (Table 4). The model obtained from ANOVA for gallic acid production showed the multiple regression coefficients (R^2) of 0.998, which indicates that the model can explain a 99.8 % variation in the gallic acid yield.

Based on the statistical calculations and predictive analysis, the optimized values of the individual parameter are obtained and depicted in Table 6. The statistical analysis of Taguchi's L_{16} experimental design data for gallic acid yield revealed that Agitation speed contributed the maximum effect of 24.32 % and initial pH exhibited the least effect of 6.03 % on the gallic acid production at optimum conditions.

The final optimum media composition (% w/v) for an increased gallic acid yield by co-culture of *Bacillus gottheilii* M2S2 and *Bacillus cereus* M1GT is, tannic acid, 1; glucose, 0; agitation speed, 120 rpm; and inoculum size, 10 % (v/v); whereas other components were

maintained (% w/v) at 0.5, NH_4NO_3; 0.05, K_2HPO_4; 0.05, KH_2PO_4; 0.05, $MgSO_4.7H_2O$; 0.05, NaCl; and optimum pH 6.0 (Table 7).

To validate the results of the experimental design matrix, trials were carried out in triplicates at the optimized conditions and observed the gallic acid yield of 578.26 µg/mL which is in good agreement with the MINITAB software predicted value of 574.08 µg/mL (Table 7). This optimum condition produced 578.26 µg/mL of gallic acid concentration in 24 h of fermentation whereas 91.04 µg/mL was observed before process optimization and hence the yield was enhanced by 6.35 fold by Taguchi's L_{16} Orthogonal array of experimental design.

---------- Table 6 and 7----------

Kinetic studies of gallic acid production at optimized process conditions

The kinetic results of the co-culture of *Bacillus gottheili* M2S2 and *Bacillus cereus* M1GT in shake flasks for gallic acid yield are shown in Figure 2. Biomass concentration was inadequate during the initial 4 hours of inoculation; this condition is most common during growth where the lag period is vital to acclimatize to the new environment. during this period the. During the lag period, the growth was amplified from 0.07g/L to 0.16g/L whereas tannin content was declined from 9.4 g/L to 9.1g/L. The lag phase is followed by the log phase, in which the tannic acid content is reduced to 2.28 g/L from 9.1 g/L; whereas biomass growth was raised to 4.18 g/L from 0.16 g/L. During the log phase, biosynthesis and excretion of gallic acid were initiated, and by the end of the growth phase after 24 h of fermentation, the maximum increase was observed to be 586 µg/mL. Relatively constant biomass and decrease in gallic acid production were observed in the stationary phase, continuing until the death phase. By these findings, it was demonstrated that the gallic acid yield was growth-associated and that both strains of bacteria were rapidly growing strains with tremendous adaptability to the fermentation medium.

----------Table 8----------

----------Figure 2----------

During the fermentation process, biomass & product yield factors were estimated (Table 8). After 24 h of fermentation, it was found that maximum $Y_{p/s}$ and $Y_{p/x}$ were 175.95 mg/g of tannic acid and 172.89 mg/g of biomass respectively. This indicates that gallic acid production followed the growth pattern of *Bacillus gottheilii* M2S2 and *Bacillus cereus* M1GT, indicating that gallic acid was the primary metabolite produced. On further analysis, it was observed 4 h after fermentation that the maximum cell growth rate was 0.701 h^{-1}. This demonstrates that both strains grew much faster during the initial exponential phase. For the production of Tannase in SSF under optimized conditions, reported biomass, product yield, and specific growth rate were 0.276 g/g, 0.177 U/g, and 0.0703 h^{-1} respectively [34]. The evaluation of the specific rate of product formation (q_p), together with maximum biomass concentration (X_m) helps in distinguishing whether the increased production rate was due to high biomass production (high X_m) or because of a very productive strain (high q_p) [35]. In this study, it was observed that gallic acid production via a co-culture of *Bacillus gottheilii* M2S2 and *Bacillus cereus* M1GT showed more productivity with an average q_p of 73.14 µg/mg.h and was also due to the maximum X_m of 4.18 mg/mL at 24 h of fermentation (Table 8). The results of this study are in good pact with the outcomes reported by Aguilar et al. [35]. Certain variations in kinetic parameters and growth rate between the previous reports [36, 34] and the current report may have been caused due to differences in fermentation conditions and microbial species. This is the first report which was discussed the impact of kinetic parameters on gallic acid production via the co-culture method.

Antibacterial activity of gallic acid produced

In the food industry, the growth of pathogenic strains of bacteria is the main reason for food spoilage. To combat the adverse effects of food spoilage on health, the search for safe,

effective, and natural preservatives have been an integral part of the industry. In this present work, the product gallic acid produced through co-culture of *Bacillus gottheilii* M2S2 and *Bacillus cereus* M1GT under submerged fermentation was shown to be natural alternative preservatives for foodstuff and also combats food poisoning, avoiding any health hazards of chemical antimicrobial agent applications. The gallic acid produced under optimized conditions was extracted via cold centrifuge to study the antimicrobial activity. Further investigation of the gallic acid extract was done to estimate the antibacterial activity using the disc diffusion method against four different pathogenic strains of bacteria causing food poisoning - two Gram-positive strains (*Bacillus* sp. and *S. aureus*) and two Gram-negative strains (*E. coli*, *and Serriatia marcescens*). The results of the estimation of antibacterial activity are shown in Table 2 and Figure 3 and indicate that the gallic acid extract is most prominent in subduing the growth of food spoilage bacteria with variable efficacy. Hence, it can be inferred that the gallic acid extract showed robust antibacterial activity and high effectiveness against pathogenic bacteria. Anabela et al. [37] reported the antimicrobial activity of gallic acid against different pathogenic bacteria like *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Listeria monocytogenes*.

----------**Table 9**----------

----------**Figure 3**----------

Conclusion

A maximum gallic acid yield of 574.08 µg/mL was produced after statistical optimization of process conditions. The results of kinetic studies indicate that production of gallic acid through

coculture of *Bacillus gottheilii* M2S2 and *Bacillus cereus* M1GT is completely growth associated and 75 % of tannic acid was degraded within 24 h of fermentation. And a stable balance between growth and yield coefficients was obtained, which indicates that the gallic acid production can be scaled up for commercial use. The fed-batch operation can be implemented to maintain the balance between growth and production. The cell-free extract (gallic acid) has proved to be effective against food spoilage pathogens and can be used as an alternative food preservative to store foodstuffs and control food poisoning diseases.

Supplementary information

Additional Table 1. Response table for Means.

Additional Table 2. The average effect of parameters at assigned levels for gallic acid production under SmF with co-culture fermentation of *Bacillus gottheilii* M2S2 and *Bacillus cereus* M1GT.

Additional Figure 1. Main effects plot for SN ratios for the production of gallic acid with co-culture fermentation of *Bacillus gottheilii* M2S2 and *Bacillus cereus* M1GT.

Additional Figure 2A. Minitab generated interaction plots for the selected factors. (a) D x B; (b) A X B, (c) A X C, and (d) A x E.

Additional Figure 2B. Minitab generated interaction plots for the selected factors. (a) E x D; (b) A X D, (c) E X B, and (d) C x D.

Additional Figure 2C. Minitab generated interaction plots for the selected factors. (a) C x E; and (b) B X C.

Acknowledgments

The authors show thankfulness to the Department of Biotechnology, Manipal Institute of Technology, Manipal Academy of Higher Education, India; for providing the facilities to carry out the research work.

Authors' contributions

SS designed the study, performed the optimization, kinetics, and antimicrobial experiments, analyzed the data, and wrote the original draft. JMA contributed to the experimental investigation, data curation, and also in the drafting of the manuscript. VRM supervised the study, and was also involved in data interpretation and drafting the manuscript. All authors read and approved the final manuscript.

Funding

Not applicable.

Availability of data

The datasets used and/or analysed during the current study are available from the first author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Aguilar ZP, Cruz MA, Montanez J, Raul RH, Jorge EWP, Ruth EB, et al. Gallic acid production under anaerobic submerged fermentation by two bacilli strains. Microb Cell Fact. 2015;14:209.
2. Chandrasekaran M and Beena PS. Tannase: source, biocatalytic characteristics, and bioprocesses for production. Marine Enzymes for Biocatalysis. Woodhead publishing limited; 2013. Chapter 11.
3. Kanpiengjai A, Khanongnuch C, Lumyong S, Haltrich D, Nguyen TH, Kittibunchakul S. Co-production of gallic acid and a novel cell-associated tannase by a pigment-producing yeast, *Sporidiobolus ruineniae* A45.2. Microb Cell Fact. 2020;19**:** 95.
4. Banerjee D, Mahapatra S, Bikas RP. Gallic acid production by submerged Fermentation of *Aspergillus aculeatus* DBF9. Res J Microbiol. 2007;2: 462-468.
5. Verma S, Singh A, Mishra A. Gallic acid: molecular rival of cancer. Environ Toxicol Pharmacol. 2013;35(3):473–85.

6. Bajpai B, Patil S. A new approach to microbial production of gallic acid. Braz J Microbiol. 2008;39(4):708-711.

7. Kroes B, van den Berg A, van Ufford HQ, van Dijk H, Labadie R. Anti-inflammatory activity of gallic acid. Planta Med. 1992;58(06):499–504.

8. Lokeswari N. Production of gallic acid using mutant strain of *Aspergillus oryzae*. J Pharm Res. 2010;3:1402–1406.

9. Lokeshwari N, Sriramireddy D, Pola S, Bobbarala V. Production of an antibacterial drug trimethoprim using novel biotechnological approach. Drug Invent Today. 2010;2(5):268-270.

10. Arshad R, Mohyuddin A, Saeed S, Hassan AU. Optimized production of tannase and gallic acid from fruit seeds by solid state fermentation. Trop J Pharm Res. 2019;18:911–918.

11. Roberta C, de Lima JS, Fonseca J, Gomes JEG. Promising substrates to increase the production of tannase under solid state fermentation (SSF) by *Penicillium* spp. Afr J Biotechnol. 2017;16:2121–2126.

12. Xiao A, Huang Y, Ni H, Cai H, Yang Q. Statistical optimization for tannase production by *Aspergillus tubingensis* in solid-state fermentation using tea stalks. Electron J Biotechnol. 2015:18:143–152.

13. Mondal KC, Banerjee D, Banerjee R, Pati BR. Production and characterization of tannase from *Bacillus cereus* KBR9. J Gen Appl Microbiol. 2001;47:263–267.

14. Saeed S, Aslam S, Mehmood T, Naseer R, Nawaz S, Mujahid H, et al. Production of gallic acid under solid-state fermentation by utilizing waste from food processing industries. Waste Biomass Valor. 2020;12:155-163.

15. Sharma KP, John PJ, Goswami P, Soni M. Enzymatic synthesis of gallic acid from tannic acid with an inducible hydrolase of *Enterobacter spp*. Biocatal Biotransformation. 2017;35(3):177-184.

16. Deschamps AM, Lebeault JM. Production of gallic acid from tara tannin by bacterial strains. Biotechnol Lett. 1984;6:237-242.

17. Belur PD, Mugeraya G. Microbial production of tannase: state of the art. Res J Microbiol. 2011;6(1):25-40.

18. Deschamps AM, Otuk G, Lebault JM. Production of tannase and degradation of chestnut tannins by bacteria. J Ferment Technol. 1983;61: 55-59.

19. Das MP, Mondal KC, Pati BR. Production of tannase through submerged fermentation of tannin-containing plant extracts by *Bacillus licheniformis* KBR6. Polish J Microbiol. 2006;55(4):297-301.

20. Selwal MK, Yadav A, Selwal KK, Aggarwal NK, Gupta R, Gautam SK. Optimization of cultural conditions for tannase production by *Pseudomonas aeruginosa* IIIB 8914 under submerged fermentation. World J Microbiol Biotechnol. 2010;26(4): 599-605.

21. Natarajan K, Rajendran A. Evaluation and optimization of food-grade tannin acyl hydrolase production by a probiotic *Lactobacillus plantarum* strain in submerged and solid state fermentation. Food Bioprod. Process. 2012;90(4):780-792.

22. Purohit JS, Dutta JR, Nanda RK, Banerjee R. Strain improvement for tannase production from co-culture of *Aspergillus foetidus* and *Rhizopus oryzae*. Bioresour Technol. 2006;97(6):795-801.

23. Abdullah, R, Naeem N, Aftab M, Kaleem A, Iqtedar M, Iftikhar T, et al. Enhanced Production of alpha amylase by exploiting novel bacterial co-culture technique employing solid state fermentation. Iran J Sci Technol Trans Sci. 2018;42:305–312.

24. Pandey A, Selvakumar P, Soccol CR, Nigam P. Solid-state fermentation for the production of industrial enzymes. Curr Sci. 1999;77:149–152.

25. Subbalaxmi S, Murty VR. Process optimization for tannase production by *Bacillus gottheilii* M2S2 on inert polyurethane foam support. Biocatal Agric Biotechnol. 2016;7:48-55.

26. Li M, Zhao X, Zhang X, Di Wu, Su Leng. Biodegradation of 17β-estradiol by bacterial co-culture isolated from manure. Sci Rep. 2018;8:3787.

27. Singh TS, Verma TN. Taguchi design approach for extraction of methyl ester from waste cooking oil using synthesized CaO as heterogeneous catalyst: response surface methodology optimization. Energy Convers Manag. 2019;182:383–397.

28. Mohan SV, Reddy MV. Optimization of critical factors to enhance polyhydroxyalkanoates (PHA) synthesis by mixed culture using Taguchi design of experimental methodology. Bioresour Technol. 2013;128:409–416.

29. Doran, PM. Homogeneous Reactions. In: Bioprocess Engineering Principles. 1st ed. California: Elsevier;1995.p.257–295.

30. Sharma S, Bhat TK, Dawra RK. A spectrophotometric method for assay of tannase using rhodanine. Anal Biochem. 2000;279(1):85-89.

31. Hagerman AE, Butler LG. Protein precipitation method for the quantitative determination of tannins. J Agric Food Chem. 1978;26(4):809-812.

32. Kannan N, Aravindan R, Viruthagiri T. Effect of culture conditions and kinetic studies on extracellular tannase production by *Lactobacillus plantarum* MTCC 1407. Indian J Biotechnol. 2011;10(3):321-328.

33. Raghuwanshi S, Dutt K, Gupta P, Misra S, Saxena RK. *Bacillus sphaericus*: The highest bacterial tannase producer with potential for gallic acid synthesis. J Biosci Bioeng. 2011;111(6):635-640.

34. Subbalaxmi S, Vytla RM. Evaluation of kinetic parameters for growth, tannic acid utilization and tannase production in *Bacillus gottheilii* M2S2 using Polyurethane foam blocks as support. 3-Biotech. 2017; 7:275.

35. Aguilar CN, Augur C, Favela-Torres E, Viniegra-Gonzalez G. Production of tannase by *Aspergillus niger* Aa-20 in submerged fermentation and solid state fermentation: Influence of glucose and tannic acid. J Ind Microbiol Biotechnol. 2001;26:296-302.

36. Selvaraj S, Natarajan K, Nowak A, Murty VR. Mathematical modeling and simulation of newly isolated *Bacillus cereus* M1GT for tannase production through semi-solid state fermentation with agriculture residue Triphala. S Afr J Chem Eng. 2021;35:89-97.

37. Anabela B, Carla F, Maria JS, Manuel S. Antibacterial activity and mode of action of ferulic and gallic acids against pathogenic bacteria. Microb Drug Resist. 2013;19(4):256-265.

Figure Legends

1. The main effect plots of all individual parameters tannic acid, glucose, agitation speed, initial pH, and inoculum size on gallic acid yield under SmF by co-culture of *Bacillus gottheilii* M2S2 and *Bacillus cereus* M1GT.

2. Kinetic profiles of the concentrations of gallic acid, tannic acid, protein, and biomass in the batch process of gallic acid by co-culturing *Bacillus gottheilii* M2S2 and *Bacillus cereus* M1GT at optimized conditions in a shake flask through submerged fermentation.

3. Antimicrobial activity test of the gallic acid extract against food spoilage bacteria. C, Control (without gallic acid extract i.e. empty well); and T, Test (with gallic acid extract).

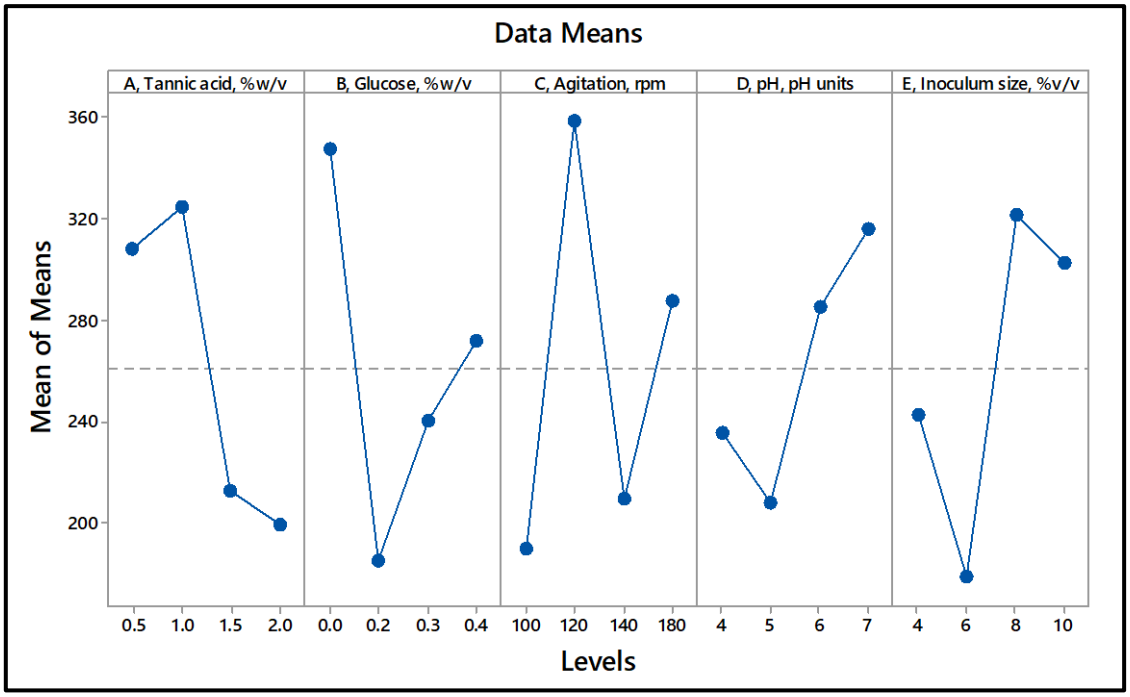

Fig.1: The main effect plots of all individual parameters tannic acid, glucose, agitation speed, initial pH, and inoculum size on gallic acid yield under SmF by co-culture of *Bacillus gottheilii* M2S2 and *Bacillus cereus* M1GT.

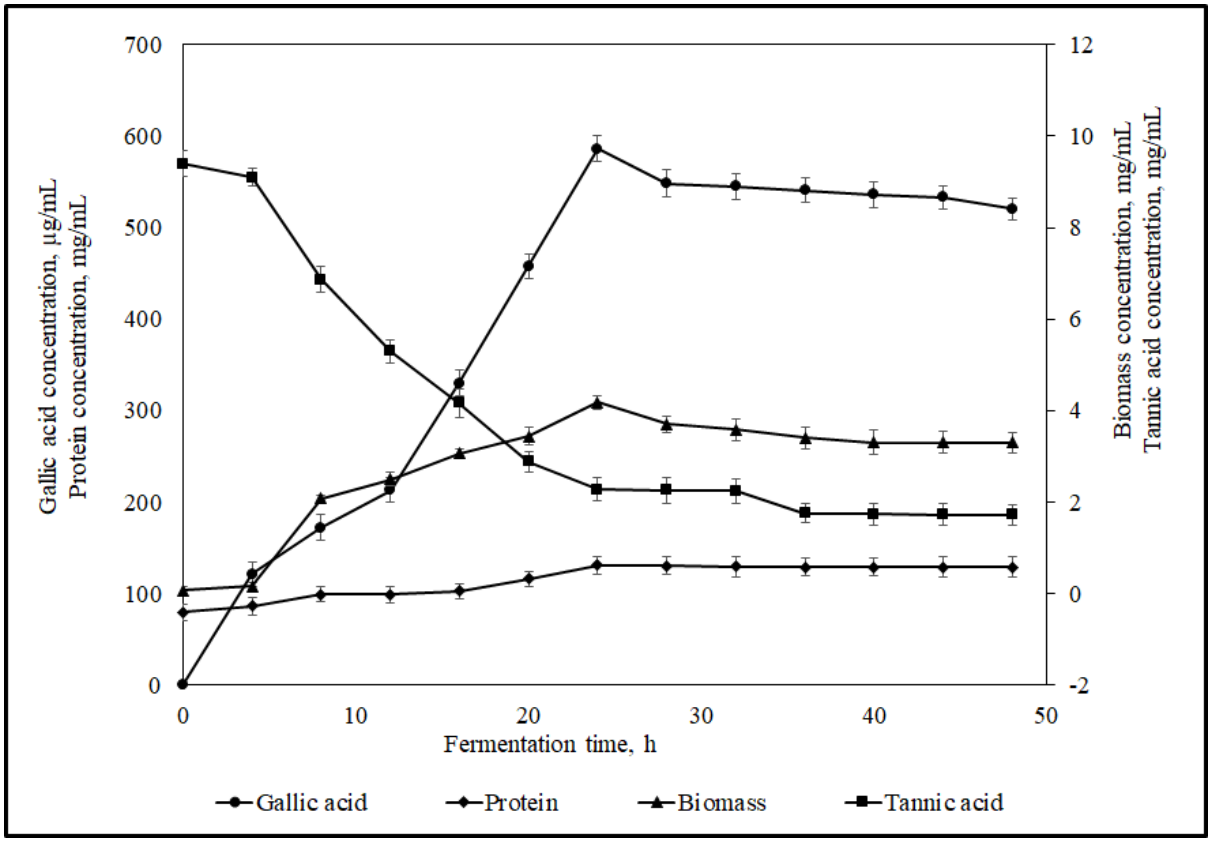

Fig.2: Kinetic profiles of the concentrations of gallic acid, tannic acid, protein, and biomass in the batch process of gallic acid by co-culturing *Bacillus gottheilii* M2S2 and *Bacillus cereus* M1GT at optimized conditions in a shake flask through submerged fermentation.

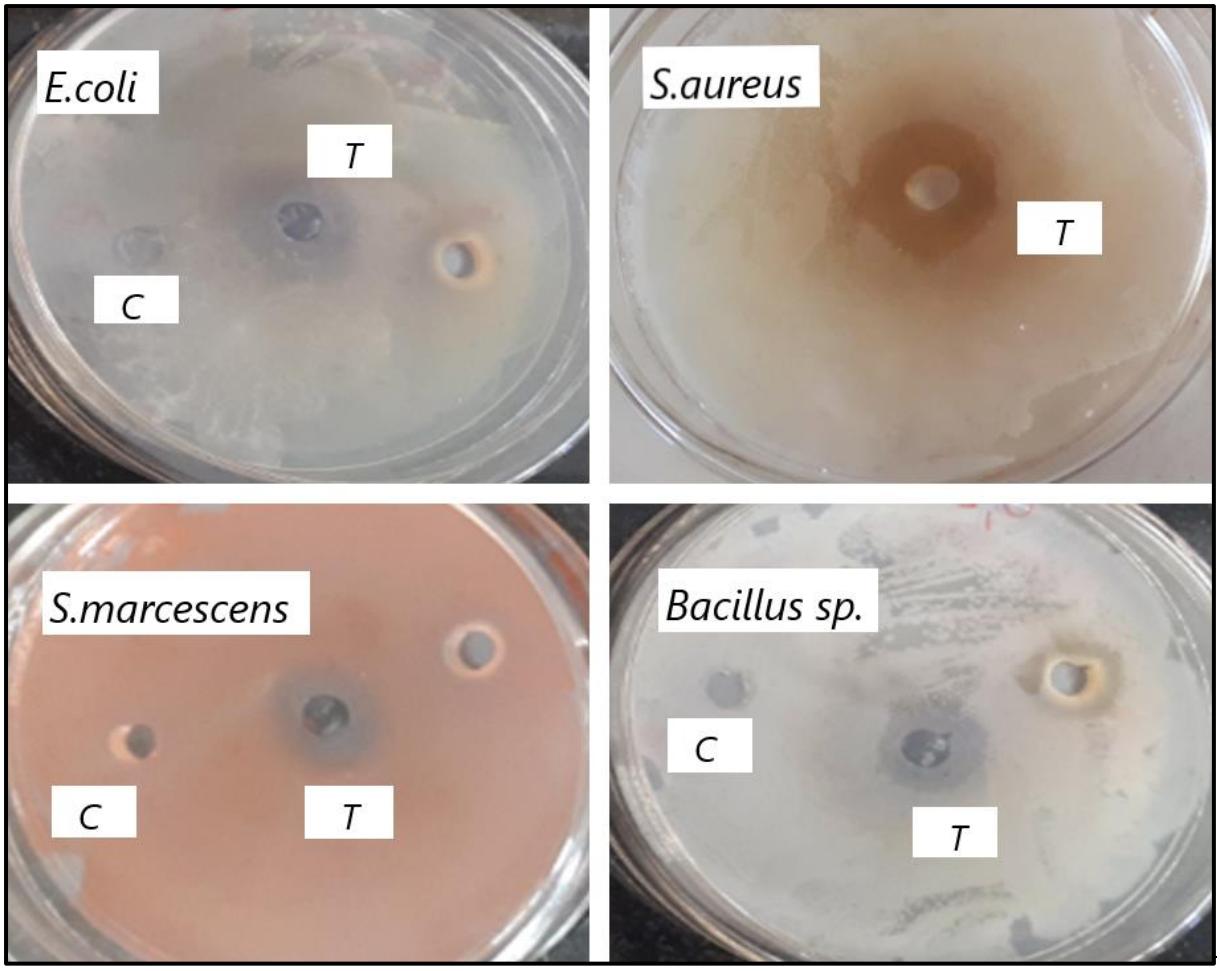

Fig.3: Antimicrobial activity test of the gallic acid extract against food spoilage bacteria. C, Control (without gallic acid extract i.e. empty well); and T, Test (with gallic acid extract).

Table 1: Parameters and their respective levels used in Taguchi's L_{16} Orthogonal array for the yield of gallic acid by co-culturing *Bacillus gottheilii* M2S2 and *Bacillus cereus* M1GT under submerged fermentation.

	Parameters	Level 1	Level 2	Level 3	Level 4
A	Tannic acid concentration (% w/v)	0.5	1.0	1.5	2.0
B	Glucose concentration. (% w/v)	0.0	0.2	0.3	0.4
C	Agitation speed (rpm)	100	120	140	180
D	Initial pH	4.0	5.0	6.0	7.0
E	Inoculum size (% v/v)	4.0	6.0	8.0	10

Table 2: Taguchi's L_{16} Orthogonal array of experimental design matrix for the production of gallic acid by co-culturing *Bacillus gottheilii* M2S2 and *Bacillus cereus* M1GT under submerged fermentation in shake flask.

Run	A	B	C	D	E	Gallic acid concentration (µg/mL)		S/N ratio
						Experimental	Predicted	
1	1	1	1	1	1	279.00 ± 0.88	279.00	48.91
2	1	2	2	2	2	193.63 ± 1.03	193.63	45.74
3	1	3	3	3	3	319.83 ± 0.93	319.83	50.09
4	1	4	4	4	4	441.70 ± 0.82	441.70	52.90
5	2	1	2	3	4	574.08 ± 0.79	574.08	55.18
6	2	2	1	4	3	292.00 ± 0.91	292.00	49.31
7	2	3	4	1	2	222.08 ± 1.12	222.08	46.93
8	2	4	3	2	1	210.95 ± 0.95	210.95	46.48
9	3	1	3	4	2	218.99 ± 0.89	218.99	46.81
10	3	2	4	3	1	168.26 ± 1.20	168.26	44.52
11	3	3	1	2	4	107.64 ± 1.42	107.64	40.64
12	3	4	2	1	3	354.47 ± 0.96	354.47	50.99
13	4	1	4	2	3	319.21 ± 0.75	319.21	50.08
14	4	2	3	1	4	87.23 ± 1.06	87.23	38.81
15	4	3	2	4	1	311.78 ± 0.69	311.78	49.88
16	4	4	1	3	2	79.18 ± 0.98	79.18	37.97

A, Tannic acid concentration; B, Glucose concentration; C, Agitation speed; D, Initial pH; E, Inoculum size.

Table 3: Response table for signal-to-noise ratios

Level	A	B	C	D	E
1	49.41	50.25	44.21	46.41	47.45
2	49.48	44.60	50.45	45.74	44.36
3	45.74	46.89	45.55	46.94	50.12
4	44.19	47.09	48.61	49.72	46.88
Delta	5.29	5.65	6.24	3.99	5.76
Rank	4	3	1	5	2

A, Tannic acid concentration; B, Glucose concentration; C, Agitation speed; D, Initial pH; E, Inoculum size.

Table 4: ANOVA for the yield of gallic acid with co-culture fermentation of *Bacillus gottheilii* M2S2 and *Bacillus cereus* M1GT.

	Parameter	DOF (f)	Sum of squares (S)	Variance (V)	Pure Sum (S)	Percent (P)%
1	Tannic acid concentration	3	49984	16661	49984	19.54
2	Glucose concentration	3	55240	18413	55240	21.59
3	Agitation speed	3	72079	24026	72079	28.18
4	Initial pH	3	28378	9459	28378	11.09
5	Inoculum size	3	50138	16713	50138	19.60
Other	Error	0				
Total		15	255818			

Table 5: Interaction effect of selected parameters on gallic acid yield with co-culture fermentation of *Bacillus gottheilii* M2S2 and *Bacillus cereus* M1GT under SmF.

Interacting parameter pairs	Column[a]	SI (%)	Col[b]	Opt[c]
pH X Glucose	4 x 2	96.81	7	[3,1]
Tannic acid x Glucose	1 x 2	81.19	3	[2,1]
Tannic acid x Agitation speed	1 x 3	73.00	5	[2,2]
Tannic acid x Inoculum size	1 x 5	66.21	9	[2,4]
Inoculum size x pH	5 x 4	60.47	13	[4,3]
Tannic acid x pH	1 x 4	59.91	6	[2,3]
Inoculum size x Glucose	5 x 2	57.50	10	[4,1]
Agitation speed x pH	3 x 4	37.48	9	[2,3]
Agitation speed x Inoculum size	3 x 5	42.34	12	[2,4]
Glucose x Agitation speed	2 x 3	23.46	6	[1,2]

[a]: locations of column to which interacting parameters are assigned.
SI: Interacting severity index (100 % for 90^{o} angle between the lines, 0 % for parallel lines).
[b]: shows the column that should be reserved if this interaction effect is to be studied.
[c]: Indicates the parameter levels desirable for the optimum conditions.

Table 6: Performance and optimum conditions for the yield of gallic acid by co-culture fermentation of *Bacillus gottheilii* M2S2 and *Bacillus cereus* M1GT.

Parameters	Levels	Level description	Contribution
Tannic acid concentration (% w/v)	2	1	20.31
Glucose concentration. (% w/v)	1	0	27.67
Agitation speed (rpm)	2	120	31.08
Initial pH	3	6	7.7
Inoculum size (% v/v)	4	10	13.23

Total contribution from all parameters = 100 %
Current grand average of performance = 261.25 µg/mL
Expected result at optimum conditions = 574.08 µg/mL

Table 7: Predicted and Experimental optimal conditions for the gallic acid production under SmF with co-culture fermentation of *Bacillus gottheilii* M2S2 and *Bacillus cereus* M1GT.

Parameters						Gallic acid
A	B	C	D	E		concentration, **µg/mL**
2	1	2	3	4	Predicted	574.08
					Experimental	578.26

A=Tannic acid concentration, B=Glucose concentration, C=Agitation speed, D=Initial pH, E=Inoculum size.

Table 8: Kinetic parameters of growth and gallic acid yield by co-culturing *Bacillus gottheilii* M2S2 and *Bacillus cereus* M1GT at optimized conditions in shake flask through submerged fermentation.

Time, h	μ, h^{-1}	$Y_{x/s}$ (mg/mg)	q_s (mg/mg.h)	$Y_{p/s}$ (µg/mg)	$Y_{p/x}$ (µg/mg)	q_p (µg/mg.h)	Gallic acid yield (µg/mL)
0	0	0	0	0	0	0	0
4	0.701	0.1176	24.57	13.32	757.50	320.423	121.20
8	0.423	0.302	1.426	25.05	082.70	34.983	172.02
12	0.298	0.473	0.914	40.27	085.20	36.039	213.00
16	0.236	0.734	0.588	79.15	107.87	45.629	330.08
20	0.194	1.194	0.362	158.94	133.07	56.288	457.76
24	0.166	1.833	0.235	257.017	140.19	59.301	586.00
28	0.140	1.637	0.264	242.478	148.11	62.649	548.00
32	0.123	1.598	0.270	243.286	152.22	64.390	544.96
36	0.109	1.943	0.222	309.04	159.07	67.284	540.82
40	0.098	1.896	0.227	308.21	162.51	68.741	536.28
44	0.089	1.910	0.226	308.22	161.58	68.349	533.22
48	0.082	1.920	0.225	302.43	157.63	66.677	520.18

Table 9: Antimicrobial activity test of the gallic acid extract against food spoilage bacteria.

Bacterial strain	Inhibition zone, mm
Serriatia marcescens	13.5 ± 0.43
Escherichia coli	20.4 ± 0.38
Staphylococcus aureus	16.5 ± 0.41
Bacillus sp.	09.4 ± 0.32

Figures

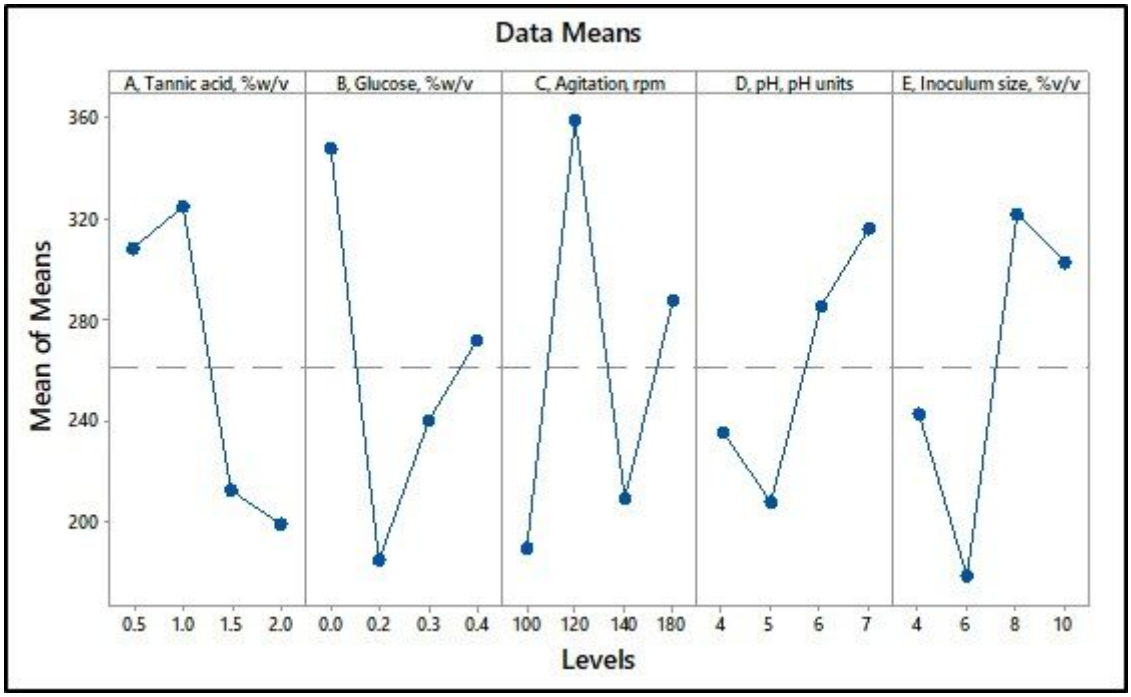

Figure 1

The main effect plots of all individual parameters tannic acid, glucose, agitation speed, initial pH, and inoculum size on gallic acid yield under SmF by co-culture of Bacillus gottheilii M2S2 and Bacillus cereus M1GT.

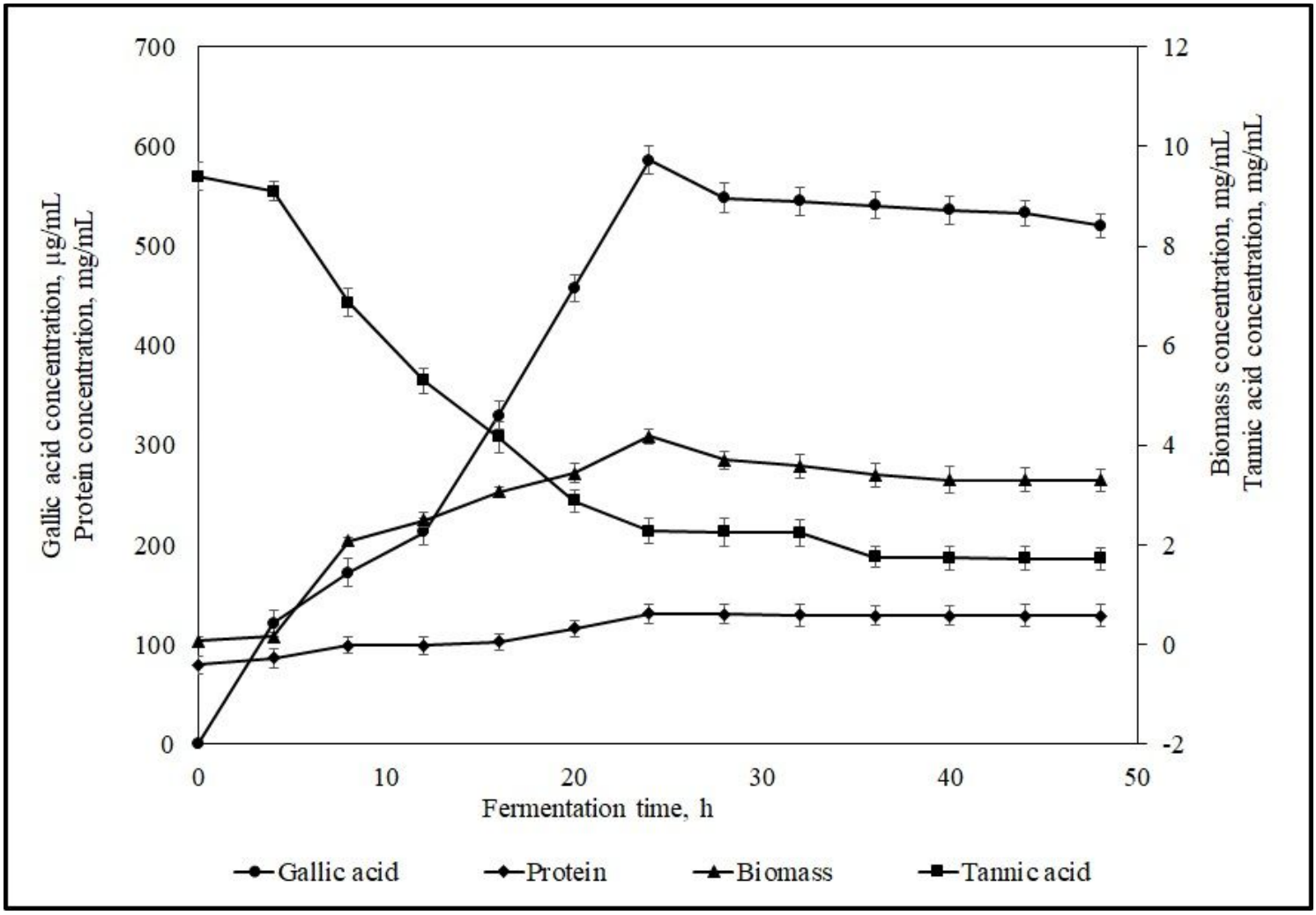

Figure 2

Kinetic profiles of the concentrations of gallic acid, tannic acid, protein, and biomass in the batch process of gallic acid by co-culturing Bacillus gottheilii M2S2 and Bacillus cereus M1GT at optimized conditions in a shake flask through submerged fermentation.

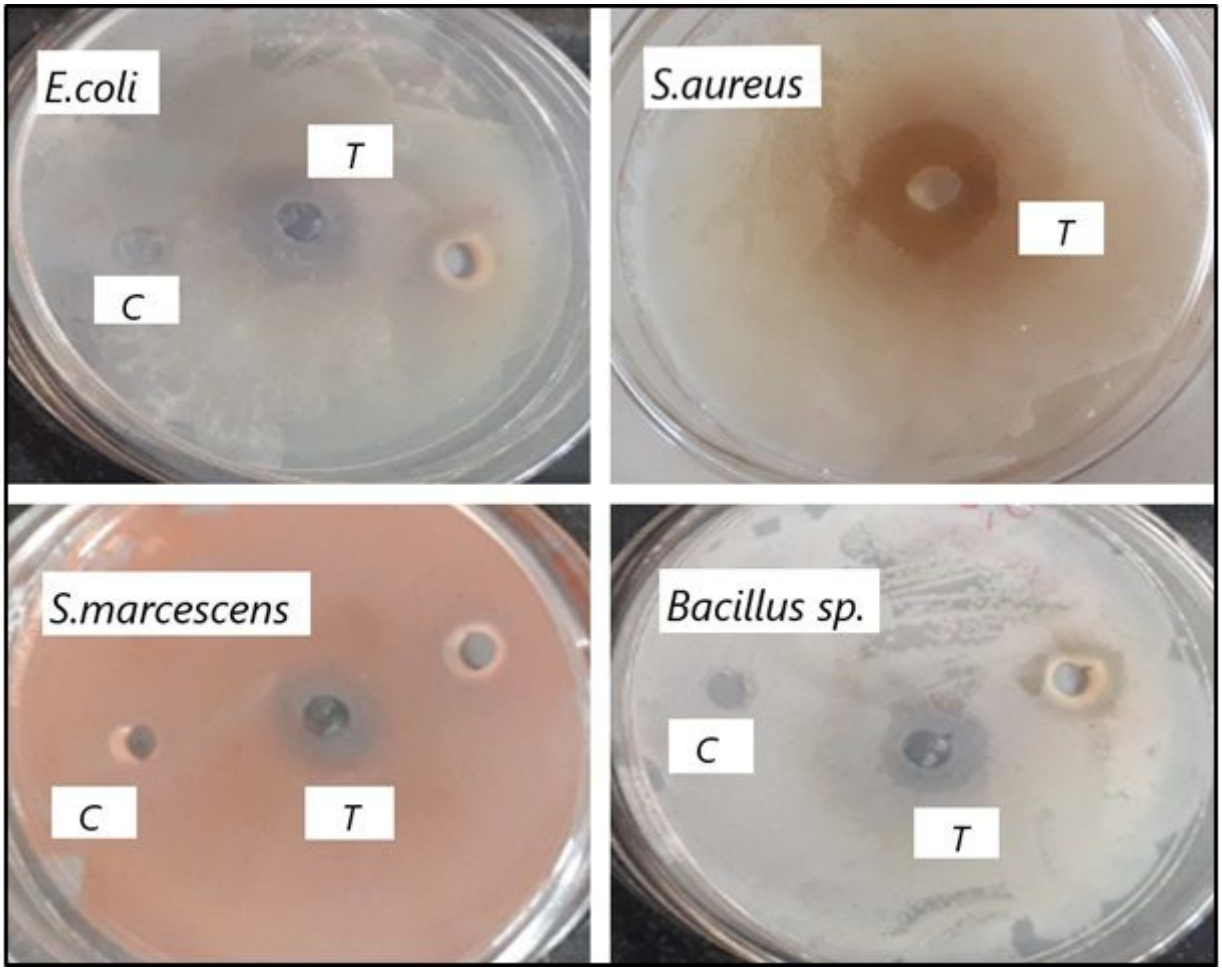

Figure 3

Antimicrobial activity test of the gallic acid extract against food spoilage bacteria. C, Control (without gallic acid extract i.e. empty well); and T, Test (with gallic acid extract).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryMaterials.docx