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Authors
Alaa A. Abdulshaheed[1]
Department of Microbiology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, Bangi 43600, Selangor, Malaysia, Department of Biology, College of Science, University of Baghdad 10071, Baghdad, Iraq.
p111456@siswa.ukm.edu.my, alaa.ali@sc.uobaghdad.edu.iq

Marlia Mohd. Hanafiah[2*]
Centre for Tropical Climate Change System, Institute of Climate Change, Universiti Kebangsaan Malaysia, Bangi 43600, Selangor, Malaysia, Department of Earth Sciences and Environment, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600, Bangi, Selangor, Malaysia;
mhmarlia@ukm.edu.my

Sahira Nsayef Muslim[3]
Department of Biology, College of Science, Al-Mustansiriyah University, Baghdad 10422,Iraq
mnt2000ther@gmail.com

Rab Nawaz[4]
Centre for Tropical Climate Change System, Institute of Climate Change, Universiti Kebangsaan Malaysia, Bangi 43600, Selangor, Malaysia
rabnawaz.utp@gmail.com

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Isolation, Identification, And Subsequent Application Of Gram-Negative Bacterial Strains For The Biosynthesis Of Tannase And Gallic Acid From Various Organic Sources

Abstract
Tannase (TN) and gallic acid (GA) are commonly produced using chemical processes that may contribute to environmental pollution since they release significant amounts of harmful effluents into the environment. This work aimed to biosynthetic method for producing TN and GA from various organic materials. Various clinical waste sources were collected and used to isolate 8 distinct gram-negative (GN) bacterial strains. Based on the screening results, Acinetobacter baumannii was chosen for further investigation due to its superior ability to produce TN and GA, as demonstrated by inhibition zone diameter tests. Among different organic materials, 0.4% amla seeds were found to be the best substrate for producing the highest amount of TN (18.276 U/mL) and GA (11.583mg/mL). To determine the most important factors influencing the process for TN and GA production, a "changing one factor at a time (COST)" approach, and response surface methods (RSM) were employed to optimise the parameters. The optimum production of TN and GA was observed at pH 5.78, temperature 38.69°C, inoculum size 10.47%, and incubation time 68.27h. According to the findings, A. baumannii can serve as excellent producers of TN and GA from organic materials with ammonium sulfate and amla seeds as nitrogen and carbon sources, respectively.

1. INTRODUCTION

Trihydroxybenzoic acid, commonly known as gallic acid (GA), is used in a variety of products, including but not limited to food, medicine, paints, inks, and dyes [1, 2]. According to reports, the 74 million US dollar global market for GA is projected to expand to $125.6 million by the end of 2026, with a compound annual growth rate of 7.8% between 2020 and 2026 [3]. Thus, more GA needs to be produced in order to meet the demand on a global scale. Chemical-based techniques commonly known as acid hydrolysis are now the most widely used methods for producing GA. However, this is not a sustainable approach since it releases significant amounts of hazardous effluents that may be a source of harmful emissions to the environment and produce low purity and limited output of the GA [4]. According to Andrade et al. [5], the enzymatic method using tannic acid is a promising method for producing GA as it is environment friendly and cost effective. However, utilizing commercial tannic acid has a number of drawbacks, including being expensive and requiring standard safety procedures (Fu et al., 2015). Naturally occurring tannic acid, such as that produced by bacteria and fungi from organic sources, is preferred because it lowers the cost and environmental impact of the production process.

Tannin acyl hydrolase (EC 3.1.1.20), commonly known as tannase (TN), is an extracellular enzyme that facilitates the breakdown of the ester bond in hydrolyzed tannin, producing glucose and GA as a byproduct [6, 7]. TN is used for a variety of purposes besides the manufacturing of GA, including the manufacture of beer, wine, coffee, tea, feed, food, pharmaceuticals, drinks, and even leather products [8]. Additionally, it is used as a catalyst in the biodegradation of tannin [9] and the removal of contaminants from the environment [10]. TN production from plants has been widely reported in the literature [11, 12]. However, using plants as a source of TN raises serious concerns because the WHO estimates that the global market for medicinal plants is currently worth around $14 billion and is anticipated to rise by 15 to 25% annually [3]. The plant species will become endangered if they are regularly exploited for such purposes. Alternative sources have been researched for their potential as TN and GA producing substrates, including Mangifera indica seed kernels [13], black plum seeds, Swietenia macrophylla [14], and a variety of leaves of tannic acid-rich plants, such as Syzygium cumini [2], Citrus sinensis, Psidium guajava, Ficus religiosa, Eucalyptus globulus, Citrus limon, and Ficus benghalensis [4]. Nonetheless, the hunt for inexpensive and waste materials to serve as a substrate in the TN and GA production and an environment friendlier biosynthetic process for TN and GA production is still ongoing.

In recent years, microorganisms have gained recognition as a source of enzyme production because of their biochemical and genetic diversity [7, 15, 16], higher final product yields, and valuable properties that make them suitable for biotechnological applications [4]. According to Kanpiengjai et al. [17], fungi exhibit enhanced TN activity due to their improved ability to biodegrade hydrolyzed tannin. For instance, Aspergillus niger was shown to be capable of producing 49.4 U/g TN and 13.3 mg/g GA under solid state fermentation from Triphala waste [3]. Nevertheless, the relative sluggish growth rate and genetic complexity of the fungi are, however, the main issues in using the fungal strain on an industrial scale. On the other hand, because some bacteria can thrive in environments with severe pH and temperature, they may contribute to the development of thermophilic TN [12, 18]. Despite the fact that bacteria can efficiently break down tannic acids and natural tannins, research on the production of bacterial TN is quite limited. Additionally, TN-producing bacteria isolation from waste sources appears to be exceedingly rare, despite the possibility of waste volatilization into TN and concurrent GA production.

By adjusting environmental factors as well as operating conditions that have a positive impact on enzyme activity, bacterial strains can produce TN and GA in large quantities. For greater yield and increased productivity, operating factors like medium formulation, microorganism producers, operating parameters, and raw materials used [19] must be optimized. Likewise, various environmental parameters like nutrients, dissolved oxygen, temperature, pH, agitation, and substrate concentration must also be taken into consideration. In addition to carbon, the medium's nitrogen sources—both organic and inorganic—have an impact on the production of TN [20]. The optimal conditions for the synthesis of an enzyme or metabolite did not appear to be the same as those for growth [21]. Studies to date have examined the impact of a few selected variables on the production of TN and GA by bacteria. Therefore, research is required to take into account as many variables as possible in order to maximize the production of valuable resources such as TN and GA while minimizing the resource input.

Historically, univariate analysis or design have been used to examine the impact of various operational factors on the efficiency of a process for the production of biochemicals [4, 5]. This suggests that the influence of independent factors on the efficiency of the process has been investigated by changing a single variable at a time (COST) approach. This approach can be useful if the aim is understanding a newly developed process. However, the issue with this kind of approach is that the data is not obtained in a random order, therefore this method could not be helpful for a well-developed process. In the absence of randomization, there's a good chance that a causal association will be found when none is there. Therefore, statistical tools or approaches must be applied in order to comprehend processes and apply a systematic strategy to improve them [18]. In order to obtain a useful and highly significant model of the TN and GA bio-production process by conducting a small number of experiments, which can save time and resources, a rigorous statistical design is therefore required. The current investigation has chosen to use the CCD model, which is a popular model in RSM since it considers both the primary impacts of individual factors and their interactions on the outcome variables.

In light of the aforementioned background information, the objective of this research was to develop a biosynthetic process by employing a methodical approach to produce TN and GA simultaneously while utilising various organic waste sources. Initially, 150 samples of

blood, sputum, urine, burn ICU, and wounds were gathered from various hospitals located in Baghdad, Iraq. Subsequently, different strains of GN bacteria were isolated from these sources of clinical waste and evaluated using zone of inhibition tests to determine their capacity to generate TN and GA. The ability of the top-performing isolate to produce TN and GA from various organic sources, such as pomegranate peel, tanninic acid, amla seed, raspberry leaves, and tamarind, was then investigated. Then, the optimal conditions for the growth of the best-performing bacteria were achieved by adjusting factors including the concentrations of carbon and nitrogen sources, an appropriate substrate, temperature, and incubation time, as well as the size of the inoculum. Lastly, RSM was employed to optimise the operating conditions for the biosynthetic process TN and GA production utilising the best-performing strains and the most appropriate substrate.

2. MATERIALS AND METHODS

2.1 Materials

Ammonium nitrate (NH4NO3) (Merck, Germany), ammonium sulphate (NH4)2SO4) and ammonium chloride (NH4Cl) (Promega, USA), bovine serum albumin (BSA) (BDH, England), brain heart infusion broth (BHIB), brain heart infusion agar (BHIA), clinical waste samples, glycerol (Oxoid, England), Nutrient broth (NB), Nutrient agar (NA), Muller Hinton agar (MHA), MacConkey agar, Monopotassium phosphate (KH2PO4) (BDH, England), tryptone, peptone, GA (Himedia, India), Sodium chloride (NaCl) (JTBaker, UK), yeast extract (Difco, USA), and distilled water was used whenever required.

2.2 Clinical waste samples collection and preservation

In sterile glass vials, 150 clinical waste samples—including sputum, blood, urine, and samples from the burn ICU—were gathered from various hospitals in Baghdad, Iraq. The samples were taken from Al-Dehwi Private Hospital, Al-Elwiya Maternity Teaching Hospital, Al-Balady Hospital, and Al-Yarmouk Teaching Hospital. The samples were collected in a span of three months between October and December of 2021. The samples were labelled and kept at 4°C in BHIA.

2.3 Isolation and identification of bacterial strains

Every bacterial isolate was cultivated for 24 h at 37°C on MacConkey agar. After incubation for 24 h, subculturing and gramme staining were carried out following refs [22, 23]. Then standard biochemical tests, such as oxidase [24] and catalase [25] were then conducted in accordance with the results of the gramme staining. All of the bacterial isolates were injected into MacConkey agar using a sterile plastic loop, and they were then cultivated for 24 h at 37°C in an aerobic atmosphere. Pure cultures of GN bacteria were obtained by continuously streaking a single colony on MacConkey agar. The purified stock cultures were either stored in BHIA at 4°C and sub-cultured every two weeks or kept for long-term preservation at –20°C in BHIB containing 20% glycerol.

The isolated bacterial strains were identified using an automated technique known as VITEK®2 as described previously [26]. Following the guidelines provided by the manufacturer, each tube containing the produced suspension was individually identified using the VITEK 2 Compact® device, VITEK® GN cards, and VITEK 2 system software (bioMeerieux, France). All tested strains underwent up to 24 h of incubation. During the triplicate study of the strains—two independent tests and three cards per strain—only the result that was replicated in two of the three cards was considered.

2.4 Estimation of tannin content

TN and GA were produced using a variety of organic substrates, including pomegranate peels, amla seeds, raspberry leaves, and tamarind seeds. The tannin content of these substrates was assessed using the method described by Saeed et al. [4] with minor modifications. The process includes allowing each substrate's seeds, leaves, and peels to dry naturally. After that, the dried material was submerged in 10 mL of 70% ethanol and allowed to dry overnight at 4°C. The sample of each substrate was then placed in a test tube, diluted with 3 mL of BSA, and incubated for 15 min. The combined sample was centrifuged for 10 minutes at 5,000 rpm. The supernatant was discarded and the pellets were allowed to react in solution of FeCl3 and SDS-triethanolamine (1:3, v/v). The test tubes were left for 15 min to react and finally the absorbance of the solution was measured at 520 nm [27] using a spectrophotometer. The absorbance data was used to estimate the tannin content of different substrates.

2.5 Screening of bacterial strains

The potential of each identified bacterial strain to produce TN and GA was examined. Numerous quantitative and semi-quantitative procedures were used to evaluate the TN and GA production by each isolate.

2.5.1 Semi-quantitative test

A slightly modified version of the method described by Muslim et al. [16] was used to prepare the tannic acid agar medium, which contained the following ingredients per 100 mL: 0.5 g tannic acid, 1.0 g peptone, 0.05 g KH2PO4, 0.2 g NH4NO3, 0.5 g NaCl, and 3.0 g agar-agar. A dark green zone around the colonies was produced after 24 h of incubation at 37°C as a result of the tannic acid nearby being hydrolyzed. The green zone was assessed and the results were compared for different strains.

2.5.2 Quantitative test

Without the use of agar, the chosen isolates were cultivated in tannic acid medium before being incubated at 37°C for 24 h on a rotating shaker at 150 rpm. The cells were extracted using a cold centrifuge at 8000 rpm for 10 min. The TN enzyme was employed with the clear supernatant. Then, the supernatant was examined to determine the amount TN and GA produced by each bacterial strain according to ref [12]. The optical density (OD) of the TN enzyme was measured at two wavelengths (254.6 and 293.8 nm) using the approach reported by Saad et al. [1] to determine the GA concentration. GA concentration was quantified using Eq. 1.

$$\text{GA concentration}\left(\frac{\text{mg}}{\text{mL}}\right) = 21.77\ (\text{OD}_{254.6}) - 17.17\ (\text{OD}_{293.8}) \quad (1)$$

2.5.3 Tannase assay

The TN activity of the bacterial isolates was evaluated in accordance with the spectrophotometric methods reported

in ref [28]. For the TN assay, two distinct reaction mixtures were prepared. In a test tube, 2 mL of the substrate containing 0.35% w/v of tannic acid dissolved in 0.1 M acetate buffer at pH 5 was combined with 0.5 mL of TN to create mixture 1 (t1). Subsequently, 2 mL of 95% ethanol solution was mixed with 20 µL of the assay reaction mixture to stop the enzymatic reaction. Following the addition of ethanol to t1 for dilution, the absorbance at 310 nm was measured using a UV-Vis spectrophotometer. Additionally, Et1 and Et2 were OD 310 nm of tannase following the addition of ethanol and after a specific amount of time of tannase reaction, respectively. The t2 mixture was withdrawn once more and measured after a specific amount of time of enzyme reaction at 37°C. The TN activity was calculated using the following equation (Eq. 2).

$$\text{TN activity} = 114 \times \frac{E_{t2} - E_{t2}}{t_2 - t_1} \quad (2)$$

A unit of TN activity is the quantity of TN, expressed as U/mL, needed to hydrolyze 1 µmol of tannic acid's ester bonds in 1 minute. The unit of measurement for specific activity was the TN activity unit per mg of enzyme protein (U/mg).

2.5.4 Protein assay

For the protein assay, 50 µL of 1 M NaOH was mixed with 20 µL of each enzyme, and the mixture was agitated constantly for two to three min. After that, 1 mL of Bradford Regent was added to the mixture. Using the standard curve as a reference, the absorbance (OD) was measured at 595 nm to calculate the protein content (mg/mL). Plotting the protein concentration against the absorbance at 595 nm resulted in establishing a standard curve (10–100 mg/L) that may be used to estimate the protein content of unidentified samples. DI water was utilized as the blank and BSA as the standard. The protein concentration was calculated using Eq. 3.

$$\text{Portien concentration} = \frac{OD_{595\ nm}}{\text{slope} \times 1000} \quad (3)$$

2.6 Optimization of growth of A. baumannii

Different growth conditions were optimized to maximize the production of GA and TN when cultivating the best-performing bacterial strain, A. baumannii. The impact of multiple factors, including culture medium, carbon source and concentration, nitrogen source and concentration, on the production of TN and GA was examined and optimized. These growth parameters were optimized through the application of the COST technique. While maintaining the other variables constant, one variable was changed within its designated range. After figuring out the first parameter's optimal value and holding the first one constant, we went on to the next one. As explained in the following sections, the optimization studies were carried out in a methodical manner.

2.6.1 Effect of culture medium

To find the best culture medium with tannin extract, diverse culture media including Brain heart infusion agar (BHIA), nutrient augur (NA), and Muller Hinton agar (MHA) were employed with various tannin extracts. The tannin extracts, including tannic acid, tamarind seeds, amla seeds, raspberry leaves, and pomegranate peels were held constant at 0.5%. The chosen isolate was infused into this medium, cultured for 24 h at 37°C, and the colony's dark clear zone was assessed. The best combination of the culture media and the extract was selected for further investigations. The selected isolate was inoculated with 0.5% inoculum size onto this media and incubated on a laboratory shaker at 150 rpm, 37°C, pH 6 for 24 h. After cooling centrifugation, a clear supernatant was used as an extract. Then, the activity of TN and GA concentration were measured.

2.6.2 Effect of carbon and nitrogen sources

The effect of carbon concentration on TN and GA production was evaluated. In place of 0.5% tannic acid, various amounts of the chosen carbon source (tannic acid, tamarind seeds, amla seeds, raspberry leaves, and pomegranate peels) ranging from 0.1–0.9% were made and applied. The A. baumannii was infused into the medium and incubated for 24 h at 37°C and pH 6. Estimates of TN and GA were made for each carbon concentration. Different nitrogen sources, including ammonium chloride, tryptone, yeast extract, peptone, sodium nitrate, and ammonium sulphate, were utilized in the medium along with the chosen carbon source at a concentration of 0.5%. The TN assay and GA levels were evaluated following a 24 h incubation period at 37°C. The study employed various concentrations of the optimal nitrogen source (0.1–0.9%) to analyze how varying nitrogen source concentrations affected the yield of TN and GA.

2.6.3 Effect of pH, temperature, incubation time and inoculum size

The impact of pH, temperature, incubation period, and inoculum size on TN and GA production were also investigated. Tannin and substrate were incubated for 24 h at various pH levels between 4.0 and 7.5 at 37 °C in order to determine the optimal pH range for the production of TN and GA. The reaction mixture was incubated at varying temperatures ranging from 10 to 45°C in order to determine the optimum temperature for the formation of TN and GA. An optimum medium with an optimal pH and temperature was produced to optimize the incubation period. At the optimum temperature, the medium was introduced with various incubation times (24–96 h). Additionally, GA concentration and TN assays were carried out. The optimal medium was produced and sterilized to maximize inoculum volume. This medium was inoculated with a range of inoculum volumes (5–12%) during the optimum incubation period and temperature. Then, GA concentration and enzyme activity were examined.

2.7 Optimization of environmental factors using RSM

A number of experiments were carried out to thoroughly analyze and optimise the most important factors for the A. baumannii-assisted bio-production of GA and TN. Statistical software (Design Expert 12.0.0, State®Eas Inc.) was used to design the experiments. It was also employed for process variable optimisation, statistical modelling, and analysis. In the current investigation, independent variables including inoculum size (X1) in percentage, pH (X2), temperature (X3) in °C, and incubation period (X4) in h were taken into account. The response variables, denoted as Y1 and Y2, were TN (U/mL) and GA (mg/mL), respectively. Using Equation

(4), the selected factors were transformed into dimensionless values and coded as A → X1, B → X2, C → X3, and D → X4 which will make comparing different components of different types and units of input variables. This will also compensate for a lack of mathematical model fit and assist lower the likelihood of statistical analysis inaccuracy.

$$x_i = \frac{x_i - X_o}{\Delta X} \quad (4)$$

where ΔX is the value assigned to the step change, xi is the dimensionless coded value assigned to the ith factor, and Xo is the centre point value of xi.

The minimum and maximum values of each input variable were assigned values of –1 and +1 correspondingly, in accordance with the CCD principle. Table 1 displays the range of the independent parameters together with their actual and coded values. The range of the selected parameters were based on the values reported in the literature [7, 29, 30]. The experimental design was based on the CCD principle. The experimental design matrix is shown in Table 3. With four (n) variables, the design had "2n" fractional factorial points, "2n" axial points, and "1" centre point. Thus, 10 axial points, 10 centre points, and 10 fractional factorial points were used in 30 tests. Three replications were collected, and the average is provided in order to compute the experimental error, reduce it, and get reliable findings.

Table 1. Description and range of chosen independent variables.

Factor	Unit	Symbol	Coded value	Range	
				Minimum (–1)	Mximum (+1)
Inoculum size	%	A	x_1	5	12
pH	—	B	x_2	4.0	7.5
Temperature	°C	C	x_3	10	45
Incubation time	h	D	x_4	24	96

An empirical model shown in Eq. (5) was used to represent the biosynthetic process of TN and GA formation. The optimal values for the input variables were identified and the nature of the output was explored using RSM.

$$Y = \beta_o + \sum_{i=1}^{n} \beta x_i + \sum_{i=1}^{n} \beta_{ii} x_i^2 + \sum_{i=1} \times \sum_{i \neq j=1} \beta_{ij} x_i x_{ij} + \varepsilon \quad (5)$$

where, Y represents TN and GA production efficiency, is the coefficient for linear effects, o represents the error, ii is the quadratic ij is interaction effects of the independent factors.

To statistically validate the model, CCD's default test of data analysis—the analysis of variance (ANOVA)—was employed. The ANOVA results were used to infer the intricate relationships between the two response and the four independent factors of the overall data scenario. The coefficient of determination (R2) shown in Eq. (6), the main result of multiple regression analysis, was used to interpret the quadratic polynomial model fitting to the output data. The results were assessed using statistical tests such as the probability test (p-value), alpha (α = 95%, 0.05), Fisher's test (F-value), and others.

$$R^2 = \frac{\sum_{t=1}^{n} (A_t - P_t)^2}{\sum_{t=1}^{n} (A_m - P_t)^2} \quad (6)$$

3. RESULTS AND DISCUSSION

3.1 Estimation of tannin content

The results showed that different plant extracts had different tannin content. Among all seeds, leaves and peels, amla seeds were reported to have the highest tannin content (45.61 mg/g), followed by pomegranate peels (39.12 mg/g), tamarind seeds (13.21 mg/g), and mulberry leaves (4.95 mg/g). The result of tannin content in amla seeds is similar to that reported in the previous report by Kumar et al. [31]. According to Kumar et al. [32], the reported tannin content of amla seeds was 40.3 mg/g which is lower than the tannin content estimated in this study. The difference in tannin content in the seeds, leaves and peels of the same plant may be due to different environmental conditions in addition to the diversity of nutrients in different soil regions.

3.2 Gram-negative bacterial isolation and identification

Eight different strains of GN bacteria of varying numbers were isolated from a total of 150 clinical samples collected from various hospitals in Iraq. GN bacterial strains were identified using the VITEK 2 compact system and the results are shown in Figure S2–S9. The strains were identified as Escherichia coli (15), Klebsiella pneumoniae (23), Enterobacter cloacae (11), Burkholderia cepacian (4), Pseudomonas aeruginosa (13), Serratia marcescens (8), Acinetobacter baumannii (25), and Acinetobacter lwoffii (23). The distribution of isolated strains from different clinical sources is shown in Figure 1. At 25% each, the GN bacteria were most prevalent in the sputum and blood samples. Urine samples contained the least number of GN bacteria (12%). The total number of isolates and their respective percentages from various clinical sources is displayed in Table S1. A. baumannii was the most prevalent bacterial strain (17), accounting for 70% of cases in the Burn ICU. This is

supported by the findings of earlier research, which found that the most common bacteria identified from burn ICUs were A. baumannii, P. aeruginosa, K. pneumoniae, and Enterococcus [33]. The highest number of A. baumannii in Burn ICU is understandable since it has more recently grown to be a significant source of concern in regions of conflict. It has gained particular interest in the most recent Iraqi desert battles, leading to the nickname "Iraqibacter" [34].

The wound samples had the highest number of A. lwoffii (11). The blood samples, on the other hand, were abundant in K. pneumoniae, with a total of 12 representing 40% of all the bacterial strains isolated from blood samples. This is in line with the results reported by Mahmoudi et al. [35]. Their results showed that K. pneumoniae was the most prevalent (27.5%) GN strains present in blood samples. Other GN bacterial strains were less prevalent in all of the clinical samples.

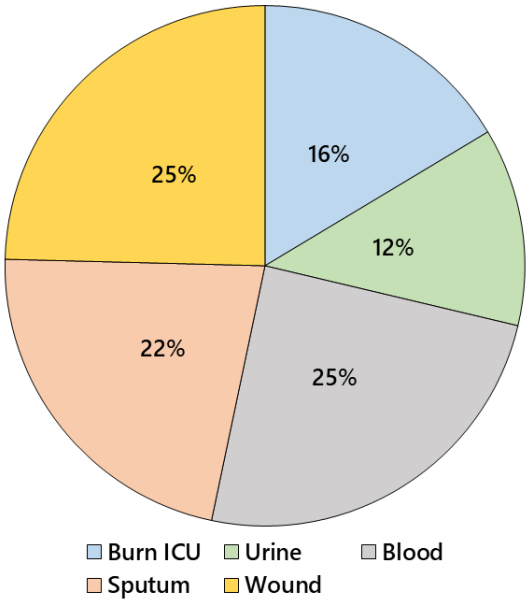

Figure 1. Proportions of isolated strains from various clinical sources.

3.3 Screening of the isolates

Following a 24-h incubation period, the ability of each bacterial isolate to produce TN and GA was assessed. Semi-quantitative and quantitative tests were used to evaluate the TN activity and GA production potential of the isolated bacterial strains. The biosynthesis of TN and GA on nutrient agar (NA) containing 0.5% tannic acid was evaluated using semi-quantitative assays. Ninety-nine of the 122 GN bacteria were shown to be able to produce TN and GA, based on the size of the dark green zone depicted in Figure 2. Detailed results of the screening of the isolated bacterial strains based on the diameter of hydrolysis zone are presented in Table S2. It can be observed that all of the bacterial isolates had the ability to produce TN and GA, with the exception of the A. lwoffii isolates, which were unable to do so. The majority of the bacteria that produced TN and GA were identified as isolates of A. baumannii, with inhibition zone diameters ranging from (14–20 mm) (Figure 2a). Table S3 shows that of the 25 A. baumannii isolates obtained from burn ICU, 17 (68%) had greater TN enzyme activity. This might be because burn patients have a high risk of infection due to their compromised skin barrier, prolonged hospital stays, and acquired immunosuppression [36].

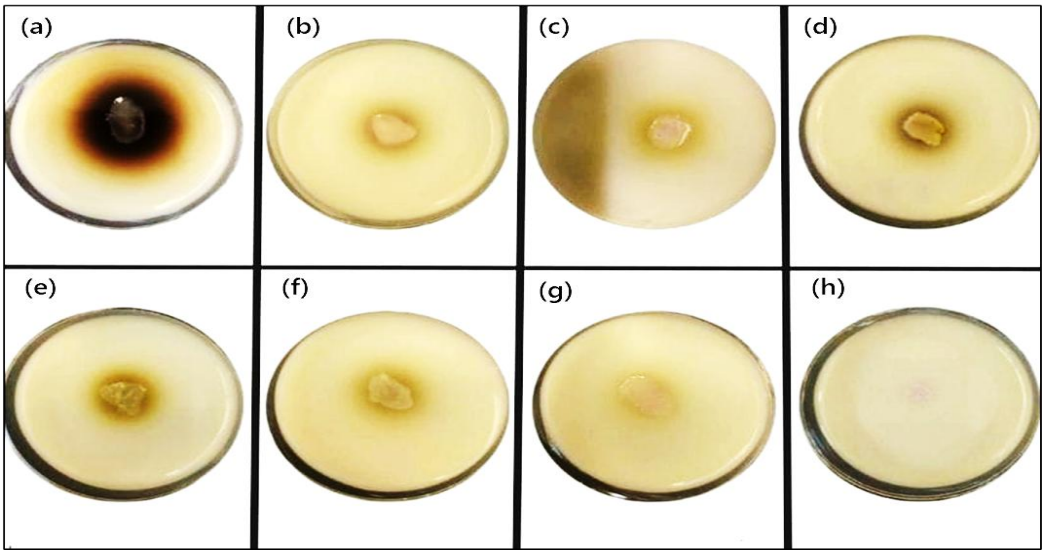

Figure 2. TN and GA produced by A. baumannii (a), K. pneumoniae (b), E. coli (c), S. marcescens (d), P. aeruginosa (e), E. cloacae (f), B. cepacia (g) and A. lwoffii (h) on NA with 0.5% tannic acid after incubating at 37°C, pH 6 for 24 h.

Based on the size of the dark zone, 85 bacterial isolates, including A. baumannii, K. pneumoniae, P. aeruginosa, E. coli, E. cloacae, S. marcescens, and B. cepacia, were found to be able to produce TN and GA using a semi-quantitative test. For the quantitative test, 25 A. baumannii isolates with the highest amounts of hydrolysis in the dark green zone were selected from a total of 85 isolates. Table 2 illustrates that all A. baumannii isolates had TN activity ranging from (5.21–8.22 U/mL), specific activity ranging from 3.27–6.68 U/mg, and GA content ranging from 1.31–4.28 mg/mL. A. baumannii 11, isolated from the burn ICU, was determined to be the most efficient producer of TN (6.68 U/mL) and GA (4.28 mg/mL) as shown in Figure S1. Thus, it was chosen for further investigation.

Table 2. Statistical analysis for A. baumannii isolates producing TN and GA.

A.baumannii isolates	Semi quantitative method	Quantitative method			Gallic acid concentration (mg/mL)
	Diameter of hydrolysis zone mm)	Tannase activity (U/mL)	Protein concentration (mg/mL)	Specific activity (U/mg)	
1	19	6.78±0.86	1.35±0	5.01±0.09	3.11±0.02
2	14	4.82±0.86	1.47±0	3.28±0.09	1.63±0.04
3	16	5.58±0.86	1.43±0	3.90±0.09	2.35±0.04
4	17	6.02±0.86	1.42±0	4.23±0.09	2.53±0.03
5	14	4.77±0.86	1.46±0	3.26±0.09	1.57±0.02
6	18	6.63±0.65	1.34±0	4.94±0.07	2.77±0.02
7	15	4.88±0.86	1.44±0	3.38±0.09	1.92±0.02
8	15	5.25±0.86	1.41±0	3.72±0.09	2.28±0.03
9	17	5.82±0.86	1.43±0	4.06±0.09	2.46±0.03
10	14	4.45±0.86	1.62±0	2.74±0.09	1.36±0.03
11	20	7.42±0.7	1.23±0	6.03±0.07	4.26±0.02
12	18	6.26±0.86	1.33±0	4.69±0.09	2.55±0.04
13	14	4.41±0.72	1.59±0	2.77±0.07	1.28±0.03
14	16	5.52±0.87	1.44±0	3.83±0.09	2.33±0.03
15	17	5.88±0.86	1.44±0	4.08±0.09	2.49±0.02
16	19	6.33±0.78	1.33±0	4.76±0.08	2.66±0.03
17	15	5.08±0.80	1.47±0	2.43±0.08	2.28±0.56
18	18	6.38±0.78	1.33±0	4.79±0.08	2.69±0.02
19	14	4.58±0.83	1.57±0	2.91±0.08	1.49±0.02
20	19	6.47±0.83	1.34±0	4.82±0.08	2.92±0.02
21	17	5.79±0.79	1.45±0	3.99±0.08	2.41±0.02
22	16	5.54±0.77	1.49±0	3.71±0.08	2.26±0.02
23	14	4.56±0.82	1.59±0	2.87±0.08	1.43±0.02
24	18	6.11±0.89	1.31±0	4.65±0.09	2.62±0.02
25	15	5.38±0.74	1.48±0	3.63±0.07	2.21±0.02

* The data is presented as mean ± standard deviation

Notably, the TN produced by A. baumannii is less than the TN activity of 8.5866 U/mL previously reported for Escherichia fergusonii that was isolated from a Uralian ram's rumen [37]. Nevertheless, A. baumannii was shown to produce TN and GA at maximum levels when a number of parameters were examined. Different environmental factors, such as culture medium, carbon source and concentration, nitrogen source and concentration, pH, temperature, incubation time, and inoculum size, were optimized to improve the performance of isolated strain (A. baumannii) for the production of TN and GA. The next sections present and discuss the findings of the impact of these parameters on the production of TN and GA.

3.4 Optimization of environmental factors (COST approach)

3.4.1 Culture medium and carbon source

Using a range of culture media containing MHA, NA, and BHIA in conjunction with tannin extracts from tamarind seeds (Tamarindus indica), pomegranate peels, amla

seeds, raspberry leaves, and tannic acid, the optimal culture medium for the production of TN and GA using A. baumannii was explored. Detailed results of the effect of culture medium on the TN and GA production are shown in Table S4. The results revealed variations in TN and GA production with change in the culture media, according to the diameter of the dark green zone. When nutrient broth (NB) was used together with tannic acid as a carbon source, the hydrolysis zone was measured to be 20 nm. Nevertheless, the generation of GA and TN was limited to 4.61±0.74 mg/mL and 8.36±0.74 U/mL, respectively (Figure 4a). When amla seeds were used as a substrate along with MHB growth medium, the largest hydrolysis zone, measuring 38 nm, was seen. When evaluated in combination with NB and MHB, pomegranate peals yielded a hydrolysis zone measuring 17 and 12 nm, respectively.

It is interesting to note that applying MHB along with amla seeds enhanced the production of TN and GA to 18.28±0.74 U/mL and 11.58±0.74 mg/mL, respectively as displayed in Figure 4(a). Similarly, the hydrolysis zone reached 27 nm when the MHB and tannic acid were tested together. Nevertheless, it is clear from 8.39±0.74 U/mL and 4.68±0.74 mg/mL, respectively, that this combination (MHB and tannic acid) was unable to increase TN and GA production. Unexpectedly, BIHB could not produce TN and GA when combined with any organic source other than tannic acid (Table S4). Similarly, no matter which culture medium was used, raspberry leaves and tamarind were unable to produce TN or GA. There is no consistency in the substrate specificity for TN synthesis because of the different quality of the substrates that were utilised and the different assay methods that were applied to determine the reaction product [38].

It can be inferred from the data in Figure 3 and Table S4 that amla seeds work better as a carbon source when combined with MHB to enable A. baumannii to produce as much TN and GA as possible. It was shown that the best carbon source for achieving the highest levels of TN (18.28±0.74 U/mL) and GA (11.58±0.74 mg/mL) was MHB in combination with amla seeds. The MHB was studied in combination with various organic sources, and Figure 3 illustrates the diameter of the hydrolysis zone under various treatments. As shown in Figure 3(d), it is evident that the combination of MHB and amla seeds produced a 38 mm zone of hydrolysis. As shown in Figure 3(b), MHB with pomegranate peals produced a hydrolysis zone measuring 17 nm.

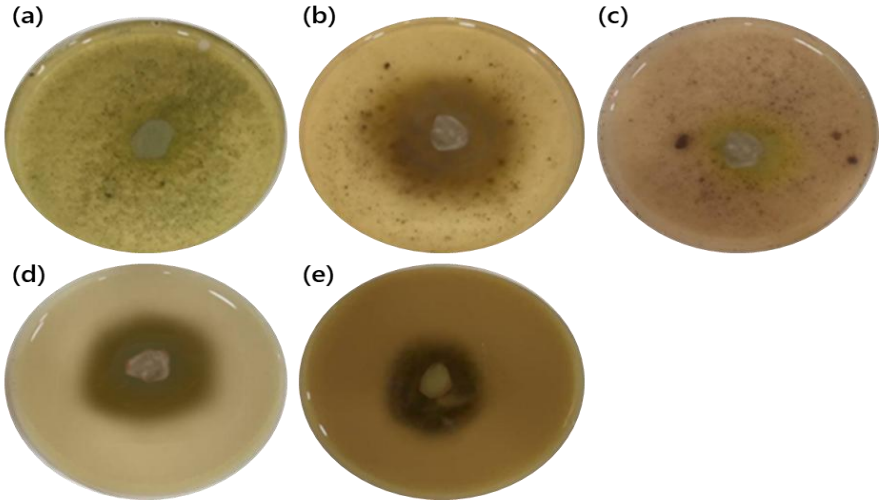

Figure 3. TN and GA production by A. baumannii on MHB with 0.5% each of (a) raspberry leaves, (b) pomegranate peels, (c) tamarind, (d) amla seeds and (e) tannic acid after culturing at 37°C for 24 hours.

On the other hand, earlier studies show that Aspergillus oryzae considerably increased ($p<0.05$) the TN (138.34 U/g) and GA (0.565 mg/g) from black plum leaves during a solid-state fermentation [4]. It was found that amla (Phyllanthus emblica) seeds provide a productive carbon source for A. baumannii, boosting the production of GA and TN activity. This is in line with earlier research [39] which reported that Phyllanthus emblica induced higher TN and GA production during submerged fermentation via Penicillium atramentosum. This indicates that the Phyllanthus emblica seed has superior potential as a substrate for producing GA and TN, independent of the method used for producing it—solid state fermentation, liquid medium reaction, or submerged system.

The performance of A. baumannii for TN and GA generation is influenced not only by the type of carbon source but also primarily by their concentration. Thus, as shown in Figure 4(b), the concentration of the optimal substrate (amla seeds) was adjusted between 0.1% and 0.9% in order to comprehend its impact on the A. baumannii-assisted TN and GA synthesis. As seen in Figure 4(b), it was proved that 0.4% amla seed was suitable for enhancing TN and GA production. With 0.4% amla seed, the TN exhibited a maximal activity of 22.556 U/mL, whereas the GA content was 15.7 mg/mL. A decrease in TN activity and GA production was observed at higher concentration of amla seeds. This may be due to the fact that amla seeds contain high tannin content which leads to toxicity or GA depletion. However, it has been previously reported that Trichoderma Harzianum produced the highest amount of TN (31.36 U/mL) when 1.0% tannic acid was used as the substrate [40].

3.4.2 Nitrogen source and concentration

The effect of nitrogen source and its concentration was evaluated on the TN and GA production by A. baumannii. In a medium containing 0.4% amla seed and MHB media, six distinct nitrogen sources—ammonium chloride, tryptone, sodium nitrate, yeast extract, peptone, and ammonium sulfate—were employed at a concentration of 0.5% each. It can be observed from Figure 4(c) that ammonium sulfate and sodium nitrate may increase TN activity and GA synthesis to levels greater than 25 U/mL and 18 mg/mL, respectively. Nevertheless, the most productive organic and inorganic source of nitrogen for increasing TN and GA yields was ammonium sulfate, with TN activity and GA concentrations of 28.69 U/mL and 21.6 mg/mL, respectively. Since ammonium sulfate was determined to be the most effective source of nitrogen, the optimal concentration for raising TN activity and GA synthesis was achieved by varying its concentration between 0.1 and 0.9%. As indicated in Figure 4(d), the optimal ammonium sulfate concentration for the production of TN (35.13 U/mL) and GA (28.03 mg/mL) was 0.2%. The result is consistent with another study that found that the optimal concentration of ammonium sulphate is 0.2% for enhanced TN activity (179.95 U/g) and GA production (0.986 mg/g) by gram negative bacteria [4]. However, employing Aspergillus niger and triphala waste as substrate, other investigations reported using a somewhat greater concentration of ammonium sulphate (0.75%) to enhance TN activity and GA production to 53.3 ± 5.3 U/g and 168.5 ± 5.0 mg/g, respectively [3]. Other studies also demonstrated detrimental effect of providing a nitrogen source on TN and GA production [2, 5, 41, 42].

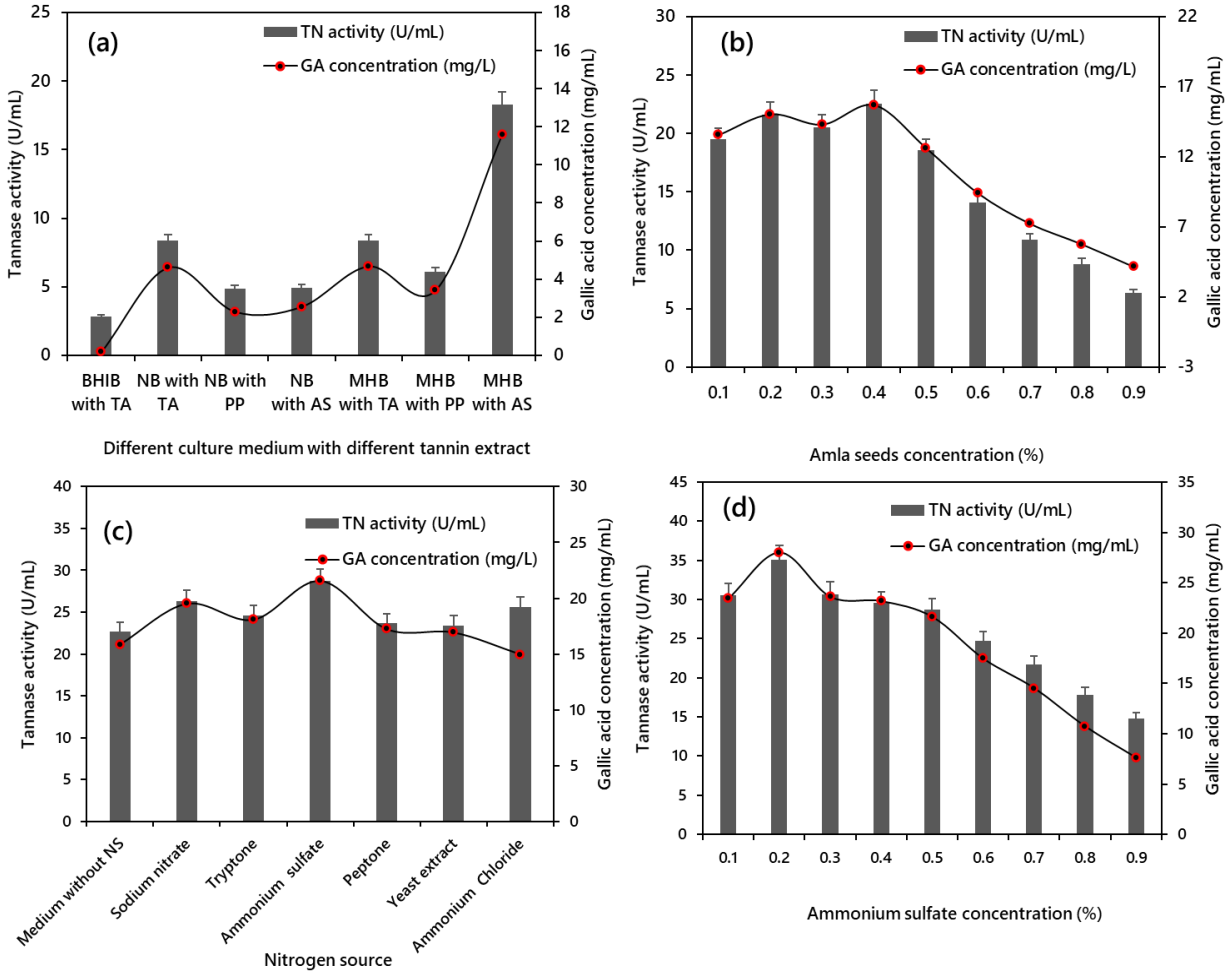

Figure 4. Effect of parameters on TN and GA production. (a) tannin extracts with different medium, (b) amla seed concentration (%), (c) additional organic and inorganic nitrogen sources, and (d) ammonium sulfate concentration (%). TA: tannic acid, PP: Pomegranate peels, AS: amla seeds.

It is important to note that the TN activity produced by P. variotii strain at optimal conditions (2.50 water: substrate ratio, 0.70 mm substrate particle size, 5.0% of ammonium sulfate concentration, incubation at 32°C, and 7.5% of tannic acid) [43] was found to be lower than the TN activity (35.13 U/mL) produced by A. baumannii at 0.4% amla seeds substrate concentration and 0.2% of ammonium sulfate in the current study. Another study has observed TN activity of 19.22 U/gds when the additional levels of tannin, glucose, (NH4)2SO4, and extract of yeast were 7.49, 8.11, 9.26, and 2.25%, respectively [44]. According to Selwal et al. [45] ammonium chloride produced the highest amount of TN by Penicillium atramentosum KM (28.9 U/mL when using amla), whereas di-ammonium hydrogen phosphate produced 31.9 U/mL when utilizing keekar leaves. Since the nitrogen source and concentration affect the TN titers in the production broth, it is obvious that these are important

parameters. It is apparent that employing various bacterial strains, different nitrogen sources have varying effects on the production of TN and GA. Consequently, while assessing the capability of bacterial strains for the generation of TN and GA from substrates of plant origin, it is imperative that particular nitrogen sources be studied in addition to other parameters.

3.4.3 pH and temperature

Apart from the various chemical components of the growth medium, physiological factors including pH and temperature also significantly impact the production process of TN by diverse microorganisms [46, 47]. Thus, by adjusting the growth medium's pH from 4 to 7.5, the effects of pH on TN and GA produced by A. baumannii were examined. This pH range was chosen to include both the upper and lower pH ranges because most bacteria are found to favor the pH range of 5.0–6.0 for the synthesis of TN [46]. The effect of pH on TN production by A. baumannii is shown in Figure 5(a). TN activity (36.753 U/mL) and GA content (30.453 mg/mL) were both shown to be higher at pH 5.5. Moving away from pH 5.5, the TN activity and GA output declined. The reduction in TN activity when pH decreases could be due to the presence of tannic salts generated throughout the process. Conversely, a rise in pH could lead to the inactivation of available tannic acid [48], hence diminishing the TN activity of A. baumannii. Since tannase is naturally acidic and produces maximal amounts of GA at pH 5.5, raising the pH of basal medium reduces the amount of GA that is produced [49]. The production of the enzyme tannase diminishes when pH rises above 5.5. Nevertheless, studies have also reported higher activity at pH higher than 5.5 [6, 50, 51]. To determine the impact of the optimal pH on the TN activity and GA production, it is critical to investigate the nature of these amino acids because the amino acids at the active site may experience protonation, deprotonation, and conformational changes as a result of the ionisations of the remaining amino acids [52].

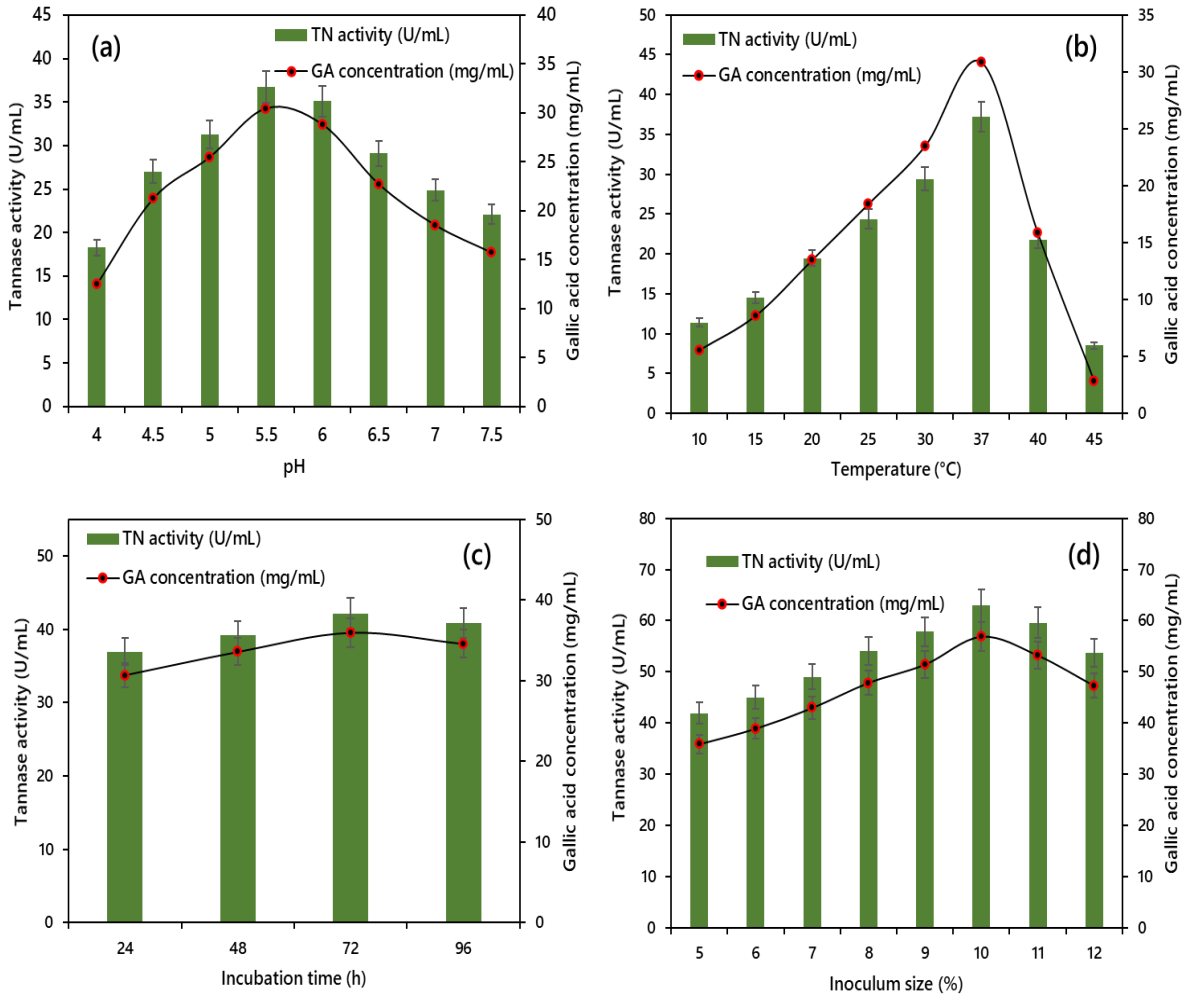

Figure 5. Effect of different parameters on TN and gallic production (a) pH, (b) temperature, (c) incubation time and (d) inoculum size.

The present study investigated the effect of temperature on the synthesis of TN and GA using isolates of A. baumannii and amla seed as a substrate. The temperature ranged from 10 to 45°C. The highest activity of TN (37.19 U/mL) and GA concentration (30.82 mg/mL) were noted at 37°C, as shown in Figure 5(b). It is evident that at temperature values below and above 37°C, the TN activity and GA production were lower. Temperature has a significant role in the biosynthesis of biomolecules because, depending on the circumstances, it can alter the metabolic activity of the microorganisms and, as a result, either boost or decrease biomolecule production. Lower temperatures result in less substrate movement through the cell, which has lowered the production of TN and GA

(Andrade et al., 2018; Dhiman et al., 2021; Kumar et al., 2016). Conversely, high temperatures caused denaturation of metabolic activity, which raised the energy required to sustain cell growth and hence decreased the production of TN and GA [53]. Due to denaturation of the distinctive globular TN structure of TN at high temperatures, a progressive decrease in TN activity was seen as temperature increases [43, 54]. These findings align with research [38, 55, 56] which also reported higher TN activity at 37°C. Although the current investigation's temperature of 37°C resulted in higher TN activity, other research also finds higher TN activity at 35°C [7].

3.4.5 Incubation time and inoculum size

By adjusting the incubation duration from 24 to 96 h, the impact of incubation time on the generation of TN and GA was investigated. The highest TN activity (42.18 U/mL) and GA production (35.98 mg/L) were attained after 72 h of incubation, as shown in Figure 5(c). These findings are in line with those of previous studies, which showed that the maximum amount of TN was produced after 72 h of incubation [2, 6, 55]. However, research indicates that as incubation durations increase, the TN activity and GA production diminish due to the bacterial transition into a stationary growth phase, the medium losing its nutrients, and the increased production of metabolic wastes. Another possible explanation for the decrease in TN activity and GA production due to rising incubation time could be the accumulation of hazardous metabolites during the course of the extended incubation period [57]. In this study, the optimum incubation time was determined to be 72 h for 42.18 U/mL and 35.98 mg/mL of TN activity and GA production, respectively using A. baumannii which is line with the results reported by Saeed et al. [2]. In contrast, sporidiobolus ruineniae was used to produce TN and GA; nevertheless, after 42 hours of incubation, the TN activity and GA production only reached to 31.1 U/mL and 11.2 g/L, respectively [17]. Compared to the earlier values presented for 42 h of incubation [22], it is evident that the TN activity and GA concentration in the current study during 72 h of incubation are much higher.

TN and GA production was examined by changing the inoculum size's concentration from 5 to 12%. The effect of inoculum concentration on the production of TN and GA is shown in Figure 5(d). A. baumannii-mediated TN and GA production was found to be influenced by the inoculum size. At 5% inoculum size, the TN activity was only 40 U/mL and the GA output was 38 mg/mL. The TN activity and GA production increased dramatically to 62.913 U/mL and 56.883 mg/mL, respectively, after 72 hours of incubation when the inoculum size was increased to 10%. It was found that the size of the inoculum was critical for the synthesis of metabolites and that reducing the inoculum was not the right approach to encourage the synthesis and growth of enzymes. The high concentration of the inoculum supports the rapid propagation of bacterial growth and subsequent product release. Similarly, Selvaraj and Vytla [58] discovered that the highest activity of TN was produced by an inoculum size of 10%. On the contrary, 7.035% has been shown to be the optimal inoculum size for TN production [59]. It was found that the size of the inoculum played an important role in the synthesis of metabolites, and a decrease in the size of the inoculum was not suitable for stimulating enzyme synthesis and growth [60].

3.5 Biosynthesis process optimization using RSM

As the initial findings showed that the four independent variables— inoculum size (x1), pH (x2), temperature (x3), and incubation duration (x4)—have a major influence on the biosynthesis of TN and GA. Consequently, CCD-based RSM was used to further explore and optimize the individual and combine effects of each of these four variables on TN activity and GA production. The optimization tests were conducted using constant concentrations of ammonium sulphate and amla seeds (0.2% and 0.5%, respectively), which were found to be the optimal concentrations based on preliminary examination. The design of experiments (DOE) shown in Table 3 was used to conduct the optimization experiments for the synthesis of TN and GA.

3.5.1 Statistical significance of the RSM model fitting

The statistical significance of the models employed for the optimisation of TN activity and GA concentration was evaluated using analysis of variance (ANOVA). The ANOVA output results are presneted in Table 4. The overall model p-values for both response variables were 0.0001, which are less than the 0.05 significance level. As a result, the null hypothesis was disproved when it was discovered that the independent and dependent variables were correlated. Considering the p-value for the linear terms (x1, x3, and x4) of the factors and their interactions is smaller than the level of significance (Table 4), it is probable that the individual variables and different combinations of them significantly affect TN activity and GA concentration. The models pertaining to TN activity (0.2798) and GA concentration (0.0045) in Table 4 exhibit p-values that surpass the significance level, indicating a satisfactory fit.

The quadratic models with the independent variables given in Eqs. (7) and (8) showing a substantial effect on the TN activity (Y1) and GA concentration (Y2) since the absence of model fit values for both response variables were insignificant. The magnitude and pattern of the association between the independent variables, and TN activity, and GA concentration, are demonstrated by the sign and value of the coefficients, respectively.

Table 3. Design matrix formulations of the four independent factors (x1: Inoculum size (%), x2: pH, x3: temperature (°C) and x4: incubation time (h) and two dependent factors (Y1: TN and activity and Y2: GA production).

Std run	Run no.	Factors				TN activity (U/mL)	Std	Run no.	Factors		GA concentration (mg/mL)

		x_1	x_2	x_3	x_4	Actual	Predicted			x_1	x_2	x_3	x_4	Actual	Predicted
5	1	10.00	5.50	37.00	72.00	62.91	55.50	15	1	5	7.5	45	96	32.37	31.19
3	2	5.00	7.50	10.00	24.00	11.11	10.77	9	2	3	4	10	96	17.34	18.99
4	3	12.00	7.50	10.00	24.00	56.34	55.31	29	3	5	6	27.5	60	25.93	22.82
14	4	12.00	4.00	45.00	96.00	54.66	57.46	10	4	1	4	10	96	37.33	35.37
26	5	8.00	5.75	20.00	60.00	44.22	44.97	11	5	9	7.5	10	72	36.45	36.34
8	6	12.00	5.50	45.00	24.00	48.33	53.45	19	6	8	2	27.5	60	26.55	26.47
11	7	5.00	7.50	30.00	96.00	12.11	15.63	30	7	8	6	27.5	60	31.88	32.73
13	8	5.00	4.00	45.00	96.00	33.33	33.33	3	8	5	7.5	10	24	32.76	32.23
19	9	6.00	2.25	20.00	72.00	38.22	40.29	22	9	9	6	40	60	50.66	51.90
21	10	8.00	5.75	40.00	60.00	39.44	45.90	8	10	7	7.5	45	24	40.11	41.65
22	11	7.00	5.75	62.50	48.00	38.68	39.18	4	11	6	7.5	10	24	35.11	35.16
20	12	8.00	9.25	20.00	48.00	26.77	26.02	2	12	6	4	10	24	31.44	31.98
25	13	8.00	5.75	27.50	72.00	48.67	45.72	13	13	5	4	45	96	37.11	36.71
12	14	12.00	7.50	10.00	96.00	55.66	56.55	12	14	1	7.5	10	96	32.88	33.08
7	15	5.00	7.50	45.00	24.00	14.66	13.97	14	15	7	4	45	96	36.65	37.97
28	16	10.00	5.75	37.00	72.00	60.99	55.49	6	16	4	4	45	24	32.88	31.90
30	17	8.00	5.75	25.00	72.00	42.68	45.51	23	17	10	5.5	37	72	56.88	53.12
18	18	9.00	5.50	25.00	72.00	47.68	50.91	21	18	9	6	25	60	36.45	39.18
23	19	9.00	5.75	27.00	24.00	44.88	45.96	18	19	4	6	27.5	60	25.22	23.55
9	20	7.00	4.00	10.00	96.00	45.88	41.45	16	20	9	7.5	45	96	41.55	42.62
2	21	12.00	4.00	10.00	24.00	54.91	55.95	27	21	9	6	27.5	60	36.11	40.06
27	22	8.00	5.75	27.00	72.00	44.58	45.68	7	22	5	7.5	45	24	33.58	32.25
6	23	12.00	4.00	45.00	24.00	52.88	48.46	25	23	10	6	27.5	60	55.88	49.41
15	24	5.00	7.50	45.00	96.00	22.87	19.95	17	24	2	6	27.5	60	28.56	31.04
17	25	6.00	5.75	25.00	72.00	34.88	33.49	5	25	5	4	45	24	32.56	33.36
16	26	11.00	7.50	45.00	96.00	58.86	59.65	26	26	2	6	27.5	60	28.66	31.04
29	27	8.00	5.75	25.00	72.00	40.22	45.51	24	27	6	6	27.5	56	28.12	24.81
1	28	5.00	4.00	30.00	24.00	30.55	28.97	1	28	5	4	10	24	29.58	29.27
10	29	11.00	4.00	10.00	96.00	54.66	55.72	20	29	10	5.5	32	60	51.55	52.44
24	30	10.00	5.75	27.50	72.00	60.11	54.99	28	30	9	6	27.5	60	36.55	40.06

Table 4. ANOVA results for Response Surface Quadratic model for TN activity and GA concentration.

Source	Sum of Squares		DF		Mean square		F-Value		p- value > F			
	TN	GA	TN	GA	TN	GA	TN	GA	TN	Remark	GA	Remark
Model	**5709.28**	**2144.48**	**14**	**14**	**407.81**	**153.18**	**19.11**	**15.61**	**< 0.0001**	**Significant**	**< 0.0001**	**Significant**
x_1	3415.85	853.46	1	1	3415.85	853.46	160.08	86.98	< 0.0001	Significant	< 0.0001	Significant
x_2	194.39	1.08	1	1	194.39	1.08	9.11	0.11	0.0086	Significant	0.7448	Insignificant
x_3	6.39	135.47	1	1	6.39	135.47	0.3	13.81	0.5921	Insignificant	0.0021	Significant
x_4	54.87	169.4	1	1	54.87	169.4	2.57	17.26	0.1296	Insignificant	0.0008	Significant
x_1^2	22.54	493.2	1	1	22.54	493.2	1.06	50.26	0.3203	Insignificant	< 0.0001	Significant
x_2^2	94.83	20.42	1	1	94.83	20.42	4.44	2.08	0.0522	Significant	0.1697	Insignificant
x_3^2	0.41	76.35	1	1	0.41	76.35	0.019	7.78	0.8918	Insignificant	0.0137	Significant
x_4^2	9.89	20.82	1	1	9.89	20.82	0.46	2.12	0.5063	Insignificant	0.1658	Insignificant
$x_1 . x_2$	265.6	0.45	1	1	265.6	0.45	12.45	0.046	0.003	Significant	0.8329	Insignificant
$x_1 . x_3$	5.03	4.33	1	1	5.03	4.33	0.24	0.44	0.6343	Insignificant	0.5166	Insignificant
$x_1 . x_4$	4.94	67.47	1	1	4.94	67.47	0.23	6.88	0.6372	Insignificant	0.0192	Significant
$x_2 . x_3$	30.26	10.04	1	1	30.26	10.04	1.42	1.02	0.2522	Insignificant	0.3278	Significant
$x_2 . x_4$	0.11	13.54	1	1	0.11	13.54	5.26 E-03	1.38	0.9431	Insignificant	0.2585	Insignificant
$x_3 . x_4$	38.98	169.51	1	1	38.98	169.51	1.83	17.28	0.1965	Insignificant	0.0008	Significant
Residual	320.07	147.18	15	15	21.34	9.81	—	—	—	—	—	—
Lack of Fit	317.05	147.08	14	13	22.65	11.31	7.48	222.28	0.2798	Insignificant	0.0045	Insignificant
Pure Error	3.03	0.1	1	2	3.03	0.051	—	—	—	—	—	—
Cor Total	6029.35	2291.67	29	29	—	—	—	—	—	—	—	—

The practical significance test is performed using the RSM summarised output of the models displayed in Table 5. The observed R2 values for GA concentration and TN activity were 93% and 95%, respectively, while the adjusted R2 values for these variables were 90% and 87%. High R2 values for both response variables (GA concentration and TN activity) also guarantee a good model fit and a reasonable agreement between the observed and projected data, as indicated in Table 5. This demonstrates the high practical significance of the models' application. Fisher's (F-values) of 19.11 and 15.61 in Table S5 show that both models were significant for TN activity and GA concentration, respectively. Stated otherwise, there is a compelling case for the models' accuracy and a very little possibility (0.01%) that the high F-values are the outcome of random variation (p-value = 0.0001). For TN activity and GA concentration, the acceptable level of precision (AP), which measures the "signal to noise ratio," was found to be 14.96% and 15.418%, respectively (Table 5).

Table 5. RSM output model fit summary statistics for TN activity and GA concentration.

Statistical key Figure	Acronym	TN activity (Y1)	GA concentration (Y2)
Coefficient of variance	C.V.	10.81	8.88

Standard deviation	St. Dev.	4.62	3.13
Adequate precision	A.P.	14.96	15.41
Coefficient of determination	R2	0.95	0.93
Predicted – R2	Pre. R2	0.79	0.37
Adjusted – R2	Adj. R2	0.90	0.8758
Mean	Mean	42.72	35.29
PRESS	—	1254.48	1439.59

The space that had been previously defined by the CCD/RSM could be navigated using both modes when the AP value was greater than 4. For the best model fitting, errors values should be as little as feasible, and R2 values should be near to 1 [46, 61]. The fitting analysis results displayed in Table 5 show high R2 values of 0.95 and 0.93 for the TN activity and GA concentration, respectively. The predicted values for TN activity and GA concentration were compared to their observed values as shown in Figure 6(a and b) to demonstrate the models' reliability.

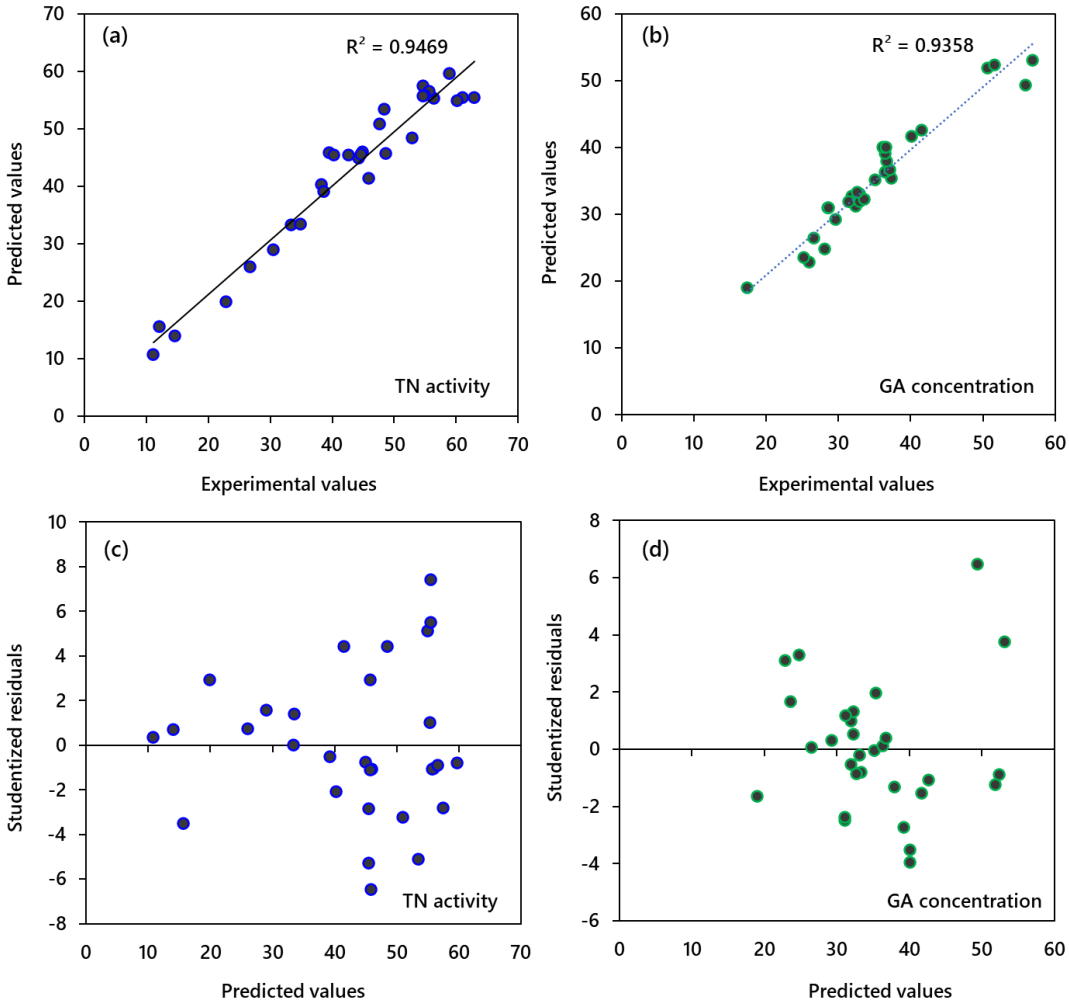

Figure 6. Experimental values and the values predicted by the models for (a) TN activity and (b) GA concentration, studentized residuals vs predicted values by the models for (c) TN activity and (d) GA concentration.

The data points exhibit linear behavior and a fairly even distribution. It appears that the proposed empirical models are suitable for predicting TN activity and GA concentration because the projected and observed results were distributed extremely closely to one another (R2 = 0.9469 for TN activity and 0.9358 for GA concentration). These values suggest that the CCD employed can more precisely predict and optimize the TN activity and GA production. The residual plots for the two models used to estimate GA concentration and TN activity are shown in Figure 6 (c and d). Plotting the residuals against the projected values makes it evident that the residuals were distributed at random and normally distributed, which is a sign of good model fitting. The normal distribution is just slightly violated by two or three points, and they might not require additional investigation.

3.5.2 Interaction effect of the input variables

The interaction effect of two or more independent variables on TN activity and GA concentration was ascertained by building 3-D view response surface plots. For a process to be cost-effective, it is often desirable to keep the factors within a range, as maintaining them at

their greatest level could be highly expensive. The response surface graphs presented in Figure 7(a) for maximizing TN activity by optimizing the pH (x2) of the medium for growth of A. baumannii AB11 and inoculum size (x1). It is clear that factors with a pH between 4 and 6 and an inoculum size greater than 8.5% had the highest TN activity (52–62 U/mL). Better GA production >52 mg/mL, as seen in Figure 8(a), was sustained when pH and inoculum size were within the same range, much like TN activity. It is noteworthy, therefore, that the impact of the medium's pH variation on the generation of TN and GA in both scenarios is minimal when combined with the inoculum size. This may be the result of the pH range (4–7.5) chosen in this study, which is ideal for A. baumannii AB11 development regardless of inoculum size. Enzymes are very sensitive to pH changes because they have a clear pH optimum and function best within a relatively restricted pH range [43]. TN activity, however, reduced when the inoculum size moved beyond of the optimal range of 8.5–10. The TN activity was only limited to 30.79 U/mL at smaller inoculum sizes, which may have been caused by fewer bacterial cells than necessary for TN production.

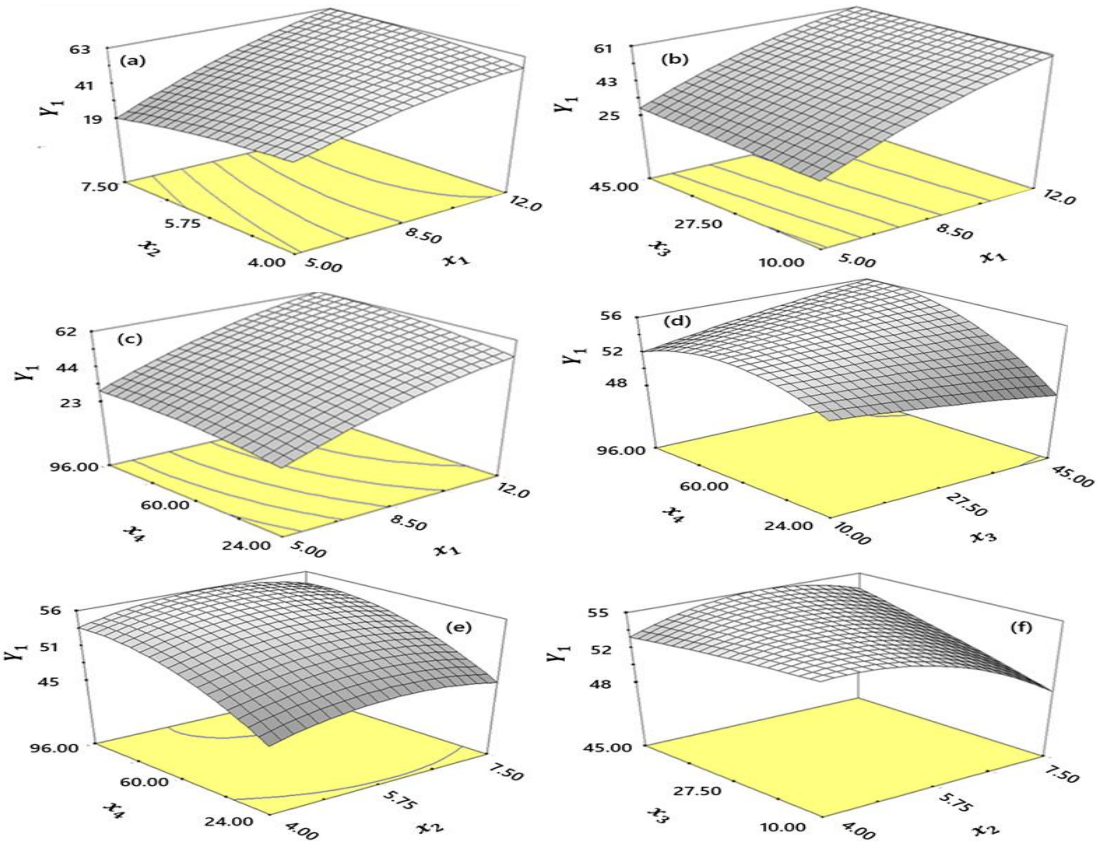

Figure 7. Response surface plots for the TN activity (Y1) by Acinetobacter baumannii AB11. Interactive effect between (a) inoculum size (x1) and pH (x2), (b) inoculum size (x1) and temperature (x3), (c) inoculum size (x1) and incubation time (x4), (d) temperature (x3) and incubation time (x4), (e) pH (x2) and incubation time (x4), and (f) between pH (x2) and temperature (x3).

The interaction effect of the temperature (x3) and inoculum size on TN activity is displayed Figure 7(b). It can be seen that when the temperature was between 27 and 38°C and the inoculum size was greater than 8.5%, the maximal TN activity was discovered to be in the region of 34–61 U/mL. The kinetic energy of the substrate and TN molecules increases with increases in temperature, influencing the rate of reaction. An increase in temperature causes TN and its substrate, amla seeds, to collide more frequently per unit of time, resulting in an increase in TN activity. Molecules' energy surged more when the temperature rises over the optimal level. However, some of the fragile bonds that hold the active proteins' three-dimensional structure together disintegrate when their chemical potential energy rises to a certain point. The TN protein becomes inactive as a result of heat denaturation. Thus, the substrate either turned denatured and inert or the catalytic rate of tannase decreased as a result of rising temperatures that exceeded the optimum range. The ionization state of proteins and the solubility of molecules in solution are similarly impacted by temperatures over the optimal range, which has been shown to lower enzyme function [62].

Figure 7(c) shows the interaction effect of inoculum size and incubation duration (x4) on TN activity. The p-value in Table 4 indicates that the interaction impact of these variables was not as strong as other factors and that the combination of these factors has no discernible effect on TN activity. A closer examination revealed that the TN activity was only slightly raised from around 50 U/mL to 61 U/mL (Figure c) with the interaction of inoculum size and incubation duration, however the TN activity rose from 30 U/mL to more than 62 U/mL with the inoculum size and temperature interaction (Figure 7a). Figure 7(d)

shows the combined influence of temperature and incubation duration on the activity of A. baumannii derived TN. The temperature and incubation duration raise the TN activity (54 U/mL), which peaks at about 65 to 70 hours and 30-35°C. Similar interaction effect of the pH and incubation time (Figure 7e) and pH and temperature (Figure 7f) can also be observed. Generally, the interaction or synergistic effect of the two or more independent variables improve the performance efficiency.

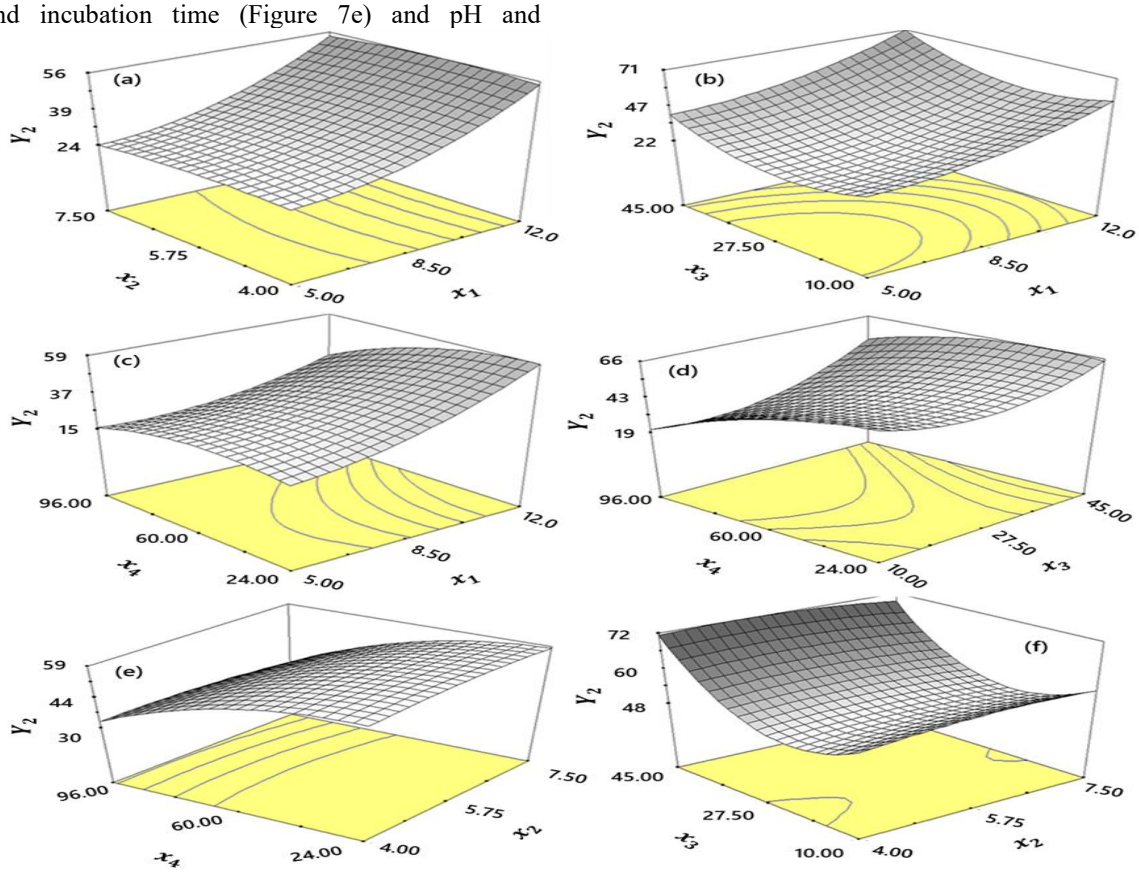

Figure 8. Response surface plots for the gallic acid (GA) production (Y2). Interactive effect between (a) inoculum size (x1) and pH (x2), (b) inoculum size (x1) and temperature (x3), (c) inoculum size (x1) and incubation time (x4), (d) temperature (x3) and incubation time (x4), (e) pH (x2) and incubation time (x4), and (f) between pH (x2) and temperature (x3).

The greatest amount of amla seeds bioconversion to GA was then ascertained by assessing the A. baumannii AB11 acid co-production capacity when it was grown in conditions with a fixed amla seed and ammonium sulfate concentration with varying pH, temperature, incubation time and inoculum size. As shown in Figure 8(a), after 72 h of cultivation, the highest amount of GA (55.1122 mg/L) from 0.4% of amla seeds was found. It has been observed that while the pH (x2) and inoculum size (x1) together have little to no effect on the production of GA, the GA concentration increases rapidly as the inoculum size increases. The GA production was minimal when the inoculum size was less than 8.5%. According to reports [63], it takes longer for cells with a smaller inoculum size to proliferate to a point where they can use the substrate to produce enzymes and subsequently produce GA from organic source. A higher inoculum concentration of A. baumannii cells would guarantee a quicker rate of proliferation and biomass yield. In order to allow for increasing GA concentration through biosynthesis by A. baumannii, a balance between inoculum size and pH would be essential.

However, when the temperature (x3) drops from 40°C to 10°C, there was a noticeable decrease in GA production from 47 mg/L to 22 mg/mL, as shown in Figure 8(b). At higher temperatures, the concentration of GA is higher. However, a temperature that is optimal for increasing GA production is required because a higher temperature may result in higher production costs because more energy will be required to produce biomolecules from the substrate. It is important note that the interaction effect of inoculum size and temperature

x1.x3 was negligible suggested by the p-value of 0.5166. Presently, the concentration of GA generated by A. baumannii AB11 surpasses the previously documented results of 11.96 g/L by Sporidiobolus ruineniae [17].

Figure 8(c) shows the combined effect of the inoculum size (x1) and the incubation time (x4) on GA bioproduction. When the inoculum size was greater than 8.50% and the duration of incubation was between 55 and 70 hours, the maximum GA concentration was observed (>55 mg/mL). The TN activity of A. baumannii AB11 is significantly influenced by the incubation period, inoculum size, and temperature, as demonstrated in Figure 7(d-f). The fact that the gallic acid content decreased in each case upon achieving its maximum level indicates that the A. baumannii strain was able to catabolize the GA through GA decarboxylase and

produce pyrogalloll, which then enters the TCA cycle as pyruvic acid, cis-aconitic acid, and 3-hydroxy-5-oxo hexanoate. The results showed that an inoculum size of 10%, pH of 5.5, temperature of 37°C, and an incubation period of 72 h were the optimal conditions for a 62.913 U/mL of TN activity.

4. CONCLUSION

Gallic acid is a valuable resource as it is used in a variety of commercial products and it is desirable to produce it in large quantities from natural sources like bacteria or fungus in order to satisfy market demand on a global level. In this study, the ability of a variety of gram-negative bacteria to produce gallic acid was examined. These bacteria were isolated from clinical waste including wounds, the burn ICU, blood, urine, and sputum. Acinetobacter baumannii, however, emerged as a top candidate for TN and GA production from the substrate of amla seeds among the different isolates. The culture media and tannic acid extract from various plant sources enhanced the production of TN and GA. Acinetobacter baumannii's bioproduction of TN and GA was found to be highly sensitive to environmental conditions including carbon, nitrogen, and inoculum concentration, pH, and temperature. Among the selected variables, inoculum size and pH significantly affected TN activity and inoculum size, temperature, and incubation time affected GA concentration significantly. Under the all-optimal conditions, Acinetobacter baumannii was able to produce 62.913 U/mL of tannase and 56.883 mg/mL of gallic acid in 72 hours from alma seeds.

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AUTHOR CONTRIBUTIONS

Alaa A. Abdulshaheed: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. Marlia Mohd Hanafiah: Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Data curation, Conceptualization. Sahira Nsayef Muslim: Writing – review & editing, Validation, Supervision, Methodology, Data curation, Conceptualization. Rab Nawaz: Writing – review & editing, Visualization, Validation, Formal analysis.

DECLARATION OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

ABBREVIATIONS/ACRONYMS

ANOVA	Analysis of variance	MHA	Muller Hinton agar
BHIB	Brain heart infusion broth	NA	Nutrient agar
BHIA	Brain heart infusion agar	NB	Nutrient broth
BSA	Bovine serum albumin	NH4Cl	Ammonium chloride
CCD	Central composite design	TN	Tannase
COST	Changing one single variable at a time	NH4NO3	Ammonium nitrate
DI	Deionized	OD	Optical density
F-value	Fisher's value	R2	Coefficient of determination
GA	Gallic acid	rpm	Revolution per minute
GN	Gram-negative	RSM	Response surface methodology
ICU	Intensive care unit	KH2PO4	Monopotassium phosphate

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