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Optimization of Medium Components for Enhancing Antibacterial Activity of Marine *Streptomyces aureofaciens* A3 through Response Surface Methodology

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Abstract

This study is the first to report on the statistical optimization of an antibacterial production medium for the marine bacterium *Streptomyces aureofaciens* A3. Glycolic and furoic acids have not been previously documented as metabolites produced by Streptomyces, especially by *S. aureofaciens* A3. The antibacterial metabolites were generated in an ISP4 medium enriched with artificial seawater with different concentrations of starch, NaCl, and ammonium sulfate. Media components were optimized through the Response Surface Method (RSM) using the Box-Behnken design (BBD) in submerged fermentation. Antibacterial metabolites produced during fermentation were extracted with ethyl acetate (EA) to obtain EA extracts. The extract activities were tested using the Kirby-Bauer method, and the extract compounds were annotated using GC/MS. The results showed that the specific concentrations of starch, ammonium sulfate, and NaCl enhanced the antibacterial activity for each tested bacteria were 11.06 - 12.07 g/L for starch and 1.39 - 1.56 g/L for ammonium sulfate and 1.76 - 2.45 g/L for NaCl. The media increased higher antibacterial activity against pathogens with rising percentages of inhibition zones of *E. coli* (62.33 %), *S. aureus* (9.41 %), *S. typhimurium* (48.69 %), *P. aeruginosa* (39.16 %) and *B. subtilis* (8.58 %), respectively. The inhibition zones of each test bacteria were 10.88 - 17.97 mm and categorized as strong antibacterial activity. GC/MS derivatization results showed that the annotated compounds in EA extracts consisted of primary metabolites such as glycolic acid, palmitic acid, stearic acid, lauric acid, myristic acid, oleic acid and secondary metabolites were furoic acid, phthalate acid, benzoic acid, methylmalonic acid. This study showed that the RSM method is efficient and effective for improving the activity of broad-spectrum antibacterial agents produced by *S. aureofaciens* A3. The application of RSM to optimize ISP4 medium enables the large-scale production of antibacterial metabolites for the pharmaceutical industry.

Keywords: Streptomyces, Antibacterial, Pathogens, Seawater, BBD, ISP4, Metabolites, Optimization

Introduction

Streptomyces is a filamentous, aerobic, Gram-positive bacteria that belongs to the Actinobacteria [1,2]. Streptomyces produce a wide range of secondary metabolites, including over 70 % of naturally derived antibiotics [3,4]. *Streptomyces aureofaciens* is widely known as a producer of tetracycline and chlortetracycline antibiotics which can eliminate 90 % of pathogenic bacteria [5,6]. The species strongly influence the production of antibacterial metabolites, components of the fermentation medium, and environmental factors [7,8]. Changes in the antibacterial production composition media might impact the quantity and abundance of antibacterial metabolites. Microorganisms require specific growth conditions and

nutrients such as carbon, nitrogen, and sodium chloride that can affect secondary production metabolites [9,10]. Thus, applying the proper media and choosing a suitable growing environment is crucial for enhancing antibiotic yields. The response surface method (RSM) can improve the antibacterial activity of compounds from *Streptomyces* spp. and the parameters required for the process [11-13].

RSM is an experimental technique that applies statistics and mathematics to optimize the parameters' process [13,14]. The RSM approach has been extensively utilized to acquire the interactions between numerous fermentation parameters and to improve the medium and environment that support the fermentation process by 3-dimensional (3D) and 2-dimensional (2D) models [13]. RSM has been effectively used to improve fermentation culture parameters in microorganisms, such as actinomycetes, bacteria, and fungi, to produce metabolites that are significant to the industry [15]. Additionally, *Streptomyces* spp. have improved their production of antibiotic metabolites through the RSM.

The optimization production of the antibacterial properties of marine *S. aureofaciens* A3 is limited and has not been reported. The recent study aims to enhance the antibacterial activity of *S. aureofaciens* A3 by modifying the ISP4 medium, consisting of starch and inorganic salts. The carbon source (starch) and nitrogen source (ammonium sulfate) in ISP4 are common components for antibacterial production [16,17]. A more precise concentration of starch and ammonium sulfate is necessary to optimize the production and activity of antibacterial metabolites. Additionally, NaCl is a crucial component for cell viability and can be a more effective antibacterial metabolite, with the bacteria requiring a specific concentration for optimal growth [18]. In addition, the changes in the ISP4 medium composition can be closely monitored to identify components that enhance antibacterial activity. In this study, medium optimization focused on starch, ammonium sulfate, and NaCl as key factors for increasing antibacterial activity.

Materials and methods

Culture collection

Streptomyces aureofaciens A3 was previously isolated from the coastal sponge *Amorphinopsis excavans*, demonstrating the strongest antibacterial activity in earlier studies. Then, *S. aureofaciens* A3 was studied extensively to optimize its antibacterial activity. The strain was maintained on International Streptomyces Project (ISP4) agar slants as a working culture.

Preparation of activated inoculum

The bacterial strain was grown in ISP4 medium (g/L) [19]: (Soluble starch - 10, K_2HPO_4 - 1, $MgSO_4 \cdot 7H_2O$ - 1, $(NH_4)_2SO_4$ - 2, $CaCO_3$ - 2, $FeSO_4 \cdot 7H_2O$ - 0.001, $MnCl_2 \cdot 7H_2O$ - 1, $ZnSO_4 \cdot 7H_2O$ - 0.001 and NaCl - 1). A fresh culture of *S.aureofaciens* A3 was inoculated into 100 mL of ISP4 medium and incubated for 3 days on a shaker at 150 rpm at 30 °C since it grows optimally at 30 °C. The inoculated flask was used as a seed culture.

Submerged fermentation and extraction of secondary metabolites

Ten % activated *S.aureofaciens* A3 in 100 mL of ISP4A (ISP4 dissolved in synthetic seawater) in a 250 mL Erlenmeyer was used as the starter culture. The seawater composition was as follows (g/L): (NaCl - 24.60, KCl - 0.67, $CaCl_2 \cdot 2H_2O$ - 1.36, $MgSO_4 \cdot 7H_2O$ - 6.29, $MgCl_2 \cdot 6H_2O$ - 4.66 and $NaHCO_3$ - 0.18 [20]. Then, the cultures were incubated in a shaking incubator at 30 °C with stirring at 150 rpm for days 0, 2, 4, 6, 8, 10, 12, and 14. Then, the cultures were centrifuged at 13,000 rpm for 10 min to separate the cells from the filtrates. Filtrates (metabolites) were extracted with 1:1 ethyl acetate) due to its low boiling point, low toxicity, and ability to dissolve lipophilic and hydrophilic compounds, and the organic ethyl acetate phase was evaporated using an evaporator at 40 °C to obtain ethyl acetate extracts (EA) [21]. 1 mg of EA extract was dissolved in 1 mL of methanol in a flask and stored at 4 °C to test the antibacterial activity. The extract was dissolved in methanol because it has high solubility for various compounds in the extract and low toxicity [22].

Antibacterial activity analysis

The agar diffusion disk method was used to analyze the antibacterial activity [23]. The pathogens used in the antimicrobial tests were *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Pseudomonas aeruginosa,* and *Bacillus subtilis*. One loop of each pathogen was inoculated in 100 mL

Nutrient Broth (NB) and incubated for 24 h at 30 °C, until the turbidity achieved 0.5 on the McFarland scale (10^8 CFU/mL). A Petri plate containing 20 mL of Mueller Hinton Agar (MHA) was inoculated with 100 µL of the bacterial suspension and 20 µL of the extract was added to paper discs attached to the surface of the MHA medium. The plates were incubated for 24 h at 37 °C and the diameters of inhibitions were observed. Streptomycin at a concentration of 1 mg/mL serves as the positive control in the test.

Media optimization for antibacterial production by BBD-RSM

The optimization medium was the ISP4A medium. The Box-Behnken experimental design (BBD) was employed with 3 independent factors (soluble starch, ammonium sulfate and NaCl) and each independent variable was tested at 3 distinct levels (−1.0, 0 and +1.0) as shown in **Table 1**. MINITAB 20.4 was the software response optimizer tool used for the RSM analysis. A quadratic polynomial equation was used to assess antibacterial activity:

$$Y = \beta 0 + \Sigma \beta i \chi i + \Sigma \beta ii \chi i2 + \Sigma \beta ij \chi i \chi j$$

The input variables χi and χj appear to have an impact on the expected response, according to the equation Y. Regression equation intercept (β0), linear (βi), quadratic (βii) and interaction (βij) coefficients were all represented. A functional model was produced from the reply data using multiple regression analysis. The variance (ANOVA) was computed by statistically analyzing the model. Significant antibacterial activity was defined as factors with a *p*-value of less than 0.05 at the 5 % significance level. Three-dimensional response surface plots illustrated that the independent variables interacted with 1 another [7].

Table 1 The level and code of independent variables for BBD.

Name	Factor	Code value (g/L)		
		−1	**0**	**+1**
X1	Soluble starch	5	10	15
X2	$(NH_4)_2SO_4$	0.5	1.25	2
X3	NaCl	1	20.5	40

Table 2 The independent factors in Box-Behnken design.

Run order	NaCl (g/L)	$(NH_4)_2SO_4$ (g/L)	Starch (g/L)
1	1	0.5	10
2	40	0.5	10
3	1	2	10
4	40	2	10
5	1	1.25	5
6	40	1.25	5
7	1	1.25	15

Run order	NaCl (g/L)	$(NH_4)_2SO_4$ (g/L)	Starch (g/L)
8	40	1.25	15
9	20.5	0.5	5
10	20.5	2	5
11	20.5	0.5	15
12	20.5	2	15
13	20.5	1.25	10

Fermentation with media optimization and metabolite extraction

Ten % of the activated culture of *S. aureofaciens* A3 was inoculated into a 250 mL Erlenmeyer containing 100 mL of ISP4A medium which was added with different amounts of starch, ammonium sulfate and NaCl according to the design in **Table 2**. The pH of the medium was adjusted to 7. The duration of the culture was incubated based on the time when it performed the highest antibacterial activity in the previous test. The culture was incubated in an incubator shaker with agitation of 150 rpm at a temperature of 30 °C. Metabolites are extracted using ethyl acetate (EA) due to its low boiling point, low toxicity and ability to dissolve lipophilic and hydrophilic compounds [24]. Metabolites were extracted following the previous process and EA extract was prepared in 1 mg/mL that was soluble in methanol. The extracts were kept at 4 °C for further tests.

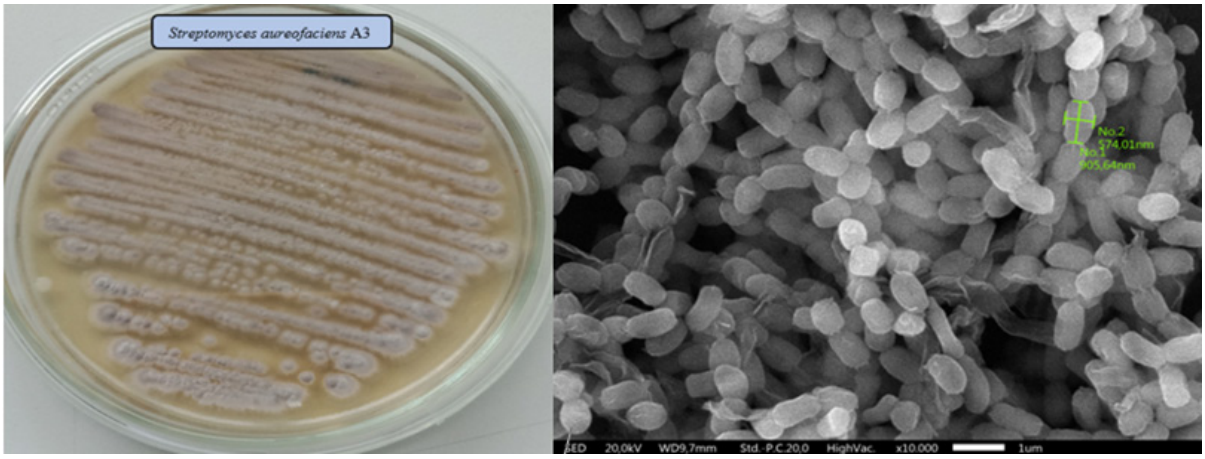
Gas Chromatography-Mass Spectrometry (GC/MS) analysis

Liquid samples were derivatized before GC/MS analysis to enhance chromatographic separation, improve peak shape and increase analyte response such as fatty acids and volatile compounds. Gas chromatography-mass spectrometry and their molecular weight (Agilent 7890B (GC) and 5977A (MSD)) were used to identify the crude extracts that were soluble in methanol. The MS (Agilent, 19091S-433: 93. DB-5MS UI 5 % Phenyl Methyl Silox) was immediately attached to the fused silica HP-5 capillary column (30 m×0.25 mm, ID, the film thickness of 0.25 µm). At the beginning of a 30 min run, the temperature varied from 40 to 300 °C. The instrument was set to start at 40 °C for 1 min, and then it went through a ramp program that increased the temperature from 40 to 325 °C at a rate of 10 °C/min while maintaining an isothermal hold for 20 min. Helium was the carrier gas and flowed at 1.2 $mL \cdot min^{-1}$. Roughly 1 µL of the sample was injected while the injector was maintained at 300 °C. The film ran for 30 to 40 min in total. Using software integrated into the GC-MS equipment, the obtained mass spectra were compared with those in the National Institute of Standards and Technology (NIST) 17/L library for matching. The concentrations of each component present were expressed as a percentage using peak area presentation.

Results and discussion

Characteristics of *S. aureofaciens* A3

The macroscopic and microscopic characteristics of *S. aureofaciens* A3 are shown in **Figure 1**. The Purple colonies were observed on Casein Starch Agar (CSA) medium after 7 days of incubation at 30 °C. The mycelia were yellow and lacked pigmentation in the medium. The spore cells measured approximately 905 by 574 nm under a 10,000-times magnification on a Scanning Electron Microscope (SEM).



(a) **(b)**

Figure 1 Macroscopic morphology (a) and spore image (b) of *S. aureofaciens* A3.

Antibacterial activity

EA extract inhibited Gram-negative and Gram-positive bacteria with moderate to strong activity (**Figure 2**). The first activity was revealed from EA extract containing antibacterial produced during the 6th-day fermentation. The inhibition zone on day 4 was categorized as no activity because the Φ inhibition zone was ≤ 5 mm. A total of 5 EA extracts showed moderate antibacterial activity (Φ 5 - 10 mm) and strong activity (Φ > 10 mm). The highest antibacterial activity was produced on a certain day of fermentation, becoming a reference for the length of fermentation in the antibacterial production optimization treatment in the RSM method. The zone of inhibition (ZOI) was classified into 4 intensities according to diameter: > 20 mm, very strong; 10 - 20 mm, strong, 5 - 10 mm, medium; and < 5 mm, no response [25].

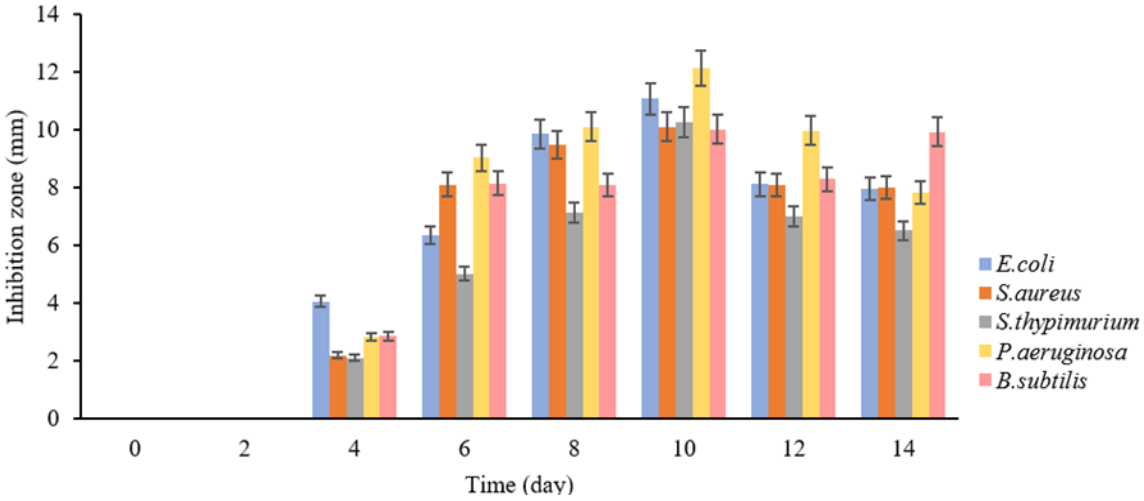


Figure 2 Antibacterial activity of *S. aureofaciens* A3 metabolites produced in ISP4A medium with 150 rpm agitation, 10 % inoculum at 30 °C for 14 days.

Box-Behnken design

The effect of different factors on bioactive metabolite production was studied using RSM. The designs obtained from the software and experimental results are shown in **Table S1**. Second-order polynomial multiple regression was used to determine the results of BBD experiments. The ANOVA regression equation in **Table 3** showed the coefficient of determination (R^2) 99.57 - 99.88 % and the coefficient of determination (R^2adj) 96.41 - 99.31 %. It indicated that the model was very good at producing antibacterial metabolites. The value of R^2 showed that the variance not predicted by

each metabolite was about 0.43 % (*E. coli*), 0.25 % (*S. aureus*), 0.30 % (*S. typhimurium*), 0.21 % (*P. aeruginosa*) and 0.12 % (*B. subtilis*. R^2 represents the proportion of the variance of the dependent variable explained by the RSM model and ranges from 0 - 100 % [7,26]. An R^2 value close to 100 % indicates that the model is more suitable. R^2adj predicts the sample size and number of predictors in the RSM model, therefore the value of R^2 is penalized if it contains null predictors. A higher percentage of R^2adj values indicates a suitable model fit [26].

Table 3 The regression coefficients (%) and equations of the second-order quadratic model.

Response	Second-order quadratic model equation (in coded factors)	S	Regression coefficients (%)	
			R^2	R^2 adj
E. coli	Y = − 48.98 + 17.84×C + 5.075×A + 21.13×B – 3.504×CC – 0.2893 AA – 10.859 BB + 0.0033 CA – 0.378 CB + 1.147 AB	0.805812	99.57	98.81
S. aureus	Y = − 37.60 + 15.284×C + 3.402×A + 18.92×B – 2.7380 CC – 0.18542 AA – 8.685 BB – 0.1050 CA – 0.922 CB + 0.6833 AB	0.408452	99.75	99.31
S. thypimurium	Y = − 53.75 + 16.078×C + 5.483×A + 27.70×B − 2,231 CC – 0.2518 AA – 9.393 BB – 0.3033 CA – 2.833 CB + 0.6767 AB	0.580696	99.16	95.21
P. aeruginosa	Y = − 64.06 + 21.539×C + 6.172×A + 33.63×B – 3.631 CC – 0.2972 AA – 13.700 AA – 0.2700 CA – 1.556 CB + 0.8033 AB	0.577386	99.41	96.64
B. subtilis	Y = − 35.075 + 10.738×C + 3.424×A + 19.895×B – 1.8509 CC – 0.16808 AA – 7.915 BB – 0.0967 CA – 0.867 CB + 0.4167 AB	0.244864	99.88	99.65

Y: Response; A: Pati; B: Ammonium sulfate; C: NaCl

S: Standard deviation of the difference between the fitted value and the data values

R^2: Coefficient of determination value of 3 responses

R^2 adj: Adjusted coefficient of determination value between experimental

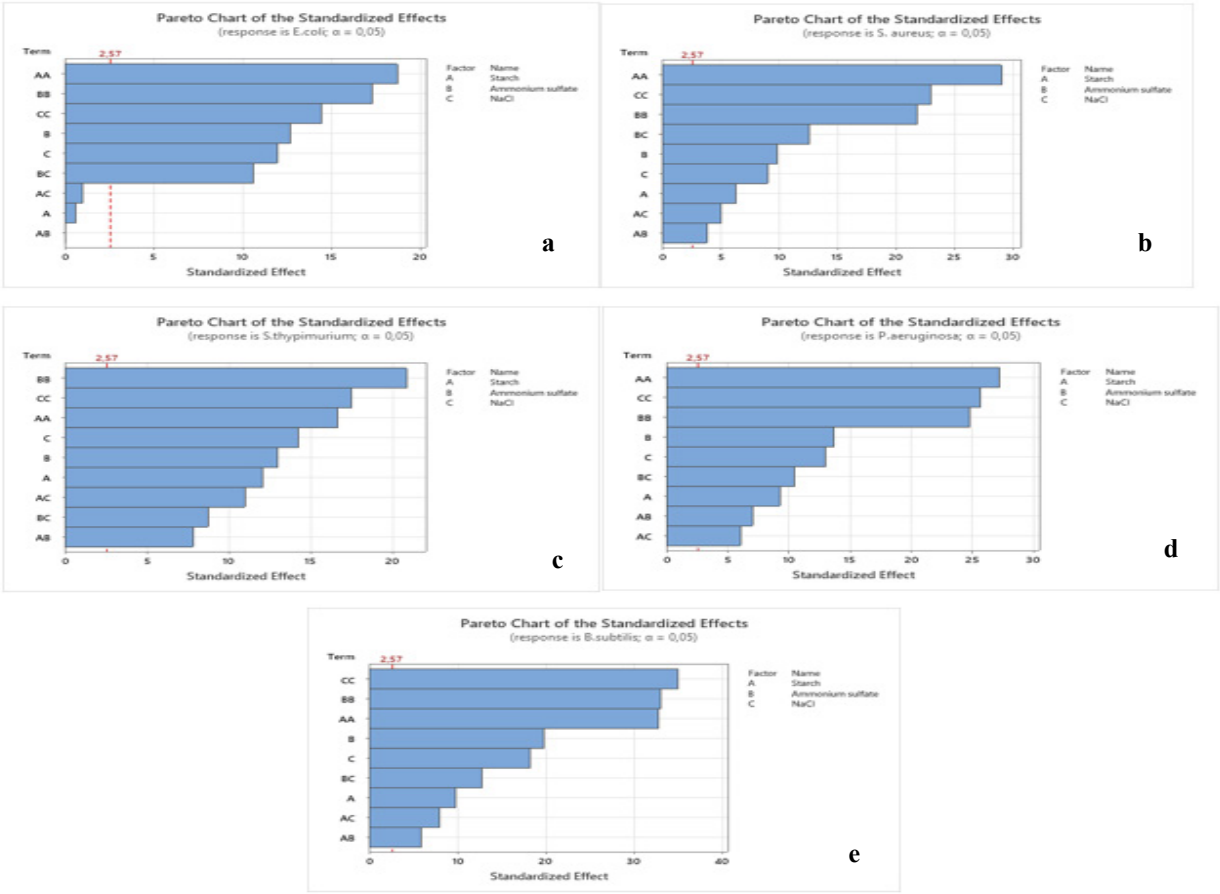


Figure 3 Pareto chart showing the effect of different variables on antibacterial compound production for (a) *E. coli*, (b) *S. aureus*, (c) *S. thypimurium*, (d) *P. aeruginosa,* and (e) *B. subtilis*.

The significance of the model equation and predicted results were assessed statistically using the F test for the ANOVA (**Table S2**). The results demonstrated that all models were highly significant with a low probability *p-value* of 0.0001 and that experimental data fit well [27]. The analysis with a low *p*-value (< 0.001) showed starch, $(NH_4)_2SO_4$, and NaCl significantly influence the production of antibacterial compounds, with starch being the most important factor, followed by ammonium sulfate and NaCl. The Pareto graph clearly illustrates the impact of factors on antibacterial compound production (**Figure 3**).

The contour plots of the response surface

The 2D contour plot and 3D response surface depict the regression equation and the interaction of 2 parameters (variables) on antibacterial activity against each pathogen (**Figure 4**). All 3D plots showed that maximum antibacterial activity occurred at lower levels of $(NH_4)_2SO_4$ and higher levels of starch and NaCl. The contour plots showed the levels of starch (10 g/L < starch < 12.5 g/L) and NaCl (2 g/L < NaCl < 3 g/L) performed the highest activity against *E. coli* (Φ > 16 mm), *S. aureus* (Φ > 12 mm), *S. thypimurium* (Φ > 15 mm), *P. aeruginosa* (Φ > 16 mm), *B. subtilis* (Φ > 10 mm) and the activities decreased with increasing starch and NaCl concentrations.

Starch and $(NH_4)_2SO_4$ increased the antibacterial effect produced by *S. aureofaciens* A3. In this study, the optimum concentration of starch in about 11.06 - 12.07 g/L, ammonium sulfate in 1.36 - 1.59 g/L (< 2 g/L), NaCl in 1.76 - 2.45 g/L (not including the concentration of NaCl in artificial seawater) revealed the highest inhibition for all pathogenic bacteria (**Figure S1**). It has never been studied that $(NH_4)_2SO_4$ concentration < 2 g/L can increase the antibacterial activity of *S.aureofaciens* A3. It indicated that limited concentrations of ammonium sulfate trigger antibacterial metabolites in *S. aureofaciens* A3 as broad-spectrum antibacterial agents. Similar studies showed the antibacterial compounds Streptonigrin produced by *S. flocculus* and SA-53 produced by *Streptomyces anandii* var. Taifiensis in media containing low $(NH_4)_2SO_4$ concentrations of 0.5 - 2 and 0.28 g/L [28,29]. Starch is a beneficial carbon

source because it promotes bacterial growth during the initial phase and enhances antibiotic production [30]. Additionally, intermediate metabolites in the primer metabolic pathway can be precursors of secondary metabolites [31].

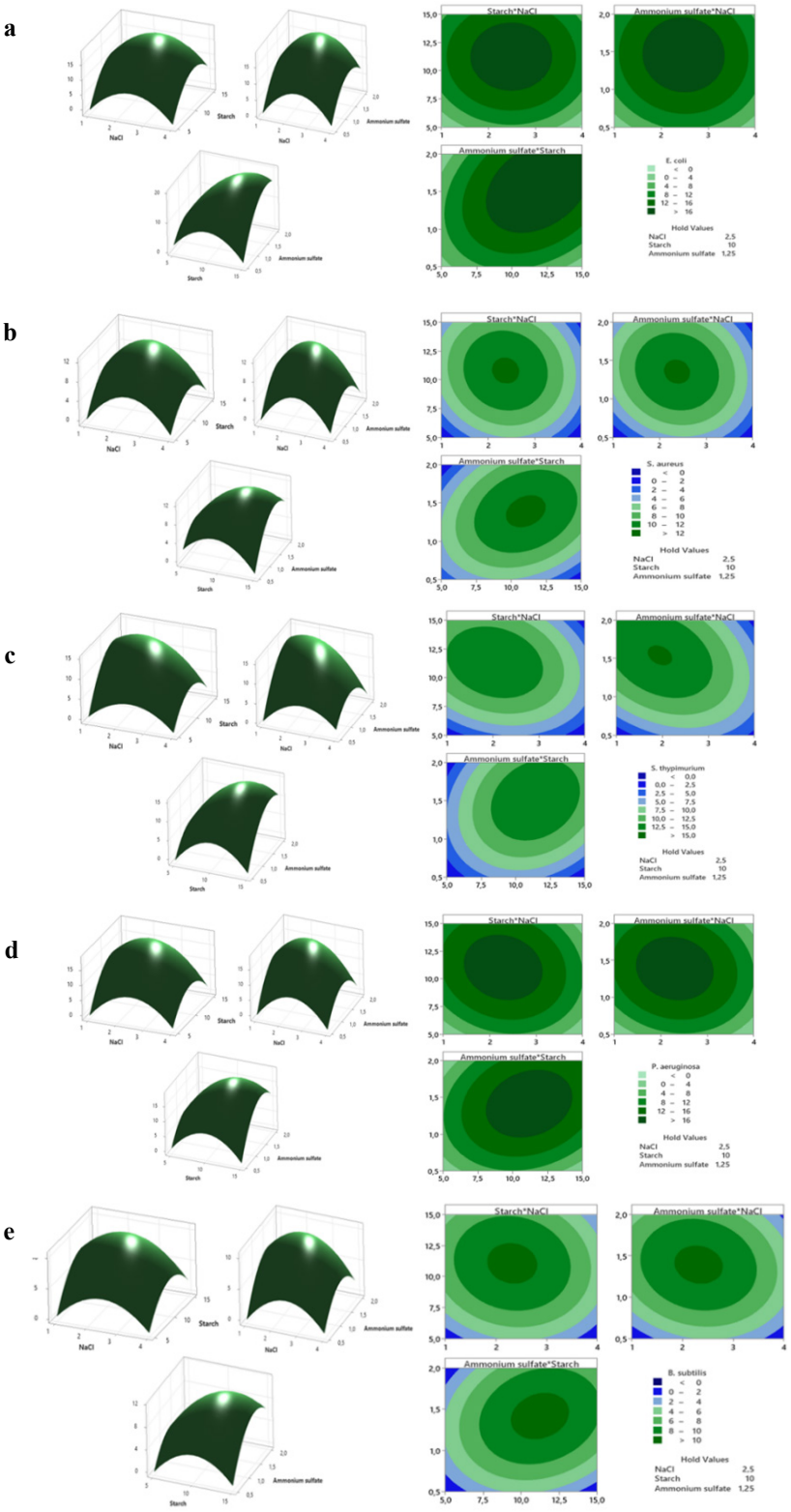


Figure 4 Response surface plots (3D) and contour plots (2D) showing the relationship between starch, ammonium sulfate, and NaCl regarding antibacterial activity against (a) *E. coli*, (b) *S. aureus*, (c) *S. thypimurium*, (d) *P. aeruginosa* and (e) *B. subtilis.*

Validation of the optimized medium

The results predicted by RSM were validated by experiments using the best-predicted composition medium (Response Optimization) for antibacterial activity against all pathogens. The EA extracts from all media demonstrated strong antibacterial activities, with inhibition diameters exceeding 10 mm. In the optimization medium (**Table S3**), the EA extract showed an increased percentage of inhibition zones compared to the ISP4A medium against a range of pathogens: *E. coli* (62.33 %), *S. aureus* (9.41 %), *S. typhimurium* (48.69 %), *P. aeruginosa* (39.16 %) and *B. subtilis* (8.58 %). These findings underscore the potent antibacterial potential of the EA extract, making it a promising candidate for further research and development. In addition, the inhibition zones for each tested bacterium were within the values predicted by the RSM, indicating good agreement between the experimental and predicted values. The experimental values of RSM are obtained directly from experiments, while the theoretical values are not directly measured but are considered correct based on theoretical principles [32].

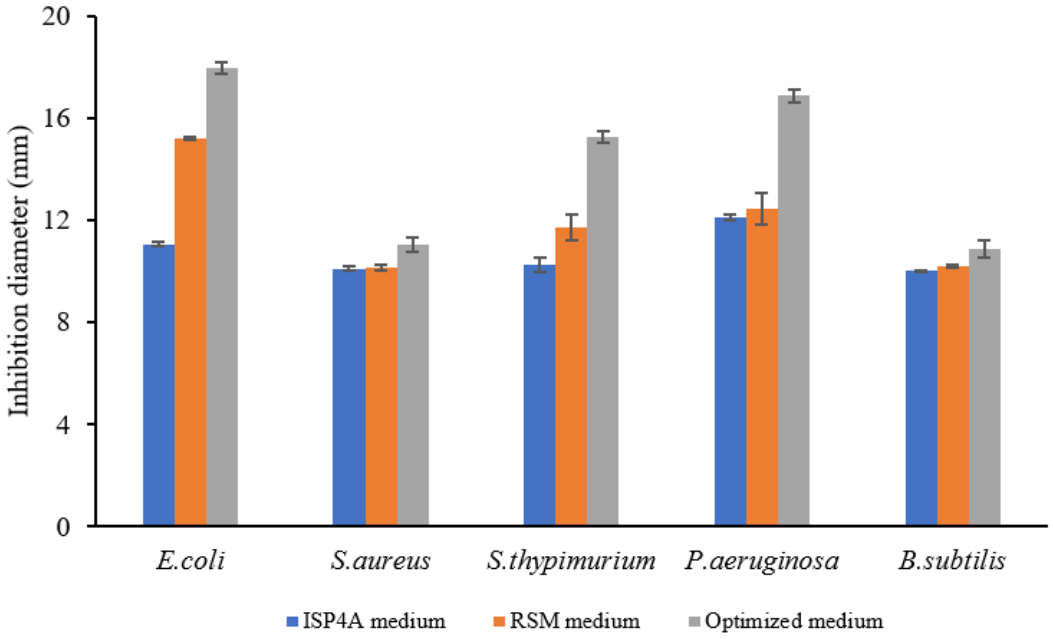


Figure 5 Antibacterial activity of EA extracts produced in various media.

Metabolite profile of EA extracts

The EA extracts produced in the optimization medium contained different or the same compounds with varying abundances. The gas chromatogram analysis in **Figure S2** revealed that the compounds identified by mass spectroscopy had specific mass fragmentation patterns. The primary metabolites were organic acids such as glycolic acid, palmitic acid, stearic acid, lauric acid, myristic acid, and oleic acid. In addition, there were secondary metabolites including phthalic acid, furoic acid, benzoic acid, and methylmalonic acid, each with varying abundance percentages as shown in **Figure 6**. Many fatty acids are normally produced during bacterial fermentation, and changes in media composition can alter the compound profile. The results showed that each extract showed a different compound profile due to variations in carbon, nitrogen, and NaCl concentrations, and all extracts showed higher antibacterial activity against all pathogenic bacteria than the metabolites produced in ISP4 media. In addition, fatty acid primary metabolites act as lipid precursors for other compounds or new compounds with diverse physiological functions [33].

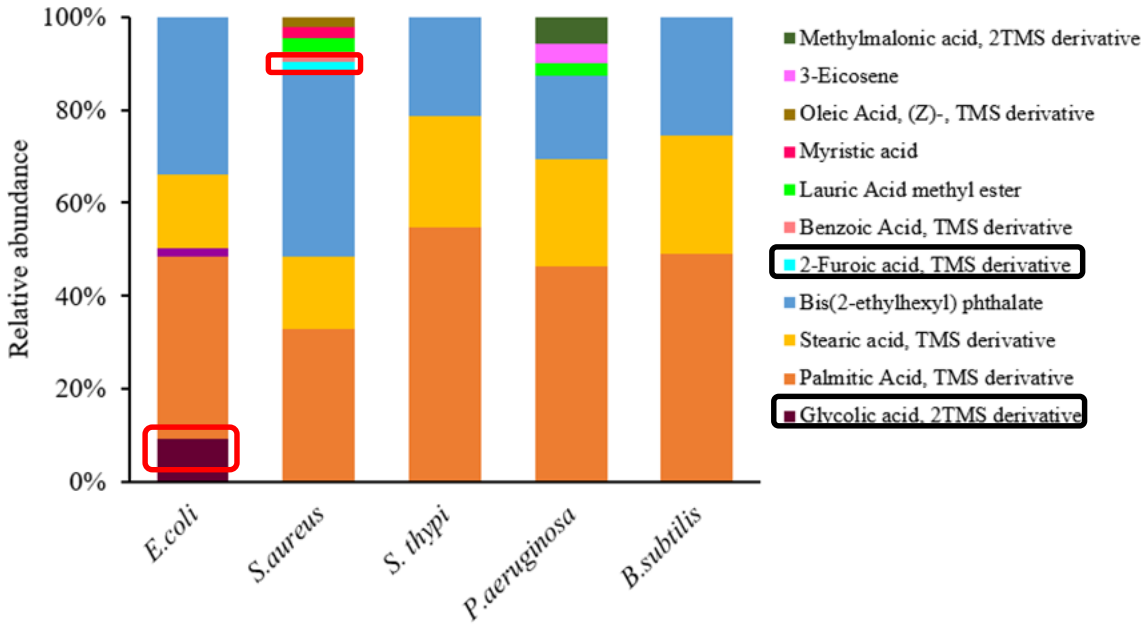


Figure 6 The relative abundance of annotated compounds of EA extracts derived from *S. aureofaciens* A3 produced in optimization media.

The specific profile of antibacterial compounds produced during the fermentation of *S. aureofaciens* A3 has not been previously identified, despite it being well-known as a producer of tetracycline antibiotics. Other compounds with low abundance from some EA extracts were glycolic acid, furoic acid, benzoic acid, methyl tetradecanoate, oleic acid, and eicosene and methylmalonic acid. It has never been known that glycolic or furoic acid has been produced from *Streptomyces* spp. Phthalic acid is a secondary metabolite with antibacterial properties against *Bacillus spizizenii*, *E. coli*, *K. pneumonia,* and *S. aureus* [34,35]. Methyl stearate and palmitic acid show antibacterial activity against *S. aureus*, *E. coli*, *K. pneumoniae* and *Salmonella* sp. [36,37]. Glycolic acid is an antibacterial agent effective against *Propionibacterium acnes*, a Gram-positive bacterium involved in the pathogenesis of acne [38]. Furoic acid (FA) is a broad-spectrum antibacterial, antiseptic, non-toxic, and environmentally friendly compound [39]. It is a secondary metabolite isolated and identified from the fungus *Coniothyrium* sp. [40]. Benzoic acid is a secondary metabolite and can inhibit the growth of bacteria such as *S. aureus, E. coli, K. pneumoniae, B. cereus,* and fungi like *Aspergillus flavus* and *A. parasiticus* [41]. Lauric acid has strong activity in inhibiting the growth of *S. aureus, Candida albicans, Vesicular stomatitis* virus (VSV), *Herpes simplex* virus (HSV), and *Venereal* sp. virus (VV) [42]. Myristic acid and oleic acid can inhibit bacterial growth in pasteurized milk and they are used as natural antibacterial food preservatives against *Listeria monocytogenes*, *B. subtilis,* and *Micrococcus kristinae* in the food industry [43,44].

There is limited published research on the ability of *S. aureofaciens* strains to produce antibacterials. The most recent study successfully isolated *S. aureofaciens* KF532950 from soil. These bacteria exhibited inhibition zones of 10 - 12 mm against *Staphylococcus* spp. and 13 - 15 mm against *Pseudomonas* spp. on ISP2 medium [45]. The activities were lower than that observed in *S. aureofaciens* A3, which produced higher antibacterial activity when grown on an ISP4 medium enriched with artificial seawater. No prior research has examined the antibacterial activity of *S. aureofaciens* derived from ISP4 medium, ISP4 medium supplemented with artificial seawater, and the optimized medium. A recent study showed *S. aureofaciens* A3 demonstrated that optimizing the medium with artificial saltwater increases its antibacterial activity in ISP2 and ISP4 media.

Conclusions

The antibacterial activity of marine *Streptomyces aureofaciens* A3 was enhanced using the Response Surface Method (RSM) with Box-Behnken design. The antibacterial extracts contained 2 organic acids:

Glycolic acid and furoic acid - the first were annotated from the *Streptomyces*. The EA extract activities were broad-spectrum and highly effective against various pathogens. The optimized medium yielded the highest antibacterial production on the 10th day of fermentation, with starch concentrations ranging from 11.06 to 12.07 g/L, ammonium sulfate concentrations of 1.36 to 1.59 g/L (< 2 g/L), and NaCl concentrations between 1.76 and 2.45 g/L, all dissolved in artificial seawater on ISP4 medium. Optimizing the *S. aureofaciens* A3 medium opens the possibility of synthesizing new compounds with potential antibacterial or antifungal properties. This provides a strategic framework to significantly enhance the ability to discover and develop new antibiotics in pharmaceutical industries. A limitation of this study is that the compounds in the extract have not been purified, which means new compounds could be discovered by determining the molecular structure using Nuclear Magnetic Resonance (NMR). Thus, the next research involves obtaining new antibacterial or antifungal compounds and their molecular structures.

Acknowledgments

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Supplementary material

Table S1 Antibacterial activity response of *S. aureofaciens* A3 using BBD.

Run order	Inhibition zone (mm)					pH
	E. coli	***S. aureus***	***S. thypimurium***	***P.aeruginosa***	***B. subtilis***	
1	0	0	0	0	0	6.83
2	0	0	0	0	0	6.79
3	7.15	4.15	9.15	8	5.15	6.65
4	2.25	1	0	0	2.25	6.35
5	0	0	0	0	0	6.87
6	0	0	0	0	0	6.79
7	9.15	4.15	9.15	8	5.15	6.41
8	1.25	0	0	0	1.25	6.75
9	1.5	2	0	0	0	6.81
10	0	0	0	0	0	6.83
11	0	0	0	0	0	6.72
12	14.25	8.25	9.05	9.05	6.25	6.63
13	15.25	10.05	11.15	12.05	10.25	6.35
14	15.15	10.05	12	12.15	10.15	6.25
15	15.25	10.15	12	13.15	10.25	6.30

Table S2 Results of the ANOVA for the regression equation.

Model	DF	Adj SS	Adj MS	F-value	*p*-value
Analysis of variance in antibacterial tests against *E. coli*					
Linear	3	197.95	65.983	101.62	< 0.001
Square	3	486.986	162.329	249.99	< 0.001
2-way interaction	3	74.685	24.895	38.34	< 0.001
Lack-of-fit	3	3.241	1.08	324	0.003
Pure error	2	0.007	0.003		
Total	14	762.867			
Analysis of variance in antibacterial tests against *S. aureus*					
Linear	3	36.426	12.1421	72.78	< 0.001
Square	3	267.647	89.216	534.76	< 0.001
2-way interaction	3	33.052	11.017	66.04	< 0.001
Lack-of-fit	3	0.827	0.276	85.75	0.012
Pure error	2	0.007	0.003		
Total	14	337.959			
Analysis of variance in antibacterial tests against *S. thypimurium*					

Model	DF	Adj SS	Adj MS	F-value	*p*-value
Linear	3	174.676	58.225	172.67	< 0.001
Square	3	297.557	99.186	294.14	< 0.001
2-way interaction	3	87.099	29.033	86.1	< 0.001
Lack-of-fit	3	1.679	0.56	167.94	0.006
Pure error	2	0.007	0.003		
Total	14	561.003			
Analysis of variance in antibacterial tests against *P. aeruginosa*					
Linear	3	146.299	48.766	146.28	< 0.001
Square	3	350.505	193.502	580.43	< 0.001
2-way interaction	3	64.953	21.651	64.95	< 0.001
Lack-of-fit	3	1.677	0.556		
Pure error	2	0	0	27.39	0.035
Total	14	793.424			
Analysis of variance in antibacterial tests against *B. subtilis*					
Linear	3	49.073	16.3577	272.82	< 0.001
Square	3	175.47	58.4902	975.51	< 0.001
2-way interaction	3	15.671	5.2235	87.12	< 0.001
Lack-of-fit	3	0.293	0.0977	29.31	0.033
Pure error	2	0.007	0.003		
Total	14	240.514			

DF: Degrees of freedom; Adj SS: Adjusted sums of squares; Adj MS: Adjusted mean squares

$p > 0.05$, insignificant difference; $p < 0.05$, significant difference; $p < 0.01$, significant difference

Table S3 Antibacterial activity of EA extracts from unoptimized and optimized medium.

Pathogens	Inhibition zones (mm)		
	Unoptimized medium	RSM medium	BBD predictor
E. coli	11.07 ± 0.08	17.97 ± 0.20	17.23 - 21.96
S. aureus	10.10 ± 0.09	11.05 ± 0.26	11.21 - 13.62
S. thypimurium	10.27 ± 0.28	15.27 ± 0.20	14.37 - 17.75
P. aeruginosa	12.13 ± 0.13	16.88 ± 0.23	17.30 - 20.69
B. subtilis	10.02 ± 0.03	10.13 ± 0.13	9.97 -11.41

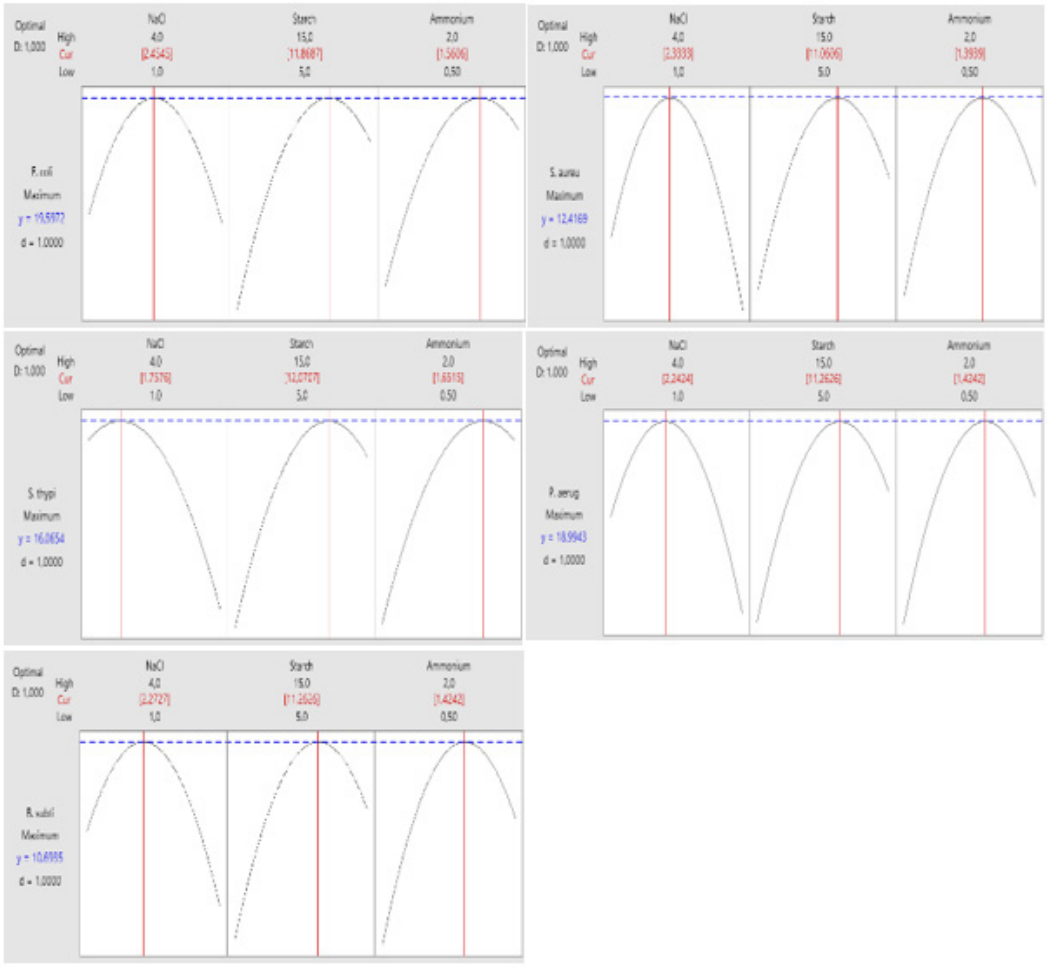
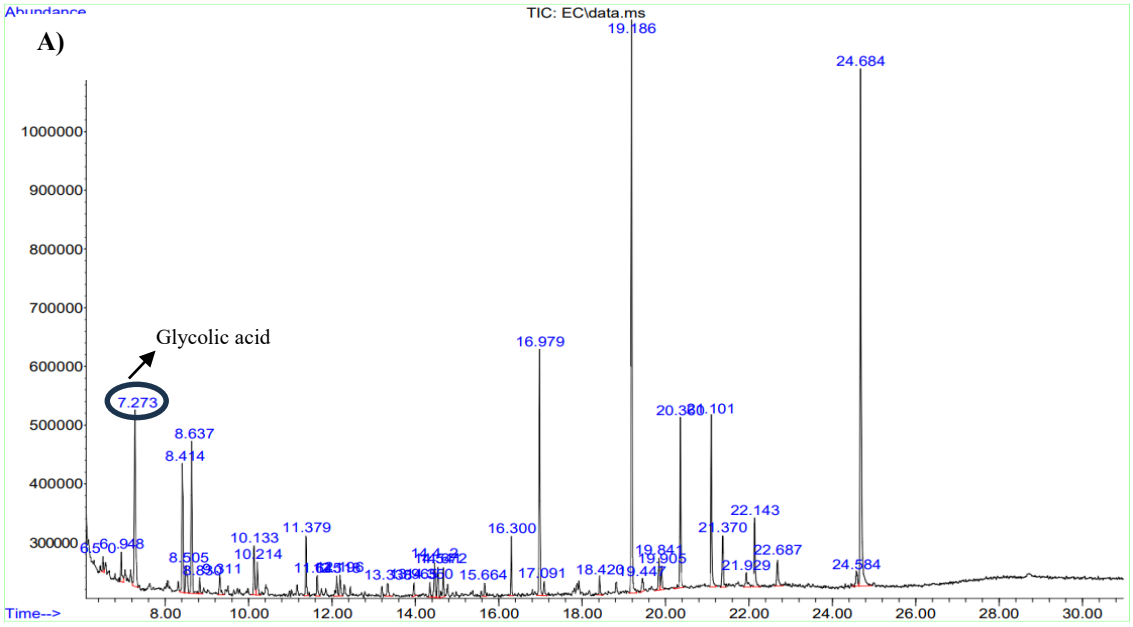
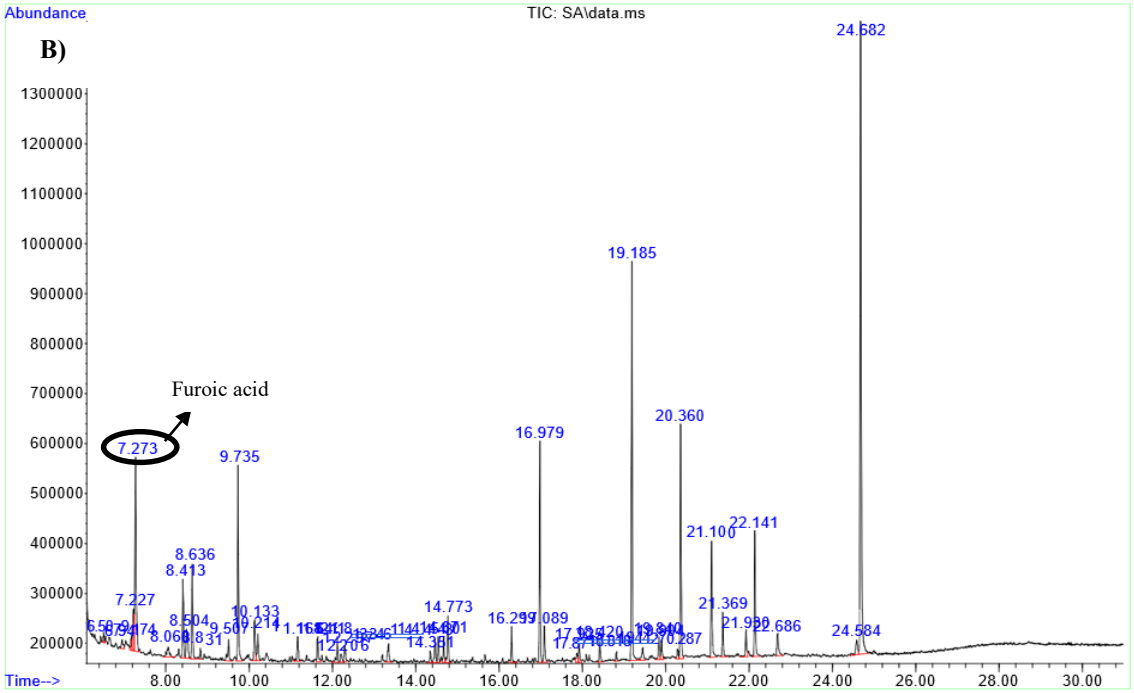
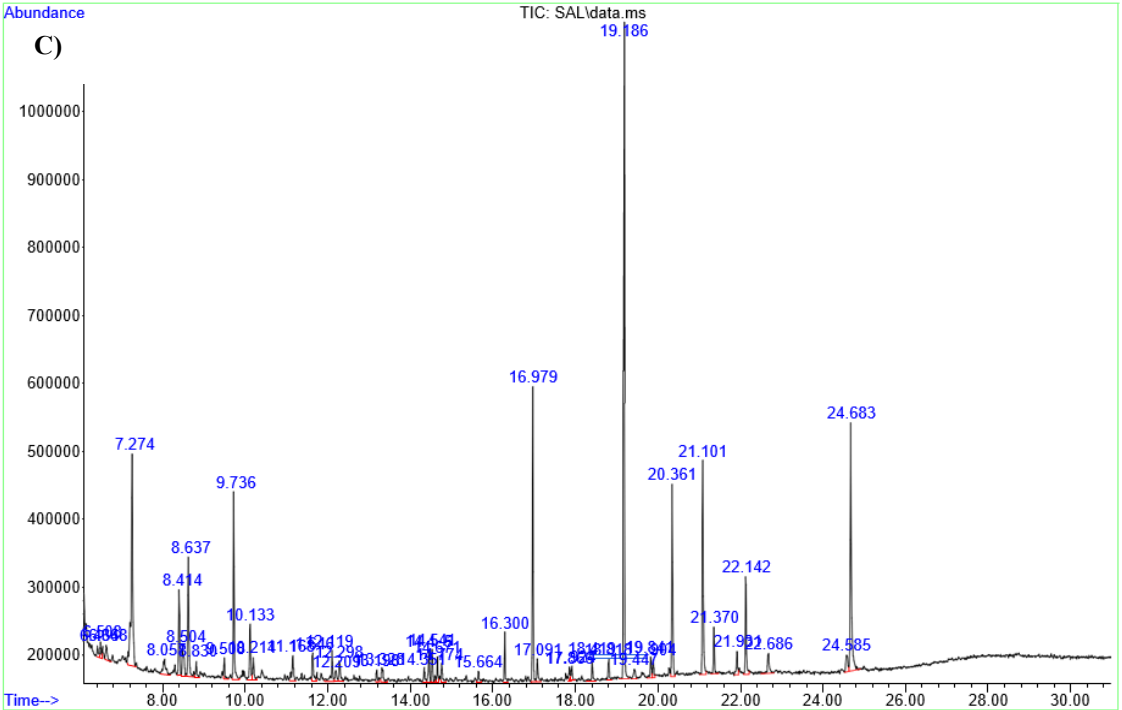


Figure S1 Response optimization chart showing the proper concentration of media components (starch, ammonium sulfate and NaCl) on antibacterial compound production for *E. coli* (A), *S. aureus* (B), *S. typhimurium* (C), *P. aeruginosa* (D) and *B. subtilis* (E).




Abundance
TIC: SA\data.ms
B)
Furoic acid
7.273
9.735
19.185
24.682
20.360
16.979
21.100
22.141
8.636
8.413
7.227
14.773
21.369
24.584
Time-->


Abundance
TIC: SAL\data.ms
C)
19.186
16.979
24.683
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21.101
20.361
9.736
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8.414
10.133
16.300
21.370
24.585
Time-->

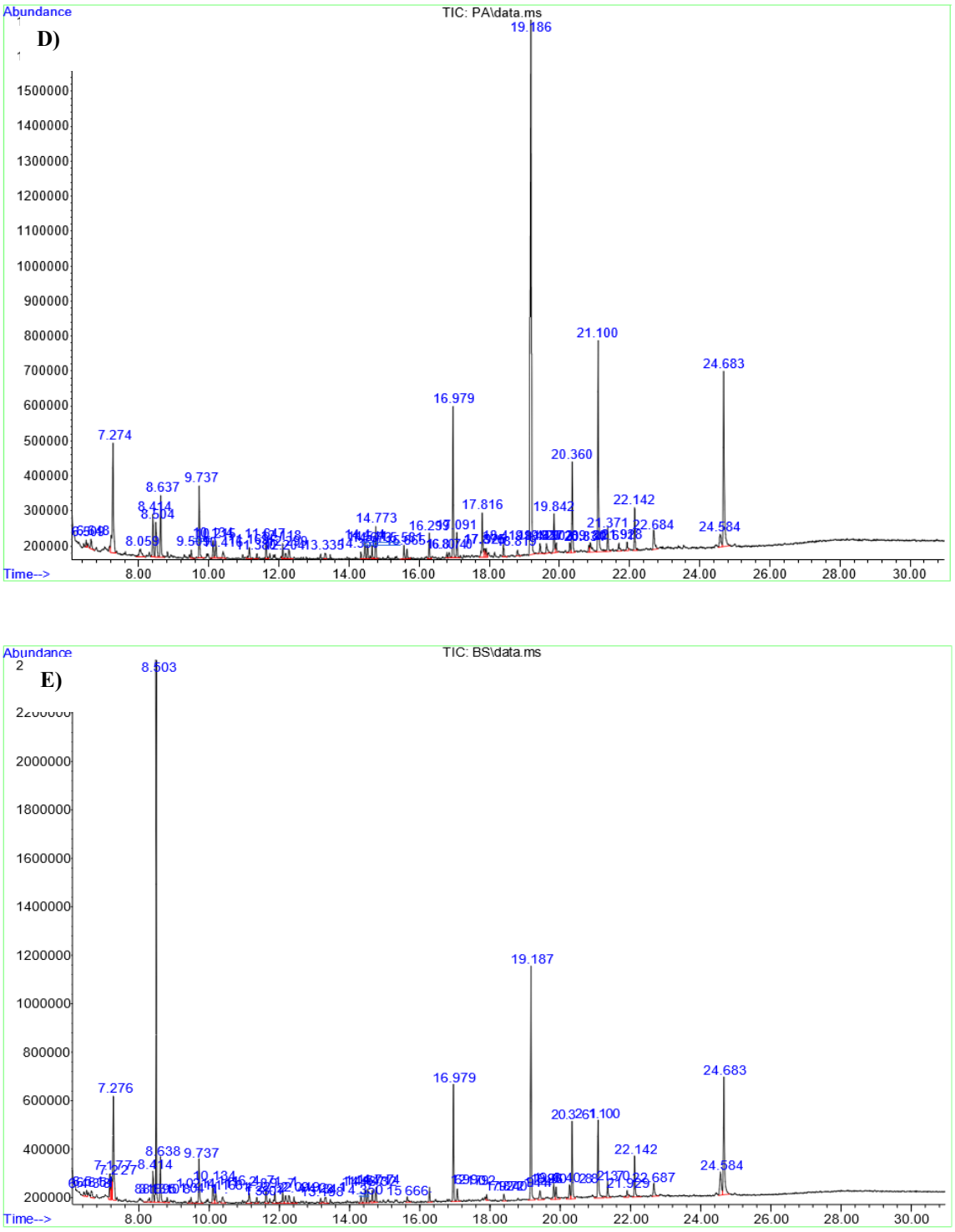

Figure S2 GC/MS spectrum of EA extracts produced in 5 optimized media. The EA extracts contain compounds against *E. coli* (A), *S. aureus* (B), *S. typhimurium* (C), *P. aeruginosa* (D) and *B. subtilis* (E).