Response-surface optimization of anti-pathogenic activity of metabolic extracts obtained from rhizobacteria of Parthenium hysterophorus

Muhammad Yousaf
Bahauddin Zakariya University
Muhammad Ibrahim
Bahauddin Zakariya University
Haq Nawaz (haqnawaz@bzu.edu.pk)
Bahauddin Zakariya University
Raheela Jabeen
The Women University Multan
Mubashir Nawaz
Bahauddin Zakariya University
Maria Kashaf
Bahauddin Zakariya University

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Abstract

The anti-pathogenic activity (APA) of the metabolic extracts obtained from rhizobacteria of *Parthenium hysterophorus* was optimized by constructing a tri-factorial central composited design in the response surface methodology. The colonies of the rhizobacteria isolated from the rhizospheric soil of *P. hysterophorus* were identified microscopically. The pink and yellow-colored colonies were cultured at five levels of three culture variables including pH (5, 6, 7, 8, and 9), incubation temperature (I_T: 24, 28, 32, 36, and 40°C), and incubation time (I_t: 24, 48, 72, 96, and 120 h). The bacterial metabolites were extracted in methanol and analyzed for their APA against *Pseudomonas aeruginosa* and *Escherichia coli*. A statistically significant linear positive effect of pH was observed on the APA of both of the bacterial extracts and quadratic negative effect on APA of yellow-colored extracts against each of the selected pathogens. The I_t showed a significant linear positive effect on APA of the pink-colored extract against *E. coli* and that of the yellow-colored extract against the selected pathogens. I_t also showed quadratic negative effects on the APA of both of the bacterial extracts against the selected pathogens. However, the I_T in the selected range showed no significant effect on the APA of the selected bacterial metabolites against the selected pathogens.

Introduction

The plant-microbe interactions are common at various stages of their lives that may either be beneficial or harmful for both of the organisms. The root exudates at the root-soil interface act as chemo-attractants as well as repellents for bacteria (Garbeva et al. 2004; Berg et al. 2014). The majority of the microbial populations are concentrated in nutrient-rich niches like the rhizosphere that have a constant supply of easily utilizable nutrients. The rhizosphere has an enormous pool of soil microorganisms and is considered the 'hot spot' for microbial colonization and activity (Mohanram and Kumar 2019).

The root exudates, consisting of plants metabolites secreted in the rhizosphere, may significantly influence the associated microbial populations in the soil. The microorganisms, in turn, release their metabolites in the soil that may affect plant health and productivity (Schirawski and Perlin 2018; Asemoloye et al. 2019). When challenged by pathogens, plants trigger a highly complex defence system, to recognize invaders and translate this signal into defence such as the expression of defence response genes. The plants use the bacterial metabolites possessing anti-pathogenic potential against the pathogenic attack (Goh et al. 2013; Fiorilli et al. 2020). These microorganisms also produce some medicinally important products that play a significant role in increasing human life expectancy (Davies 2013). Some of the microbial products possessing antiviral, anthelmintic, enzyme inhibitory, hypocholesterolemic, antiparasitic, herbicidal, pesticidal, insecticidal, and plant growth regulatory activities are also used in the pharmaceutical field at the commercial level (Demain 2014; Rehman et al. 2020). Natural compounds such as amino acids, enzymes, vitamins, organic acids, and alcohol obtained from microorganisms have also proved their value in nutrition, agriculture, and healthcare (Nannipieri et al. 2007; Mohanram and Kumar 2019). These anti-pathogenic metabolites, if extracted and isolated properly from these bacteria, can be applied as antibiotics against human pathogens.

Parthenium hysterophorus is an annual herb with a deep-penetrating taproot system and an erect shoot system. It produces some toxic materials such as sesquiterpene lactone, parthenin, and other phenolic compounds that possess allergic, antimicrobial, antifungal, antifeedant, phytotoxic, anticancer, and other pharmacological activities (Patel 2011; Dahiya and Jakhar 2015; Lalita and Kumar 2018). It is used in the treatment of ulcerated sores, wounds, dysentery, fever, anemia, heart troubles, and hepatic amoebiasis (Bagachi et al. 2016). The most frequently found bacterial genera in the rhizosphere of *P. hysterophorus* include Acidobacteria, Betaproteobacteria, Arthrobacter, Azospirillum Enterobacter, Azotobacter, Bacillus, Bradyrhizobium, Burkholderia, Flavobacterium, Klebsiella, Mesorhizobium, Alcaligenes, Pseudomonas, Rhodococcus, Streptomyces, Serratia, and Nitrospirae (Jothibasu 2012). It has been also reported that *Agrobacterium aurantiacum* and *Paracoccus carotinifaciens* produce pink-colored colonies by producing astaxanthin pigment while *Flavobacterium* spp, *Paracoccus zeaxanthinifaciens*, *Bacillus subtilis, and Bacillus cereus* and *Xanthomonas oryzae* form yellow-colored colonies by producing xanthomonadin pigment (Patel 2011; Usman et al. 2017).

Previously, studies have been reported on the types, isolation, and characterization of rhizoflora of *P. hysterophorus* and medicinal and pharmaceutical properties of the metabolic extracts of rhizobacteria from different sources (Jothibasu 2012; Yadav et al. 2012; Usman et al. 2017). However, limited data have been found on the anti-pathogenic activity of the primary

metabolites of bacteria present in the *P. hysterophorus*. Therefore, the present study was designed to isolate and identify the bacterial species from the rhizosphere of *P. hysterophorus* and optimize the effect of some culture conditions including including pH, incubation temperature, and incubation time on the anti-pathogenic activity of their metabolic products. The study would help to obtain the optimal production of the anti-pathogenic metabolites from bacteria for the pharmaceutical application.

Material And Methods

Rhizosphere soil sampling

The *P. hysterophorus* plants were collected from the different localities of district Multan. The plants were uprooted up to a depth of 12-15 cm and the soil adhered with the roots was collected on the sanitized plastic sheets using a sterilized spatula. The soil samples were homogenized well and stored in plastic bags at 4°C.

Isolation, identification, and culturing of rhizobacteria

The soil sample (10 g) was mixed with the sterile solution of Luria-Bertani nutrient broth (90 ml) and incubated in a shaker incubator at 35±3°C for 48 h. The bacterial colonies were picked by the sterile loop and transferred to the freshly prepared agar medium using the streak plating method (Zhou et al. 2019). The purified colonies of bacteria were identified microscopically based on their morphological characteristics. The purified cultured bacteria were preserved and stored in the glycerol broth at 4℃ for further analysis.

Identification of bacterial isolates based on 16S rRNA gene sequence

The deoxyribonucleic acid (DNA) of bacterial isolates producing pink and yellow-colored colonies in Luria-Bertani broth culture was extracted by previously reported method using commercially available DNA extraction kit (Vivantis kit; catalog: GF-BA-100 preps, USA) (Ibrahim et al. 2020). The amplification of bacterial specific 16S ribosomal RNA gene was done using universal primer derived from 16S rRNA sequence of *Escherichia coli* in a thermal cycler polymerase chain reactor (Agilent, Sure Cycler 8800, USA) (Porteous and Armstrong, 1993). The process of amplification was started with denaturation of DNA at 94°C for 5 min followed by 35 cycles of denaturation and annealing at 55°C (each for 30 s). The process was continued with the initial extension of DNA strands at 72°C for 1 min and final for for 10 min. The amplification was carried out using universal primers: forward primer 5′CAGCAGCCGCGGTAATAC3′ and reverse primer 5′ACGGGCGGTGTGTACAAG3′. The process of amplification was completed following the experimental protocols as reported by Ibrahim et al. 2020 (Ibrahim et al. 2020).

The 16S ribosomal RNA gene sequences were determined by Sanger sequencing from Macrogen Inc. (Korea) using respective primers. The NCBI database by BLAST was used to compared the manually aligned sequences against recent homologous sequences of 16S ribosomal RNA (Altschul et al. 1997). The Mega 7.0 software was used to construct the phylogenetic trees (Kumar et al. 2016) and the evolutionary history was concluded using the neighbor-joining method (Saitou and Nei 1987).

Experimental design for optimization of anti-pathogenic activity of metabolic extracts

The effect of culture conditions on the anti-pathogenic activity (APA) of metabolic extracts of bacteria obtained from the rhizosphere of *Parthenium hysterophorus* was optimized using the response-surface methodology. A tri-factorial response-surface central composite design (CCD) was constructed using five levels of each of the selected culture variables including pH (5, 6, 7, 8, and 9), incubation temperature (I_T: 24, 28, 32, 36, and 40℃), and incubation time (I_t: 24, 48, 72, 96, 120 h). The rhizobacteria were extracted from the soil collected from the rhizosphere of *Parthenium hysterophorus* and cultured in Luria-Bertani nutrient broth (LB-NB). The bacterial colonies were identified based on their morphological characteristics. The colonies producing pink and yellow colored pigments were isolated from the culture plates and cultured again in LB-NB at different levels of culture variables as selected by CCD. The bacterial metabolites were extracted from the media and subjected to analysis of their APA in terms of zone of inhibition (ZOI) against *Pseudomonas aeruginosa* and *Escherichia coli* in Mueller Hinton agar.

Anti-pathogenic activity of bacterial metabolites

The bacterial colonies producing pink and yellow colored pigments were cultured again in Luria-Bertani nutrient broth at different levels of pH (5, 6, 7, 8, and 9), I_T (24, 28, 32, 36, and 40°C), and incubation time (I_t: 24, 48, 72, 96, and 120 h) as selected by CCD. The pH of the medium was set at the selected levels by the addition if either 1M HCL or 1M NaOH solutions. The culture was incubated at the selected levels of temperature using WFT scientific incubator. The bacterial metabolites produced in the growth media were extracted in methanol and subjected to analysis of their APA against *Pseudomonas aeruginosa* and *Escherichia coli* in terms of zone of inhibition (ZOI) by the simple disk-diffusion method in Mueller Hinton agar (Uwizeyimana et al. 2020). The *Pseudomonas aeruginosa* (CDC-54, PAO1) and *Escherichia coli* K12 (NCTC 10538) strains were obtained from the frozen stock (-80°C) of Biodiversity Collection Section (BDCS), Nuclear Institute of Agriculture and Biology (NIAB) Faisalabad, Pakistan. The frozen strains were sub-cultured in nutrient agar, transferred to nutrient broth and incubated for growth in a microbial safety cabinet at 28°C for 24 hr using VWR scientific incubator (Gul et al. 2020). The prepared culture was then used for anti-pathogenic activity of bacterial metabolites.

Statistical analysis

The effect of input variables on the response was optimized by constructing a three factorial response-surface CCD using a statistical package Design Expert Version 11 (Stat-Ease Inc. USA). The variation in the response was analyzed by a one-way analysis of variance. The significance of variance was determined in terms of lack of fit (F-ratio) and probability. The polynomial quadratic model generated the following generalized equation to calculate the predicted values of the response.

$$Y_i = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2$$

where Y_i is the predicted response β_0 is the coefficient of intercept, β_1, β_2, and β_3 are the regression coefficients for the linear effects, β_{12}, β_{13}, and β_{23} for the interaction effects and β_{11}, β_{22}, and β_{33} are the quadratic effects of the input variables. The importance, significance, reliability, and adequacy suggested model was determined by calculating the regression coefficients (R^2), coefficient of variance (CV), and adequate precision (AP).

Results

The morphological characteristics of bacteria isolated from the rhizosphere of *P. hysterophorus* are given in Table 1. The majority of the bacterial colonies showed large size (70%), irregular shape (80%), pink and yellow color (20-46%), flat elevation (60%), dull appearance (60%), and gram-positive staining (87%). Only 20% of the colonies showed the characteristic odor while the rest of the 80% was odorless.

Table- 1

Morphological characteristic of bacterial colonies obtained from the rhizosphere of *P. hysterophorus*

Tested parameter	Characteristics	Total number of isolates	Percentage (%)
Colony size	Large	23	77
	Medium	4	13
	Small	3	10
Colony shape	Circular	6	20
	Irregular	24	80
Color	White	2	7
	Off white	4	13
	Pink	14	46
	Red	2	7
	Cream	2	7
	Light yellow	6	20
Odor	Odor	6	20
	Odorless	24	80
Appearance	Dull	20	67
	Shiny	10	33
Elevation	Flat	18	60
	Raised	6	20
	Convex	5	17
	Filamentous	1	3
Gram staining	Gram +ve	26	87
	Gram -ve	4	13

Identification of bacterial isolates based on 16S rRNA gene sequence

The bacterial species of the pink and yellow-colored colonies of bacteria extracted from rhizosphere of *P. hysterophorus* were confirmed up to strain level through 16S rRNA gene sequencing. Evolutionary Genetics Analysis (MEGA7) generated the phylogenetic tree based on 7 strains of bacteria present in each of the pink-colored and yellow-colored colony (Figure 1a, b). Based on the results of 16S rRNA gene sequencing, the bacterial species present in the pink-colored colonies showed similarity with *Cronobacter muytjensii, Cronobacter universalis, Cronobacter turicensis, and Cronobacter turicensis* strains as their nearest relatives as available on Genen Bank database (Figure 1 a). The bacterial species present in the yellow-colored colonies showed resemblance with *Cronobacter sakazakii, Klebsiella aerogenes, Enterobacter cloacae, Raoultella terrigena, Cronobacter universalis, Cronobacter muytjensii,* and *Cronobacter turicensis* strains as their nearest relatives (Figure 1b).

Anti-pathogenic activity of bacterial extracts

Experimental values of APA in terms of ZOI of the bacterial extracts obtained from the selected colonies of rhizobacteria grown at various combinations of culture variables including pH of the growth medium, I_T, and I_t are presented in Table 2. The APA of PCBE against *P. aeruginosa* and *E. coli* in terms of ZOI ranged from 5 to 12 and 7 to 13 mm with the mean ± SD of 9.55 ± 1.84 and 11.35 ± 0.98 mm respectively. The APA of YCBE against *P. aeruginosa* and *E. coli* in terms of ZOI ranged from 4 to 14 and 5 to 14 mm with the mean ± SD of 10.80 ± 2.14 and 10.35 ± 1.47 mm respectively.

Table 2

Experimental values of anti-pathogenic activity of the extracts obtained from bacterial isolates of the rhizosphere of *Parthenium hysterophorus* against *P. aeruginosa* and *E.coli*

Std.	Run	Culture variables			Anti-pathogenic activity in terms of ZOI (mm)			
		pH	I_T* (°C)	I_t (h)	PCBE		YCBE	
					P. aeruginosa	*E. coli*	*P. aeruginosa*	*E. coli*
1	15	6	28	48	5	8	7	6
2	20	8	28	48	8	9	9	9
3	9	6	36	48	6	9	8	7
4	10	8	36	48	9	10	10	9
5	2	6	28	96	5	12	6	6
6	8	8	28	96	11	12	10	12
7	14	6	36	96	8	11	9	9
8	18	8	36	96	13	14	12	14
9	17	5	32	72	9	10	6	7
10	12	9	32	72	12	13	11	12
11	7	7	24	72	11	13	13	11
12	6	7	40	72	7	10	8	6
13	16	7	32	24	6	7	4	5
14	1	7	32	120	9	11	13	10
15	5	7	32	72	12	13	15	14
16	11	7	32	72	12	13	15	14
17	3	7	32	72	12	13	15	14
18	4	7	32	72	12	13	15	14
19	19	7	32	72	12	13	15	14
20	13	7	32	72	12	13	15	14
Mean±SD					9.55±1.84	11.35± 0.98	10.80±2.14	10.35± 1.47

*I_T: Incubation temperature, I_t: Incubation time, PCBE: Pink-colored bacterial extract, YCBE: Yellow-colored bacterial extract, ZOI: Zone of inhibition

Response-surface analysis and optimization of results

One-way analysis of variance of experimental data showed a statistically significant main effect (F = 3.39-9.83 and p = 0.0007-0.0354) of the selected culture variables on the APA of both of the bacterial extracts against each of the selected pathogen (Table 3). The pH showed a significant linear positive effect (F=6.03-19.57, p=0.0012-0.0334) on APA of both of the bacterial extracts against each of the selected pathogen. I_t also showed a significant linear positive effect (F=6.03-28.96, p=0.0003-0.0334) on APA of PCBE against *E. coli* and that of YCBE against both pathogens. Each of the selected culture variables showed a non-significant interaction effect (p>0.05) on APA of both of the bacterial extracts against each of the selected pathogen. Each of the

selected culture variables showed a significant quadratic effect on APA of each of the bacterial extracts against both of the selected pathogens except that of pH on the APA of PCBE against *P. aeruginosa*. The main, linear, interaction, and quadratic effects on APA of PCBE and YCBE against *P. aeruginosa* and *E.coli* are graphically presented as 3-dimensional response-surface plots in Figure 2a-f and Figure 3a-f. The values of the regression coefficient (R^2 = 0.753-0.898) indicated that 70-89% of the variability in the APA of PCBE and YCBE against *P. aeruginosa* and *E.coli* could be explained by the suggested response-surface model. The values of coefficient of variation (CV) and adequate precision (AP) for the selected bacterial extracts against *P. aeruginosa* and *E.coli* were found to be 8.56-19.79% and 5.95-10.321 respectively.

Table 3

Results of one-way analysis of variance in the anti-pathogenic activity of bacterial extracts against *P. aeruginosa* and *E.coli*

Source	PCBE*						YCBE					
	P. aeruginosa			*E. coli*			*P. aeruginosa*			*E. coli*		
	CE	*F-value*	*p-value*	CE	*F-value*	*p-value*	CE	*F-value*	*p-value*	CE	*F-value*	*p-value*
Model	11.70	3.39	0.0354	12.886	7.83	0.0017	14.682	4.90	0.0103	13.841	9.83	0.0007
A-pH	1.437	9.77	0.0108	0.688	7.95	0.0182	1.3125	6.03	0.0334	1.625	19.57	0.0012
B- I_T (°C)	-0.062	0.02	0.8946	-0.188	0.59	0.4598	-0.187	0.12	0.7329	-0.25	0.46	0.5116
C-I_t (h)	0.938	4.16	0.0688	1.312	28.96	0.0003	1.312	6.03	0.0334	1.25	11.58	0.0067
AB	-0.125	0.037	0.8514	0.375	1.18	0.3024	-0.125	0.03	0.8719	-0.25	0.23	0.6407
AC	0.625	0.92	0.3592	0.125	0.13	0.7246	0.375	0.25	0.6304	0.75	2.08	0.1794
BC	0.375	0.33	0.5769	-0.125	0.13	0.7246	0.375	0.25	0.6304	0.5	0.93	0.3585
A^2	-0.522	2.03	0.1846	-0.432	4.93	0.0507	-1.784	17.52	0.0018	-1.204	16.87	0.0021
B^2	-0.897	5.99	0.0344	-0.432	4.93	0.0507	-1.284	9.08	0.0130	-1.454	24.64	0.0005
C^2	-1.272	12.04	0.0060	-1.057	29.51	0.0003	-1.785	17.52	0.0018	-1.705	33.83	0.0002
R^2	0.753			0.876			0.815			0.898		
CV (%)	19.26			8.56			19.79			14.2		
AP	5.958			10.321			6.459			8.968		

*AP: Adequate precision, CE: Coefficient estimates, CV: Coefficient of variance, I_T: Incubation temperature, I_t: Incubation time, PCBE: Pink colored bacterial extract, R^2: Regression coefficient, YCBE: Yellow-colored bacterial extract

The suggested response-surface model yielded the following polynomial regression equations to explain the relationship between the selected culture variables and APA of the extracts of rhizobacteria against *P. aeruginosa* and *E.coli*.

APA of PCBE against *P. aeruginosa*

$$ZOI\ (mm) = 11.70 + 1.44X_1 - 0.0625X_2 + 0.9375X_3 - 0.1250X_1X_2 + 0.6250X_1X_3 + 0.3750X_2X_3 - 0.523X_1^2 - 0.89X_2^2 - 1.27X_3^2$$

APA of PCBE against *E. coli*

$$ZOI\ (mm) = 12.89 + 0.6875X_1 - 0.01875X_2 + 1.31X_3 + 0.3750X_1X_2 + 0.1250X_1X_3 - 0.1250X_2X_3 - 0.4318X_1^2 - 0.4378X_2^2 - 1.06X_3^2$$

APA of YCBE against *P. aeruginosa*

$$ZOI\ (mm) = 14.68 + 1.31X_1 - 0.1875X_2 + 1.31X_3 - 0.1250X_1X_2 + 0.3750X_1X_3 + 0.375X_2X_3 + -1.78\,X_1^2 - 1.28X_2^2 - 1.78X_3^2$$

APA of YCBE against *E.coli*

$$ZOI\ (mm) = 13.84 + 1.63X_1 - 0.2500X_2 + 1.25X_3 - 0.2500X_1X_2\ 0.7500X_1X_3 + 0.5000X_2X_3 - 1.20X_1^2 + 1.45X_2^2 - 1.70X_3^2$$

The above regression equations were used to calculate the predicted responses that were plotted against the experimental values. The plot showed a good correlation between the experimental predicted values with good signs of correlation coefficients (R^2= 0.753-0.898) (Figure 4a-d).

The optimum levels of culture variables to achieve optimal values of APA of bacterial extracts are presented in Table 4. The observed optimum levels of pH of the growth medium were 8.84 and 7.94 for PCBE against *P. aeruginosa* and *E.coli* and 7.56 and 7.45 for YCBE against *P. aeruginosa* and *E.coli* respectively. The optimum levels of I_T were 30.57 and 32.37°C for PCBE against *P. aeruginosa* and *E.coli* and 31.95 and 29.81 for YCBE against *P. aeruginosa* and *E.coli* respectively. The optimum levels of I_t were found to be 87.88 and 87.10 for PCBE against *P. aeruginosa* and *E.coli* and 77.96 and 75.3 for YCBE against *P. aeruginosa* sand *E.coli* respectively.

Table 4

Optimum levels of culture variables to achieve optimal response of anti-pathogenic activity of metabolic extracts of rhizobacteria of *Parthenium hysterophorus*

Variables	Bacterial extract	Pathogen	Goal	Lower limit	Upper limit	Optimum levels				
						X_1	X_2	X_3	Y	Desirability
pH			in range	5	9					
I_T (°C)*			in range	24	40					
I_t (h)			in range	24	124					
ZOI (mm)	PCBE	*P. aeruginosa*	maximize	5	13	8.84	30.57	87.88	12.21	1.000
	PCBE	*E. coli*	maximize	7	14	7.94	32.37	88.10	13.04	0.948
	YCBE	*P. aeruginosa*	maximize	4	15	7.56	31.95	77.96	12.60	1.000
	YCBE	*E. coli*	maximize	5	14	7.45	29.81	75.63	12.19	1.000

*I_T: Incubation temperature, I_t: Incubation time, PCBE: Pink colored bacterial extract, YCBE: Yellow colored bacterial extract, ZOI: Zone of inhibition

Discussion

The morphological characterization of bacteria isolated from the rhizosphere of *P. hysterophorus* showed that most of the bacterial isolates formed colonies of large size with an irregular shape that produced pink and yellow colored pigments. The results also showed that the majority of the colonies showed flat elevation, dull appearance, and gram-positive staining. The

most frequently found bacterial genera in the rhizosphere of *P. hysterophorus* include Acidobacteria, Betaproteobacteria, Arthrobacter, Azospirillum Cronobacter, Enterobacter, Azotobacter, Bacillus, Bradyrhizobium, Burkholderia, Flavobacterium, Klebsiella, Mesorhizobium, Alcaligenes, Pseudomonas, Rhodococcus, Streptomyces, Serratia, and Nitrospirae (Jothibasu 2012). It has been also reported that *Agrobacterium aurantiacum* and *Paracoccus carotinifaciens* produce pink-colored colonies by producing astaxanthin pigment while *Paracoccus zeaxanthinifaciens*, *Bacillus subtilis, Bacillus cereus*, and *Xanthomonas oryzae* form yellow-colored colonies by producing xanthomonadin pigment (Usman et al. 2017; He et al. 2020; Korumilli et al. 2020; Behera et al. 2021). The correlation of present results with the previous findings suggests that the bacterial isolates from the rhizosphere of *P. hysterophorus* mostly contain *Agrobacterium aurantiacum, Paracoccus carotinifaciens, Flavobacterium species*, *Paracoccus zeaxanthinifaciens*, *Bacillus subtilis, Bacillus cereus*, and *Xanthomonas oryzae* along with other genera.

The sequencing of the 16S ribosomal RNA gene of bacterial species present in pink-colored colonies obtained from the rhizosphere of *P. hysterophorus* showed that the these bacterial species are similar to various trains of Coronobacter as their nearest relatives. However, the bacterial species present in yellow-colored colonies obtained from the rhizosphere of *P. hysterophorus* showed similarity with Coronobacter and Klebsiella, Enterobacter, and Raoultella strains as their nearest relatives.

The results indicate that bacterial extracts obtained from both of the selected bacterial colonies showed considerable anti-pathogenic activity against *P. aeruginosa* and *E.coli*. A relatively higher value of zone of inhibition indicates the more significant activity of an antibiotic or an antimicrobial agent against human pathogens. In the present study, the values of ZOI (9.55 ± 1.84–11.35 ± 0.98 mm) for the bacterial extracts obtained from the selected bacterial colonies against the studied pathogens may be considered significant. Therefore, the extracts obtained from the selected bacterial species may have practical applications in the pharmaceutical field.

The response-surface analysis of the experimental data resulted in a significant main effect of the selected culture variables on the APA of the bacterial extracts obtained from the rhizosphere of *P. hysterophorus* with a relatively higher F-value and lower p-value. Both the pH and I_t showed significant linear positive effects on the APA of both of the bacterial extracts against both of the pathogens. The pH and I_t-dependent increase in APA of the extracts may be attributed to the increased growth of the bacteria at alkaline pH and incubation period. However, the I_T and I_t showed quadratic negative effects on the APA of the bacterial extracts that may be due to the high temperature and the time-dependent depletion of nutrition in the culture medium.

The regression coefficient measures the degree of fitness of the experimental data on the regression line while adequate precision and coefficient of variation determine the signal to noise ratio in the experimental data and precision and reliability of the experimental model respectively (Anderson and Whitcomb 2016). Based on the very good signs of the regression coefficient, coefficient of variation, and adequate precision, it can be suggested that the employed polynomial quadratic response-surface model is suitable for explaining the relationship between the studied culture variables and APA of the bacterial extracts against both of the selected pathogens. Relatively higher values of R^2 and AP and lower values of CV indicate the better applicability, precision, and reliability of the response-surface models. A good agreement between the experimental and predicted values of APA also advocated the accuracy and validity of the suggested model.

The pH and time-dependent linear increase in APA of the bacterial extracts suggest that the rhizobacteria *P. hysterophorus* produce the pink and yellow colored anti-pathogenic metabolites at alkaline pH with relatively longer incubation time. However, the quadratic negative effect of pH and I_t may be attributed to the decrease in growth and metabolic activities of the bacteria above the optimal pH and depletion of the nutrient after a long time incubation. The present findings report the optimum levels of pH, I_T, and I_t for the production of anti-pathogenic metabolites from rhizobacteria that may help in optimal production of these compounds for pharmaceutical application The study also highlights the importance of *P. hysterophorus* rhizosphere as a source of bacterial colonies that can produce anti-pathogenic metabolites.

In conclusion, the bacteria isolated from the rhizosphere of *P. hysterophorus* showed large-sized and irregular-shaped colonies that produced and released mainly pink and yellow colored pigments in the medium that showed good APA against *P. aeruginosa* and *E.coli*. A statistically significant linear positive effect of pH and I_t and quadratic negative effect of I_T and I_t was observed on

the APA of the extracts obtained from the bacterial culture. The data would be a valuable contribution to the literature regarding the research on the production and APA of microbial metabolites.

Declarations

Acknowledgment

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Conflict of Interest

The authors declare no conflict of interest regarding this study.

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Data availability statement

All the data supporting this study has been provided in the manuscript.

Author's contribution

Conceptualization and data analysis: Haq Nawaz, Methodology, investigation and writing of the original draft: Muhammad Yousaf and Mubashir Nawaz, Reviewing, editing and supervision: Muhammad Ibrahim. All authors have read and approved the published version of manuscript.

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Figures

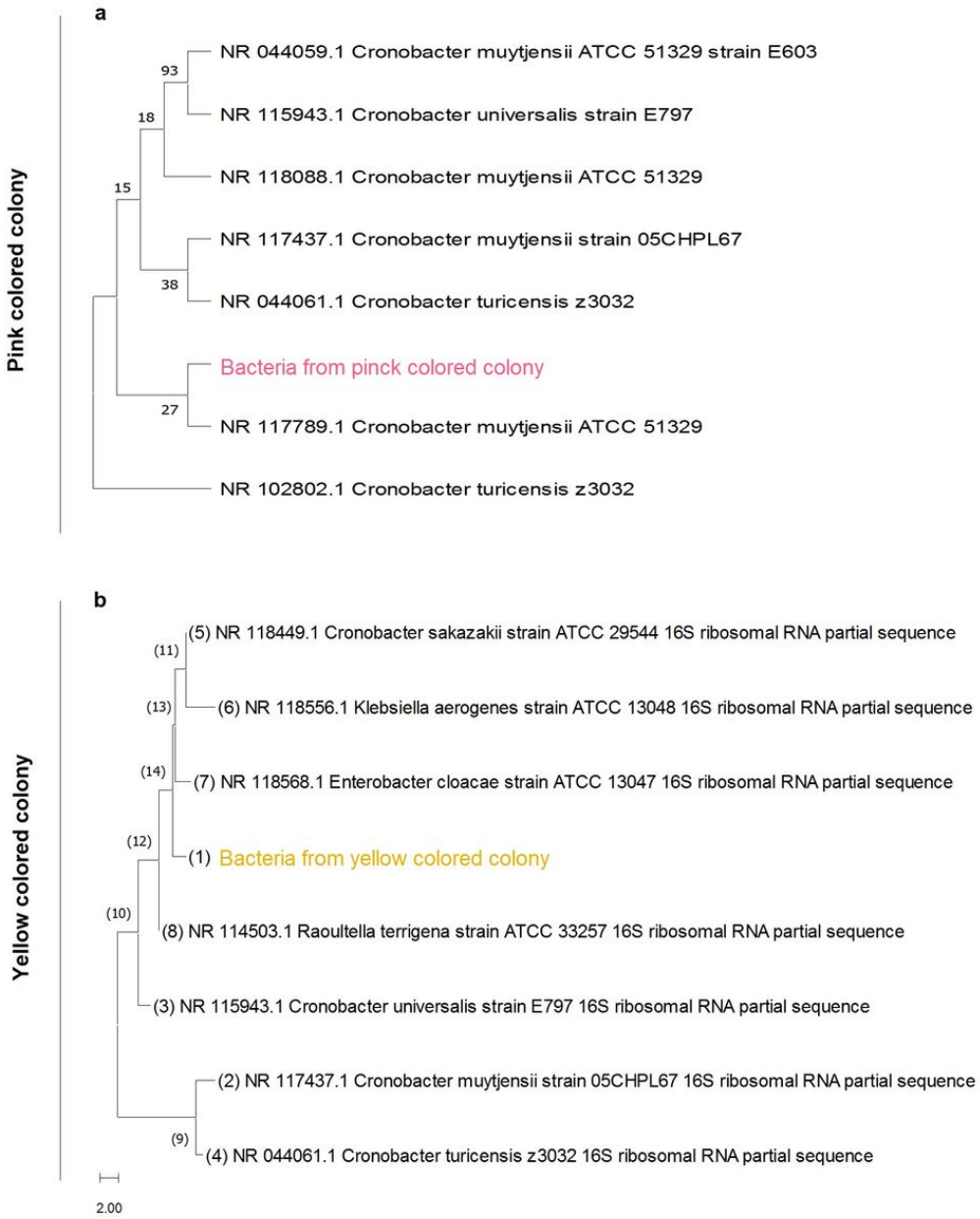

Figure 1

Phylogenetic relationship of bacterial isolates obtained from the rhizosphere of *P. hysterophorus*.

a) Bacterial species present in pink-colored colony, b) Bacterial species present in yellow-colored colony

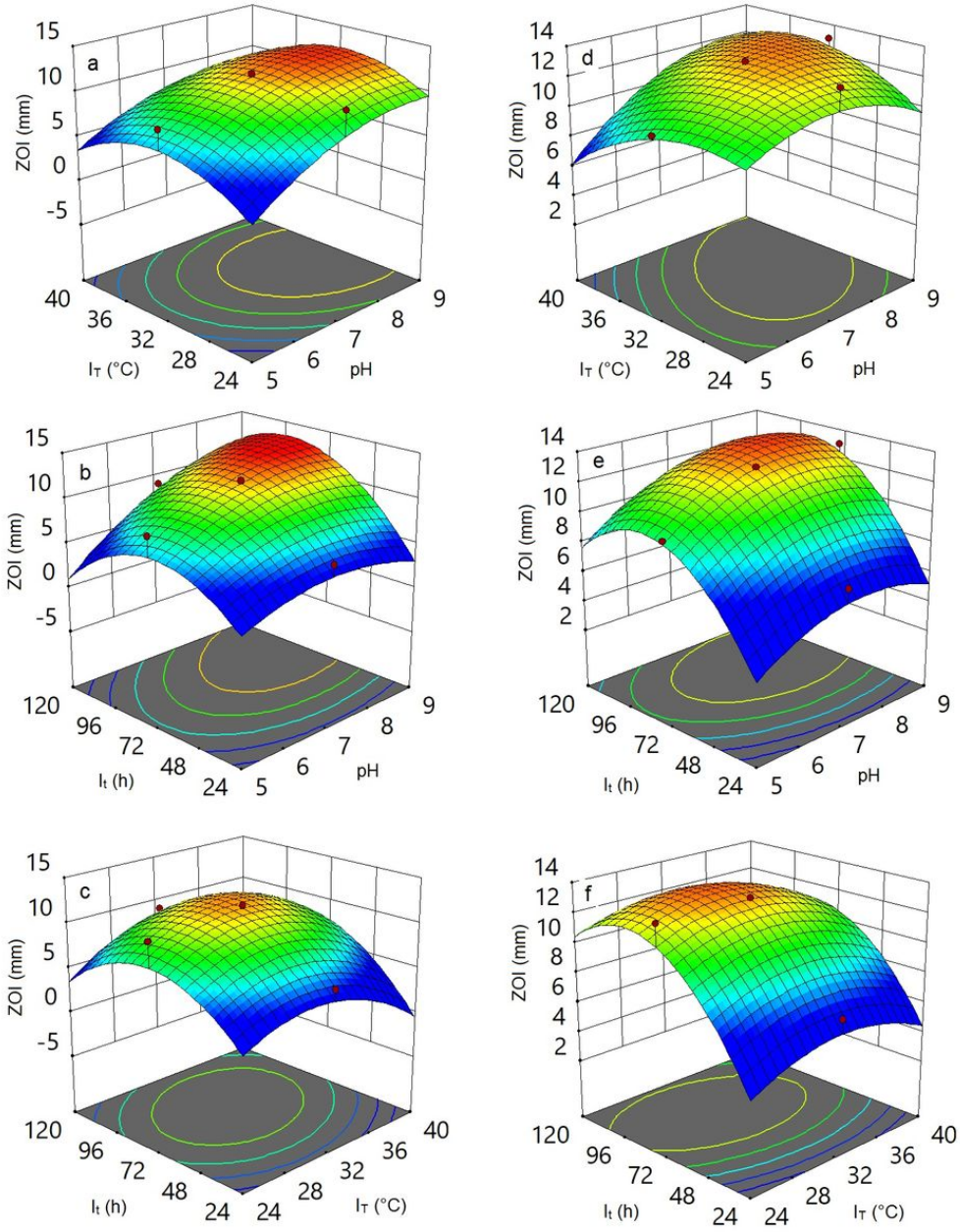

Figure 2

Three -dimensional response-surface plots of anti-pathogenic activity of metabolic extracts of rhizobacteria of *P. hysterophorus* at selected levels of culture variables

a-c) Pink-colored bacterial extracts against *P. aeruginosa*

d-f) Pink-colored bacterial extracts against *E. coli*

*IT: Incubation temperature, It: Incubation time

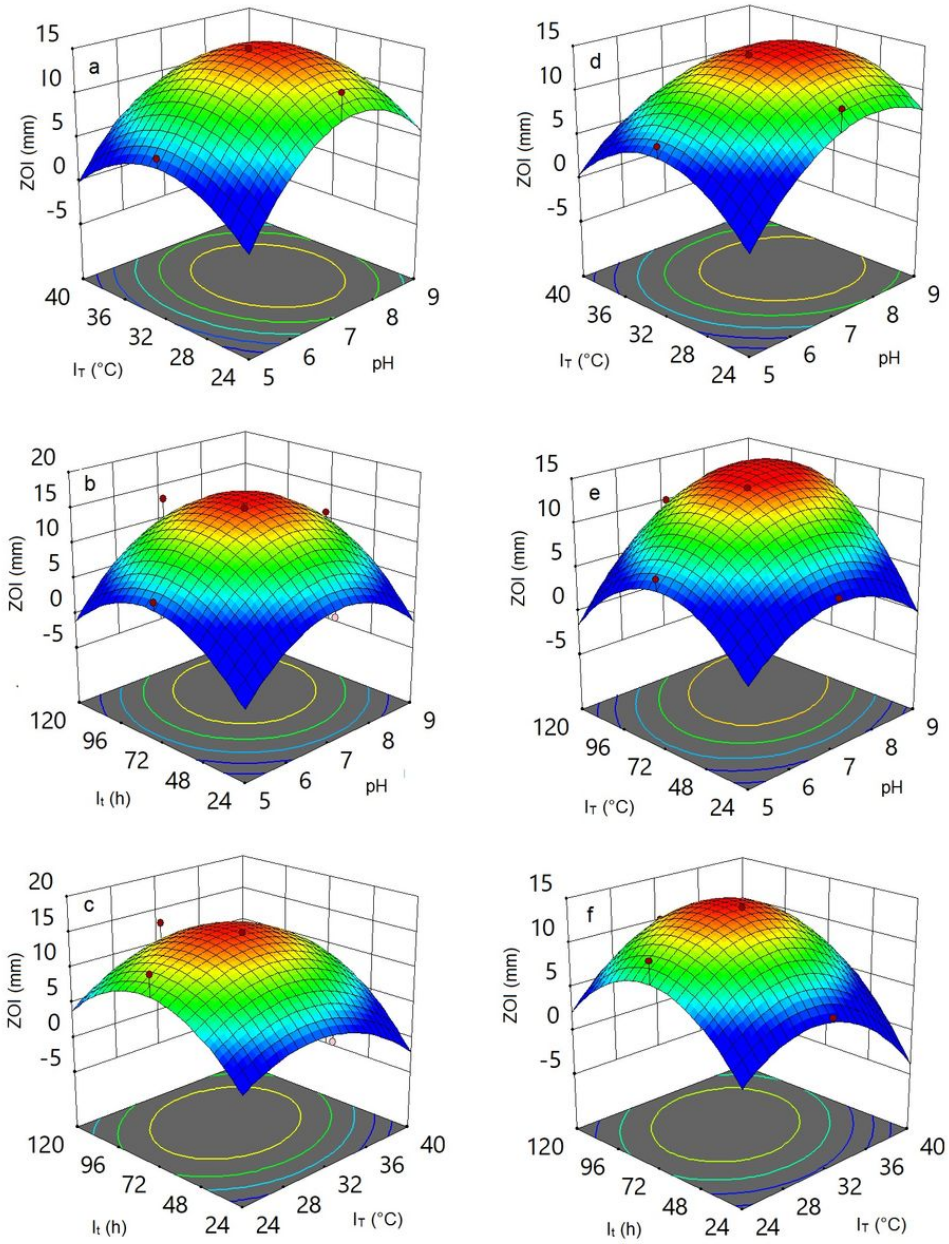

Figure 3

Three-dimensional response-surface plots of anti-pathogenic activity of metabolic extracts of rhizobacteria of *P. hysterophorus* at selected levels of culture variables

a-c) Yellow-colored bacterial extracts against *P. aeruginosa*

d-f) Yellow-colored bacterial extracts against *E. coli*

*I_T: Incubation temperature, I_t: Incubation time

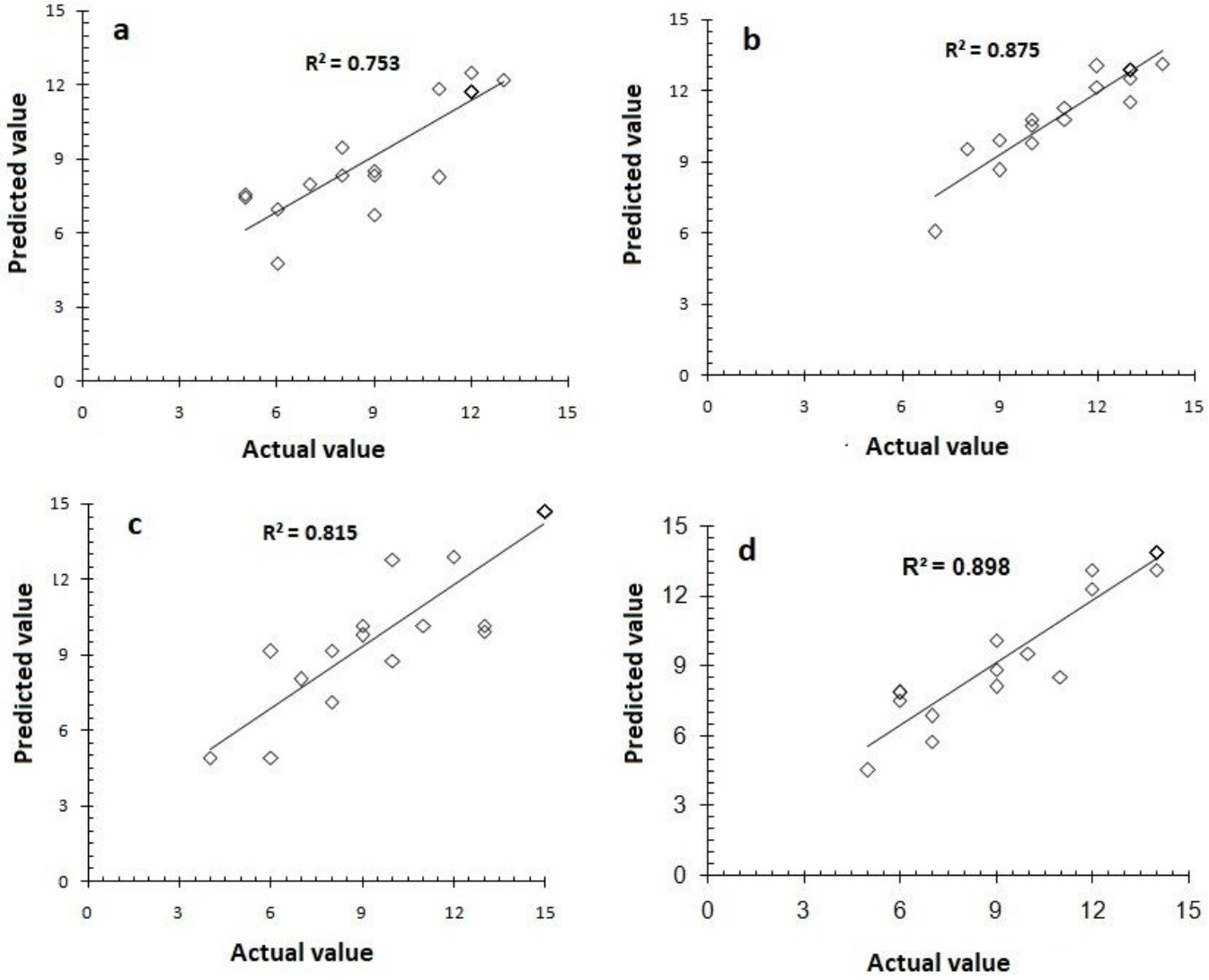

Figure 4

Correlation between the actual and predicted values of anti-pathogenic activity of metabolic extracts of rhizobacteria

a) Pink-colored bacterial extracts against *P. aeruginosa*

b) Pink-colored bacterial extracts against *E. coli*

c) Yellow-colored bacterial extracts against *P. aeruginosa*

d) Yellow-colored bacterial extracts against *E. coli*