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Medium Optimization Studies for the Production of Anti-*Xanthomonas Axonopodis* Compounds by *Bacillus* GS1

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Abstract
In all 87 bacterial isolates were obtained from different soil samples out of which nine *Bacillus* isolates inhibited the growth of bacterial pathogen of oily spot disease, *Xanthomonas axonopodis*. A potent *Bacillus* isolate, GS1 produced 34 mm of inhibition zone against the pathogen. Production optimization was done on the basis of pH, temperature, aeration and agitation. Medium optimization was done by Plackett-Burman design using a basal medium (level 0) that consisted of ingredients (g%, w/v): malt extract, 2; peptone, 0.3; K_2HPO_4, 0.25; glucose, 1; yeast extract, 0.2. The response was recorded in terms of inhibition zone diameter (mm) produced by the respective inoculated cell free supernatant. Analysis of the data was based on determination of parameter estimate values of each factor and Pareto chart. Two medium ingredients viz., K_2HPO_4 (+37.5) and malt extract (-15) showed highest estimate values evidenced by Pareto chart. Thus, these two most influential factors were further tested using full factorial study, where K_2HPO_4 and malt extract settings were tested in an increasing and decreasing order, respectively. This study revealed the optimum concentrations of malt extract and K_2HPO_4 to be 1.0 and 1.5 g %, respectively. The antibacterial compounds from the culture supernatant were extracted using different solvents and highest recovery was obtained in chloroform. These antibacterial compounds would be useful for the preparation of formulations that may be effective in the management of bacterial blight of pomegranate. This study is important towards development of biocontrol agents for its effective application in agriculture.

Keywords
Factorial design; Plackett-Burman; Bacterial blight; Biocontrol

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INTRODUCTION
Microbial disease of fruit crops is one of the major global problems of the fruit crop industry. There are several fruit crops grown in India that has market potential. Pomegranate is one of such cash crops grown in various parts of India and other regions of the world. Nowadays, pomegranate growers from India are facing serious problems of occurrence of fungal

and bacterial diseases. The species of *Aspergillus*, *Penicillium*, *Fusarium*, *Cladosporium*, *Chaetomella*, *Colletotrichum*, *Rhizopus* etc. are responsible for the disease such as fruit rot and fruit spot [1,2]. This leads to decrease in the value of pomegranates in the market. Bacterial blight of pomegranate is one of such severe infections of pomegranate that has caused huge loss to pomegranate industry in India. It is also called as oily spot disease which is caused by *Xanthomonas axonopodis* [3] and possibly by *Pseudomonas* sp. [4]. Farmers generally use synthetic chemicals for the management of these diseases; however, excessive use of it leads to environmental pollution and toxicity [5] and antibiotic resistance is a gaining problem too.

A recent approach gaining lots of importance nowadays is biological control of plant diseases. Biocontrol is the use of eco-friendly antagonistic microbes for the management of diseases of plants. It is an eco-friendly technology that is safe for the environment and most effective in the control of bacterial and fungal diseases of various crops. Several bacteria have been successfully used in the fields as biocontrol agents [6]. Most frequently used biocontrol agents are the species of *Bacillus*, *Pseudomonas*, *Enterobacter*, *Lysobacter*, *Lactococcus* etc. [7, 8]. Their antagonistic ability is due to the synthesis of antimicrobial substances such as organic acids, antibiotics and cyclic dipeptides [8, 9].

Considering the above-mentioned background, *Bacillus* sp. were isolated and screened for anti-*X. axonopodis* activity followed by medium optimization studies for the production of antimicrobials.

MATERIALS AND METHODS

Isolation of *Bacillus* sp. from soil samples

Soil suspensions were streaked on sterile nutrient agar plates by four quadrant method. The plates were incubated aerobically at 30°C for 24 h. After incubation, plates were observed for the presence of isolated colonies and twenty percent of the total colonies obtained on each of the nutrient agar plates were randomly picked on the basis of their morphology. Individual colonies were streaked on sterile nutrient agar slant and following incubation they were stored at 4°C in refrigerator for further study.

Screening for antibacterial microbes

The screening was also done by crowded plate technique and the colonies showing zone of inhibition were streaked separately on nutrient agar slant and stored till further use. These isolates and the isolates obtained as above were tested for antibacterial activity against *X. axonopodis* that was previously isolated from infected pomegranates. Agar overlay technique was used to determine the antibacterial activity of the bacterial isolates. Further, the selected potent isolates were grown in nutrient broth for 24 h at 30°C separately. The cell free supernatant (CFS) was prepared following centrifugation of inoculated broth at 10000 rpm for 10 min and filtration through 0.45 µm filter. The antibacterial activity of CFS was determined by disc diffusion method. After incubation, the inhibition zone diameters were measured.

Characterization of *Bacillus* sp.

The characterization of selected potent anti-*Xanthomonas Bacillus* isolates was done according to Bergey's Manual of Determinative Bacteriology.

Optimization of process parameters

Production optimization was done on the basis of pH, temperature, aeration and agitation as described earlier [7].

Medium Optimization

Screening Design - Medium optimization was done for the production of anti-*Xanthomonas* substances by selected strain of *Bacillus*. The optimization was done by Plackett-Burman (PB) design [10] using a basal medium (level 0) that consisted of following ingredients (g %, w/v): malt extract, 2; peptone, 0.3; K_2HPO_4, 0.25; glucose, 1; yeast extract, 0.2. In present investigation, PB design comprising of two-level factorial scheme was selected using MINITAB software. It consisted of eight trials for the evaluation of five factors (medium ingredients). The factor settings selected for the said investigation are shown in table 1 and eight trial PB design is shown in table 2. All the trials were carried out in triplicates. Eight different combinations of medium ingredients were prepared in a volume of 100 ml each in 250 ml flask. The flasks were inoculated with 0.1 ml of bacterial cell suspension (10^6CFU/ml). All the flasks were incubated at 30°C on shaker for 24 h. The CFS was prepared as described above and its antibacterial activity was determined using *X. axonopodis* as indicator bacterium by disc diffusion assay. The response of each trial was

determined in terms of inhibition zone diameter (mm) produced by CFS obtained from each trial.

Statistical analysis of the data – The response data was analysed by the determination of parameter estimate values of each factor and construction of Pareto chart. These results are also authenticated by statistical F-test to determine the effect of each factor.

One-factor-at-a-time – After the identification of most overriding factors, one-factor-at-a-time approach was used for further optimization studies. This involved testing of different concentrations of the identified factors and measuring the response values as described above.

Extraction of antibacterial compounds

The extraction of antibacterial compounds was attempted using different solvents viz., chloroform, ethyl acetate and ethanol. CFS in a volume of 50 ml was taken in a separating funnel of 250 ml capacity and equal volume of solvent was added separately. After vigorous mixing for one hour, the mixture was allowed to settle so as to obtain two layers. The organic phase was separated from it and concentrated in oven at 40^{o}C till complete dryness. One milliliter of respective solvent was then added to it and its antibacterial activity was determined by well diffusion assay. Respective solvent was used as control.

RESULTS

Isolation of *Bacillus* sp. from different sources

Bacterial cultures were isolated from aerial parts of different soil sources on nutrient agar. Bacterial colonies were selected on the basis of colony characters and Gram positive, rod shaped, and catalase positive cultures were maintained on nutrient agar slant at 4°C for further screening. In all 87 bacterial isolates were obtained. Out of these, 35, 13, 18 and 21 were isolated from garlic rhizosphere, coconut rhizosphere, pomegranate rhizosphere and garden soil, respectively.

Screening of B*acillus* sp. for antibacterial activity

The antibacterial activity was determined by well diffusion assay. Two most potent isolates, GR1 and GS1 produced maximum inhibition zones viz., 32 mm and 34 mm, respectively.

Medium optimization studies

The basal medium used for the production and factor settings are shown in table 1. An eight trial PB design was selected for the said analysis (Table 2). The response values (inhibition zone diameter, mm) of each trial are shown in table 3. The highest response value obtained was 34 mm.

The response curves for each factor were constructed. The medium ingredients, K_2HPO_4 and malt extract showed the maximum effect on response. The parameter estimates values of K_2HPO_4 and malt extract was significant. Other medium ingredients did show any significant effect on the response as shown in Fig. 2.

Further the Pareto chart of the estimates was constructed. The values of estimates are converted to percent and plotted as shown in figure 3. The sum of estimates was 55, which was considered as 100%. Thus, using Pareto chart the factors can be ranked as K_2HPO_4, malt extract and glucose, however, glucose is the least significant. These results were also confirmed by statistical F test as shown in table 4.

One-factor-at-a-time approach

Based on the results of PB design, the media were designed using settings for malt extract and K_2HPO_4 as shown in table 5. The other factors were kept at '0' level as shown in table 1. The trials were conducted using different combinations of these factors and response was evaluated as described earlier. From the response values as mentioned in table 5, it is obvious that the final medium composition should contain malt extract, 1 g % and K_2HPO_4, 1.5 g % for the maximum production of antibacterial compounds *Bacillus* GS1. The antibacterial compounds were extracted using several solvents as shown in table 5. Maximum extraction of antibacterial compounds was noticed in chloroform extract (table 5).

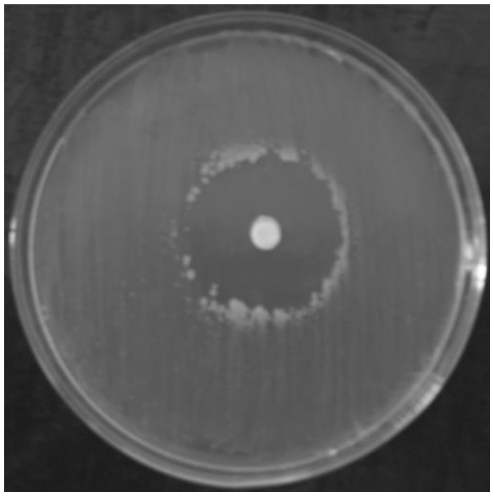
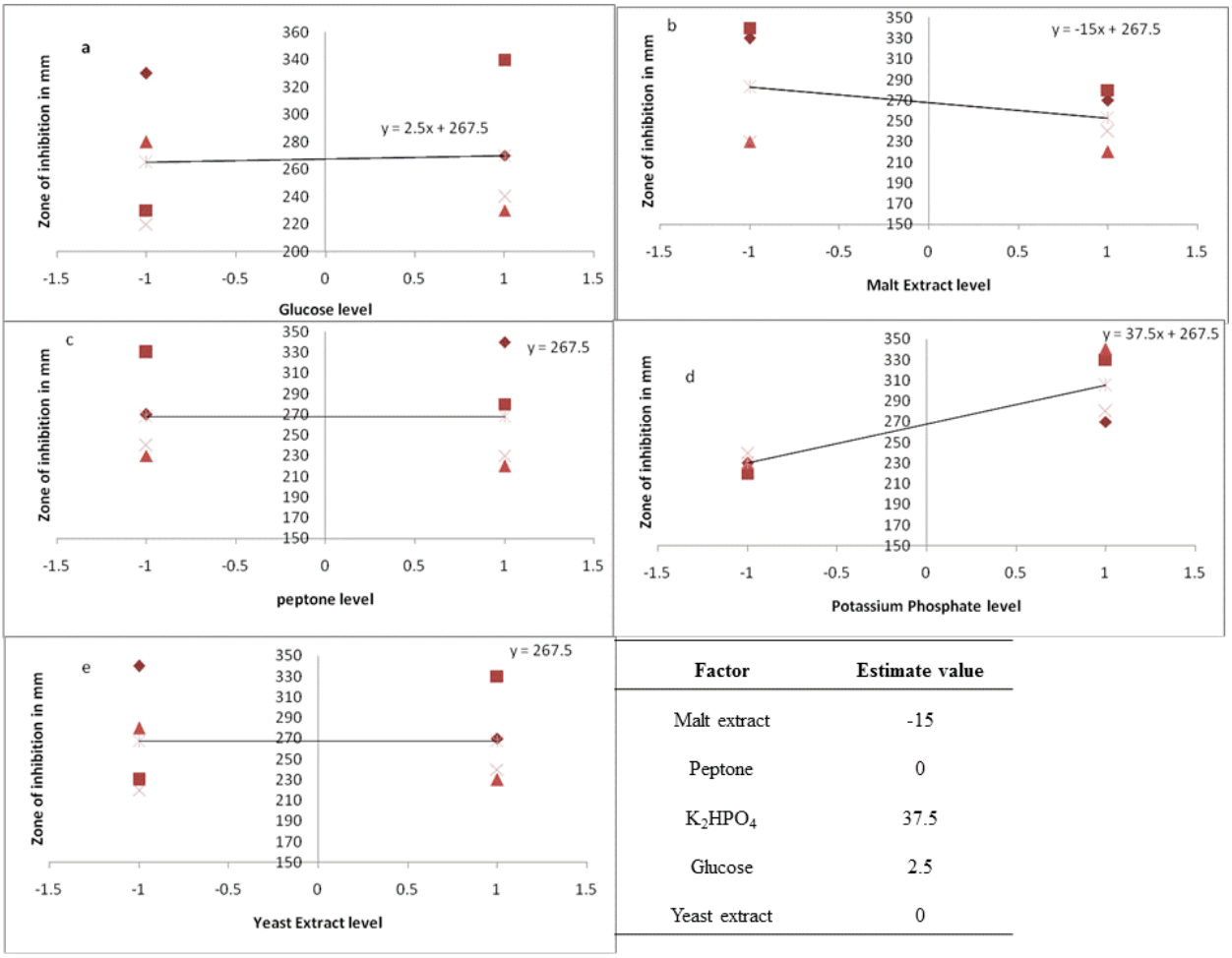


Figure 1: Zone of inhibition produced by CFS from *Bacillus* GS1 against *X. axonopodis*.



Factor	Estimate value
Malt extract	-15
Peptone	0
K_2HPO_4	37.5
Glucose	2.5
Yeast extract	0

Figure 2: Graph of inhibition zone diameter (response) verses glucose (a), malt extract (b), peptone (c), K_2HPO_4 (d), yeast extract (e) levels in Plackett-Burman study for antibacterial compounds production by *Bacillus* sp. GS1.

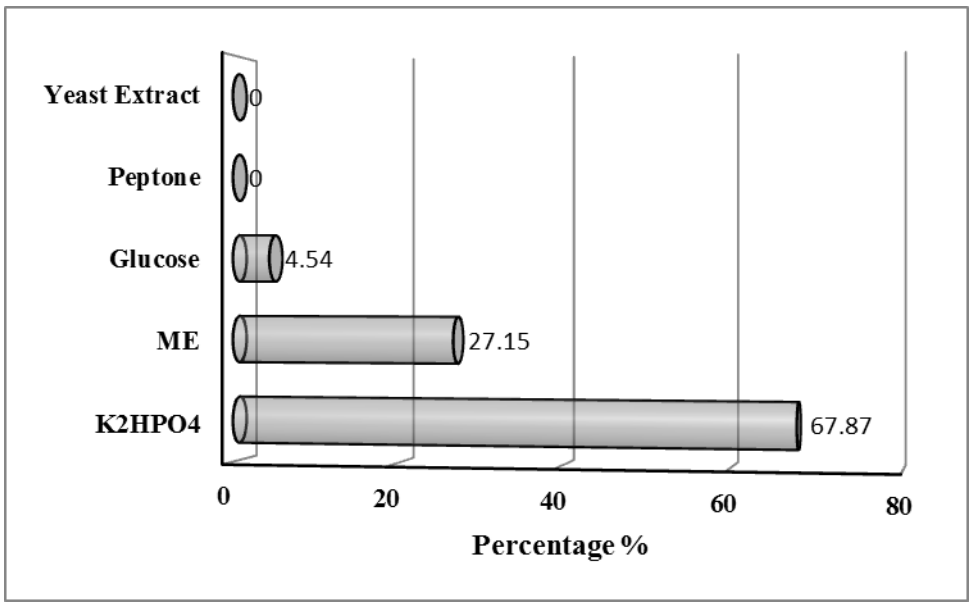


Figure 3: Pareto chart for Plackett-Burman parameter estimates for study of antibacterial compounds production by *Bacillus* sp.

Table 1: Factor settings selected for Placket-Burman design

Factor	Levels (g %) *		
	-1	0	+1
Malt extract	0.5	2	8
Peptone	0.075	0.3	1.2
K_2HPO_4	0.063	0.25	1
Glucose	0.25	1	4
Yeast extract	0.05	0.2	0.8

Table 2: Eight trial Plackett-Burman design for the study of five factors in antibacterial compounds production by *Bacillus* sp.

Trials	Malt Extract	Peptone	K_2HPO_4	Glucose	Yeast Extract
1	+1	-1	+1	+1	+1
2	-1	-1	+1	-1	+1
3	-1	+1	+1	+1	-1
4	-1	-1	-1	-1	-1
5	+1	+1	+1	-1	-1
6	+1	+1	-1	-1	+1
7	-1	+1	-1	+1	+1
8	+1	-1	-1	+1	-1

Table 3: Response of Plackett-Burman design for antibacterial compounds production by *Bacillus* GS1

Ingredients (g%) → Trials ↓	Malt Extract	Peptone	K_2HPO_4	Glucose	Yeast Extract	Response*
1	8.0	0.075	1.000	4.00	0.80	27
2	0.5	0.075	1.000	0.25	0.80	33
3	0.5	1.200	1.000	4.00	0.05	34
4	0.5	0.075	0.063	0.25	0.05	23
5	8.0	1.200	1.000	0.25	0.05	28

6	8.0	1.200	0.063	0.25	0.05	22
7	0.5	1.200	0.063	4.00	0.80	23
8	8.0	0.075	0.063	4.00	0.80	24

*** Output of a trial was determined in terms of inhibition zone diameter (mm) produced by corresponding CFS.**

Table 4: Analysis of responses of Plackett Burman study for *Bacillus* GS1.

	Malt Extract	Peptone	K_2HPO_4	Glucose	Yeast Extract
∑(H)	1010	1070	1220	1080	1070
∑(L)	1130	1070	920	1060	1070
Difference	-120	0	300	20	0
Effect	-30	0	75	5	0
Mean Square	1800	0	11250	50	0
F-Test	1800	0	11250	50	0

Table 5: Response data of one-factor-at-a-time approach.

Malt Extract (g %)	K_2HPO_4 (g %)	Response*
0.5	0.5	21
	1.0	21
	1.5	30
1.0	0.5	21
	1.0	33
	1.5	36
1.5	0.5	22
	1.0	21
	1.5	31

*** Inhibition zone diameter (mm)**

Table 5: Extraction of antibacterial compounds from *Bacillus* GS1 by different solvents

Zone of Inhibition (mm)*					
Solvent extract			**Solvent control**		
Chloroform	**Ethyl Acetate**	**Butanol**	**Chloroform**	**Ethyl Acetate**	**Butanol**
35	20	0	8	6	10

*** Determined by well diffusion assay using *X. axonopodis* as indicator**

DISCUSSION

It is well documented that *Bacillus* genus has ability of production of various antimicrobial substance [11, 12]. *Bacillus* can be isolated from various sources such as compost [13], soil [14] and rhizosphere. In presence study, we isolated bacterial cultures from garden soil and aerial surfaces of Pomegranate, Garlic, Gram, Coconut root which were identified as *Bacillus* sp. after preliminary examination. The antibacterial activity was determined by well diffusion assay using *Xanthomonas axonopodis* as indicator organism. *Bacillus* GS1 produced 34 mm of inhibition zone and was identified as the most potent strain.

Further medium optimization studies were carried out using Plackett-Burman design [10]. Plackett-Burman designs comprise one type of two-level screening design. When more than five independent variables are to be investigated, the Plackett-Burman design is used to find the most important variables in a system, which is then optimized in further studies by one-factor-at-a-time approach or full factorial design. In present investigation, eight trial design for the evaluation of five variables was selected using a basal medium as shown in table 1. The response curves generated in figure 9 revealed the maximum effect of K_2HPO_4 and malt extract on the response. The parameter estimate value for K_2HPO_4 was +37.5, which

means the response (inhibition zone) increases with increase in the concentration of this ingredient from 0.063 to 1 g %. However, the parameter estimate value for malt extract was found to be -15, which means the response increases with decreases in the concentration of this ingredient from 8 to 0.5 g %. Pareto chart of estimates were constructed to judge the relative importance of the factors (figure 3). Pareto chart displays the magnitude (relative importance) of each factor and it is the convenient way to view the results of Plackett-Burman experiment. Thus, from figure 3, the factors can be ranked as K_2HPO_4, malt extract, glucose, peptone and yeast extract. However, glucose, peptone and yeast have negligible effect. Thus, further optimization was done by increasing the concentration of K_2HPO_4 and decreasing the concentration of malt extract using one-factor-at-a-time approach as mentioned in table 5. However, these studies may be further extended for the evaluation by constructing contour plots using response surface methodology (RSM) [15].

Pomegranate fruit crop is cultivated in different regions of India and other parts of the world. Pomegranates from Maharashtra have export value. Recently, pomegranate industry is in trouble due to a disease, bacterial blight, commonly called as oily spot disease. It has caused major losses in current years in Maharashtra and Karnataka, India [1]. *X. axonopodis* attacks all the aerial plant parts that includes leaves, fruits and stem which results in huge production and market loss. Considering above background, this bacterial pathogen was selected as an indicator organism for the screening of antibacterial *Bacillus* sp. Thus, production optimization studies are important for the mass production of antibacterial substances by *Bacillus* GS1. These product formulations may be useful in the management of bacterial blight of pomegranate under field conditions.

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