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SHORT COMMUNICATION

Studies and Enhancement of Natural Bioactive Compounds Content through Media Optimization

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Abstract

In these studies, endophyte bacteria were isolated from a medicinal plant root of *Adhathoda beddomei (adosa).* The antimicrobial activity was demonstrated against different pathogenic and nonpathogenic bacteria. Identification of endophytes was done based on external morphological characteristics with the help of a Scanning Electron Microscope (SEM). another challenging problem in this regard is that the efficiency of the endophytic bacteria to produce bioactive compounds is not as high as expected because, in many previous works in literature, it has been reported that yields are generally meagre. Through this study, we have tried to enhance the production of bioactive metabolites using media optimization and Resonance Surface Methodology (RSM). These are generally from µg/l to less than mg/l and are therefore not yet appropriate for fermentative production on an industrial level.

Keywords: Antimicrobial Properties, Media Optimization, Scanning of Electron Microscopy, Secondary Bioactive Metabolites

1. Introduction

1.1 Secondary Bioactive Metabolites

Secondary bioactive metabolites are organic substances made by bacteria, fungi, or plants. These bioactive metabolites do not directly show signs of an organism's typical growth, development, or reproduction. A small number of species within a phylogenetic group are frequently the only ones that can produce certain specific secondary metabolites. Secondary metabolites frequently play a significant role in plants' defence against herbivores and other intermittent motions, according to numerous observations[1]. Secondary metabolites have been used by humans as a variety of medications, tastes, colours, and recreational drugs for many years. Secondary bioactive metabolites support a host in crucial ways like defence, competition, and interspecies interactions, but they are not crucial for survival. Secondary metabolites have significant specificity in characterizing quality. Secondary metabolites typically belong to a single species[2]. According to research, secondary metabolites have varying effects on various species. *Adhatoda beddomei* was chosen as the endophytic bacteria from the medicinal plant since the current investigation was largely focused on the examination of the properties of bioactive metabolites. It was also described as a bioactive metabolite that may have some pharmacological potential[3]. This fact is supported by the claim that endophytic bacteria, of which *A. beddomei* were chosen, defend their host plants from a variety of harmful situations[4]. To study differences in biological activity, the metabolites from the small-scale cultures of the bacterial isolates were extracted using the solvents ethyl acetate and n-hexane[5].

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2. Materials and Methods

2.1 Bioactive Metabolites' Antimicrobial Activity against Various Microorganisms

The Department of Microbiology Institute of Medical Science at BHU Varanasi in India measures the zone of inhibition that endophyte bacteria have been able to achieve against a variety of pathogenic bacteria (*Escherichia coli, Pseudomonas aeroginosa, Staphylococcus aureus, Penicillium chrysogenum, Lactobacillus lactis, Bacillus subtilis, Candida albican, Staphylococcus aureus, Bacillus megaterium, Helicobacter pylori, Corynebacterium amycolatum, Streptococcus pyogenes, Mycobacterium tuberculosis, Salmonella typhimurium, Streptococcus pneumonia, Staphylococcus epidermidis, Vibrio* sp., *Clostridium difficile,* and *Klebsiella pneumonia*)[6,7]. Endophyte bacteria produce highly antibacterial and antifungal bioactive compounds. Based on the outcomes of the first investigations, the top bacterial isolates were chosen as suitable strains for additional study. To prevent measurement error, three replicates of each experiment were kept[8].

2.2 Endophyte Bacteria Identification by Morphology

Taking advantage of the Scanning electron microscope (Hitachi S-570 SEM, Hitachi High Technologies, Tokyo, Japan) endophyte bacteria were investigated in the present study[9]. A single colony of the bacteria endophytes was placed in a 2-3% Glutaraldehyde solution for 3–4 hours at room temperature, washed for 15 minutes with 0.1 M phosphate buffer, and the process was repeated three times. The bacteria cell was dehydrated in various grades of ethanol (20, 40, 60, 70 and 85%) three times throughout 15 minutes in the following phase[10]. Finally, acetone is used to wash the bacterial cell for 3 minutes. Following this, the cells were dried with CO_2 assistance, and mounted on aluminium stubs, and various images of bacteria were created using a scanning electron microscope connected to a computer to investigate its shape[11].

2.3 Optimization Conditions of Bioactive Microbial Metabolites Formation

Attempts were optimizing the culture conditions such as incubation period, pH, temperature, carbon-nitrogen sources, with different concentrations, and RSM. Through various incubation times, different temperatures, and pH, it has also tried to explain what effect they all have on the production of metabolites. Various carbon sources such as glucose, maltose, sucrose, starch, fructose, and lactose influence the production of bioactive metabolites and cell biomass formation of endophytic bacteria. In this, a separate set was prepared for each carbon. Each set had a particular content, these contents are as follows (g/L) (Starch 10.0g, Glucose 10.0g, Beef extract 2.0g, Soyabean meal 10.0g, NaCl 4.0g, K_2HPO_4 0.5g, $MgSO_4.7H_2O$ 0.5g, $CaCO_3$ 2.0g, Casein 3.0g). Only one carbon source was used in each set. Here carbon compounds were added to starch casein medium in a 1% (w/v) concentration, with $NaNO_3$ (3g/L) being used as a supplement. In this medium, 10% of the bacteria inoculum (seed media, which had been prepared beforehand) was added. After adding the inoculum, each set was kept at 35**°C** temperatures and 180 rpm for 92 hrs. The final pH of the medium was adjusted to 7.2[12]. After the complete incubation period, centrifuged the cell at 10,000 rpm for 5 minutes and separated the bacterial cells. After centrifuging, the bacterial cell was dried at a temperature of 70°C until the cell was completely dried. Then measured dry cells in g/L and different glucose concentrations were used in the medium of this experiment. (0.5, 1, 1.5, 2.0, 2.5%. w/v). Fermentation was performed in 1L Erlenmeyer flasks (for each concentration) with constant shaking at 180 rpm for 92 hrs. with the same media composition (Each set had the same media composition but the concentration of glucose was different). The final temperature and pH of the medium were adjusted to 35**°C**, 7.2. and different types of Carbone sources were used, such as, KNO_3, $NaNO_3$, NH_4Cl, NH_4NO_3, beef extract, Peptone and Yeast extract were used. In this also have used the same condition and composition of media. The differential concentration was used of $NaNO_3$; these concentrations ranged from 0.1 to 0.7% (v/w) in 1L it was found that different levels of $NaNO_3$ produce bioactive metabolites from different levels and form cell biomass. And pH levels of the starch casein broth media were adjusted from 3 to 12 and the isolates were grown for 92 hrs. at 180 rpm and 35**°C**. In this study also, different sets were prepared for different pH, that to from the same media composition and condition. The process

was adopted as described above to produce biomass and bioactive metabolites. (Effect of different $NaNO_3$ concentrations on biomass and bioactive metabolite production)[13,14]. Similarly, the optimum temperature for the antimicrobial bioactive metabolite production was determined by incubating the endophytes bacterial strain at temperatures ranging from 20 to 60°C, while maintaining all other conditions at optimum levels[15]. The isolates were inoculated into the starch casein broth medium and incubated for up to 08 days in a rotary shaker at 180 rpm at 35°C and centrifuged the first set a day later and determined the quantity of cell biomass and bioactive metabolites by the method given above. The sets of the second, third and eighth days were also determined in this way respectively. Through these studies, the minimum number of experimental runs, a complete factorial Central Composite Face-Centered Design (CCFD) with four free changeable and their combinations were used to optimize the response with the region of three-dimensional observation spaces. Design proficient software (Version 7.0 State-Ease, Inc., USA) was used to design the experiments for bioactive secondary metabolites production. Using RSM, most variables (A, B, C and D) at their optimum levels were recognized for the highest response in conditions of antimicrobial activity of bioactive metabolites considered as the diameter of the zone of inhibition. The four independent variable parameters are as follows, Incubation Temperature, pH, Carbon source, and Nitrogen source. A total of 32 experiments were obtained using the following equation that has 24 full factorial CCD for four variables comprising 16 factorial points, 08 axial points, and 06 replicates:

$$N = 2n + 2n + nc = 24 + 2\ X\ 5 + 6 = 32$$

Where, N is the total number of experimental runs to be performed, n is the number of variables (factors), and nc is several replicates at center points. The central coded value of all variables is zero. The low and high range of every variable used in RSM and the absolute experimental plan with values in the definite and coded form are indicated. Statistical analysis: To determine the analysis of variation, a statistical analysis of the model was performed (ANOVA). Correlation coefficients (R2), adjusted determination coefficients (Adjusted-R2), root mean square errors (RMSE), and Absolute Average Deviations (AAD) between experimental and predicted data were performed to assess the fit and prediction accuracy of the model created[16].

The subsequent degree polynomial equation was used with every changeable to generate an empirical model which correlated the response (bioactive metabolites production) to four variables.

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \left(\sum_{i=1}^{n} \beta_{ii} X_i\right)^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{ij} X_i X_j$$

where, Y is the predicted response, β_0 is the intercept coefficient, β_i is the linear coefficient, β_{ij} are the interaction coefficients, β_{ii} are the quadratic coefficients, and X_i and X_j are coded values of the five additive variables.

3. Results and Discussion

3.1 Bioactive Metabolites' Antimicrobial Activity against Various Microorganisms

Figure 1 and Table 1 show the results of the antibacterial activity and the diameter of the inhibition zone of the bioactive metabolites of the 24 human pathogenic bacteria mentioned above. The maximal zone of inhibition against the bacillus subtilis is indicated by the metabolites of the endophyte's bacteria (EFB-3)[17]. It is the root of many infectious diseases that affect people. *E. coli O157:H7 (200.8)*, which is the cause of numerous illnesses. The bioactive metabolite of EFB-03 was coded as 1 in this experiment, while the bioactive metabolite of EFB-6 was coded as 2.

3.2 Identification of Endophyte Bacteria

The form of endophyte bacterial cells is often between a coccus and a rod, and they are colonized on Nutrient Agar Media (NAM). Microscopical (scanning electron microscope) examinations of the bacterium isolate revealed slightly rough cell surfaces, greyish-black cell mass and the size of bacterial cells is 8.5µm[18]. Figure 2 in the text below illustrates morphological traits. These typical characteristics have allowed for partial identification of the bacteria (EFB-3).

3.3 Media Optimization

3.3.1 Effect of Different Carbon Sources on the Formation of Bioactive Metabolites

To optimize the production of antimicrobial agents, after the end of the incubation period, it was found

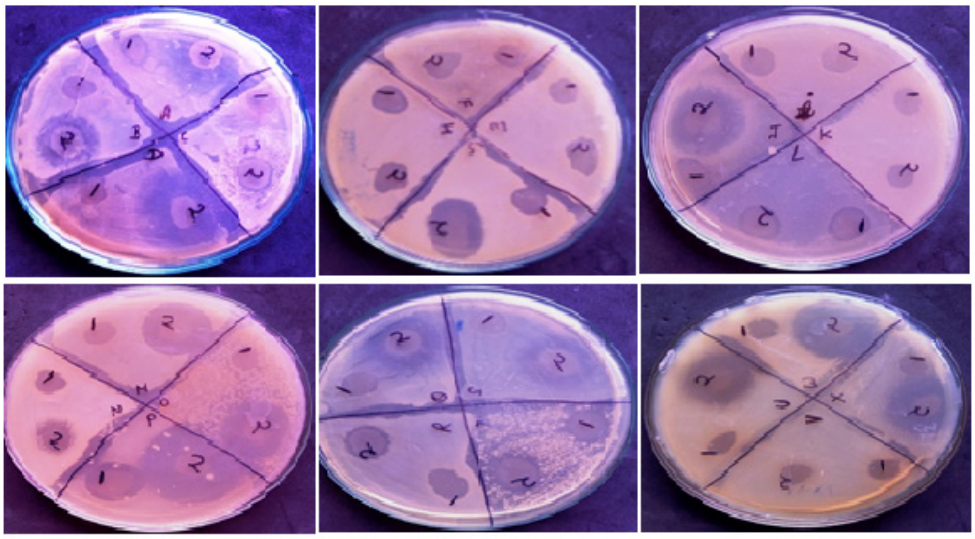

Figure 1. Bioactive metabolites of 2 strains [(1- EFB-03) and (2- EFB-06)] were tested against 24 human pathogenic bacteria's (in each plate, have 04 pathogenic bacteria).

Table 1. Sizes of the bioactive metabolite's inhibitory zone around various human pathogenic microorganisms

Pathogenic Bacteria's	The Size of the inhibitory zone in (mm)	
	EFB-02	EFB-03
E. coli O157:H7	18±0.8	20±0.8
B. cereus	1.01±0.8	4.0±0.7
B. subtilis	12±0.9	21±0.4
Megatherium	8.6±0.4	16.0±0.2
Staphylococcus aureus	06±0.5	19±0.6
Helicobacter pylori	07±0.21	14±0.8
Multiple drug resistant *S. aureus*	01±0.33	03±0.3
Streptococcus pyogenes	03±0.31	07±0.6
Corynebacterium amycolatum	08±0.1	14±0.5
Chlamydia pneumonia	12±0.4	17±0.6
S. epidermis	09±0.4	16±0.7
Mycobacterium tuberculosis	07±0.13	11±0.11
Pseudomonas aeruginosa	3.43±0.7	5.31±0.9
Clostridium difficile	10±0.51	14.50±0.83
Klebsiella pneumonia	16±0.31	20±0.32
Corynebacterium diphtheria	14±0.48	17.05±0.71
Vibrio sp.	09±0.64	11±0.11
Streptococcus pneumonia	12±0.25	16±0.63
Staphylococcus epidermidis	14±0.86	18±0.5
S. epidermis	14±0.84	18±0.51
Salmonella typhimurium	13±0.63	15±0.58
Salmonella typhi	09±0.49	11±0.17

that glucose is an excellent carbon source under which endophyte bacteria were producing the maximum bioactive metabolites. The bioactive metabolites produced in the presence of glucose were bioactive metabolites showing the maximum zone of inhibition against various bacteria and fungi. Achieving the maximum area of inhibition against various bacteria and fungi by bioactive metabolites means that the maximum production of bioactive metabolites is occurring, and it also shows that the maximum formation of cell biomass (2.2 gm/L) also occurs in the presence of glucose. Production of bioactive metabolites and cell biomass formation was found to be very poor in the presence of fructose. Because the bioactive metabolites produced in the presence of fructose received very little zone of inhibition against various bacteria and fungi and there is also a minimum formation of cell biomass (0.6 gm/L) (Figure 3).

3.3.2 *Effect of Different Glucose Concentrations on the Production of Bioactive Metabolites*

In this experiment, the glucose concentration of 15gm/L showed remarkable results followed by 20 gm/L and 22.5 gm/L concentrations. At 1.5% concentration of glucose gets maximum bioactive metabolite production and formation of cell biomass. The bioactive metabolites produced in the presence of 1.5% glucose concentration give a maximum zone of inhibition against different bacteria (The 23 mm zone of inhibition is that of

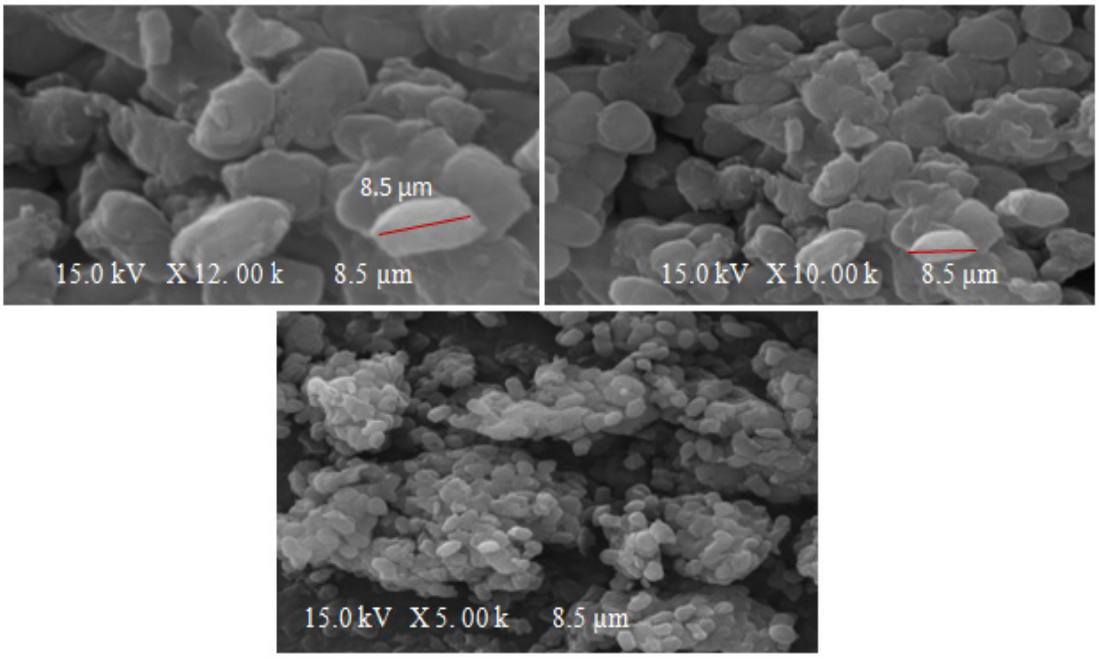

Figure 2. Endophytes bacteria (EFB-03) were taken in this image using a Scanning Electron Microscope (SEM).

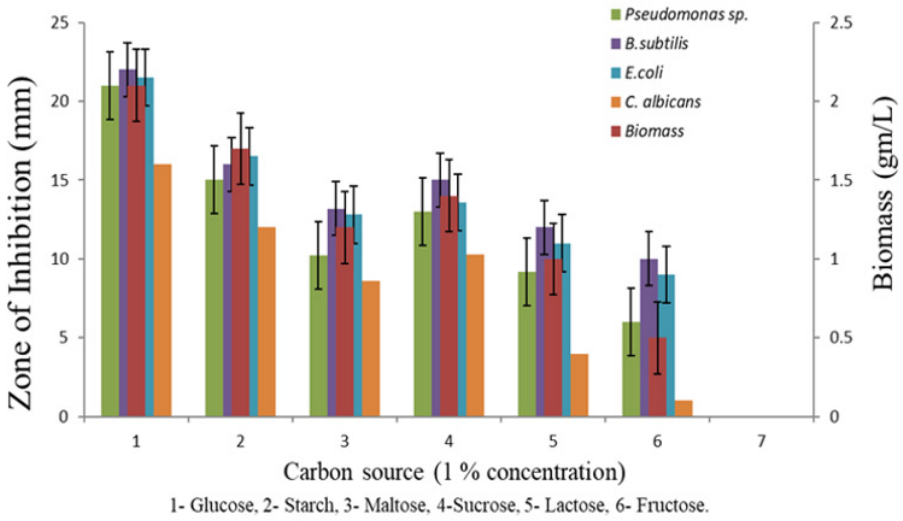

Figure 3. Effect of different carbon sources on for the production of bioactive metabolites.

B. subtilis) and fungi (the 17mm zone of inhibition is *C. albicans*). At 0.5 and 2.25% concentration of glucose, very little bioactive metabolite was produced and cell biomass was formed (Figure 4).

3.3.3 *Effect of Different Nitrogen Sources and Concentration for the Formation of Bioactive Metabolites*

From these studies, sodium nitrate ($NaNO_3$) is the best nitrogen source for improved production of antimicrobial metabolites, followed by potassium nitrate and ammonium chloride (Figure 5). At a concentration of 0.5% of $NaNO_3$ the maximum zone of inhibition (maximum zone inhibition means maximum bioactive metabolites production and cell biomass formation) was observed against *B. subtilis* and *E. coli* (Figure 6). At 0.5% concentration of $NaNO_3$, maximum bioactive metabolites were produced and cell biomass was formed. Whereas at 0.2, 0.3 and 0.6% concentrations of $NaNO_3$, endophyte bacteria were

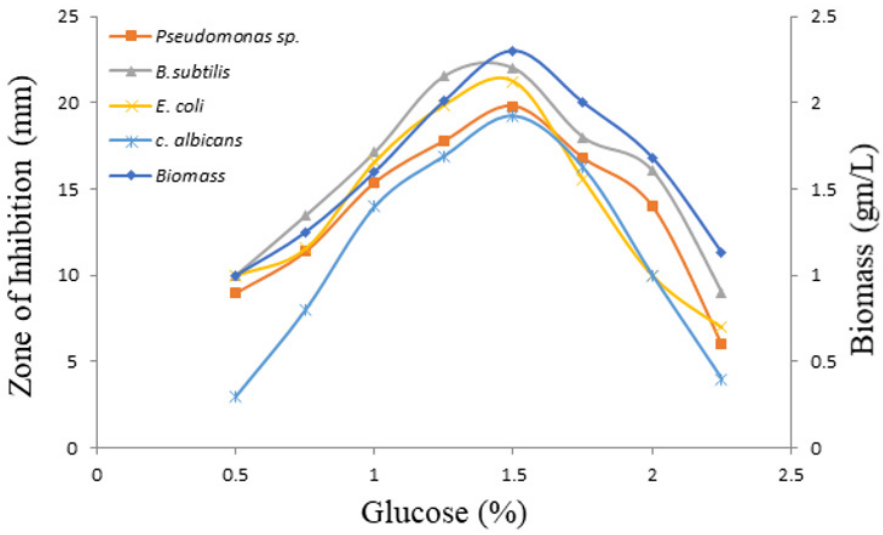

Figure 4. Production of bioactive metabolites and formation of cell biomass at various glucose concentrations.

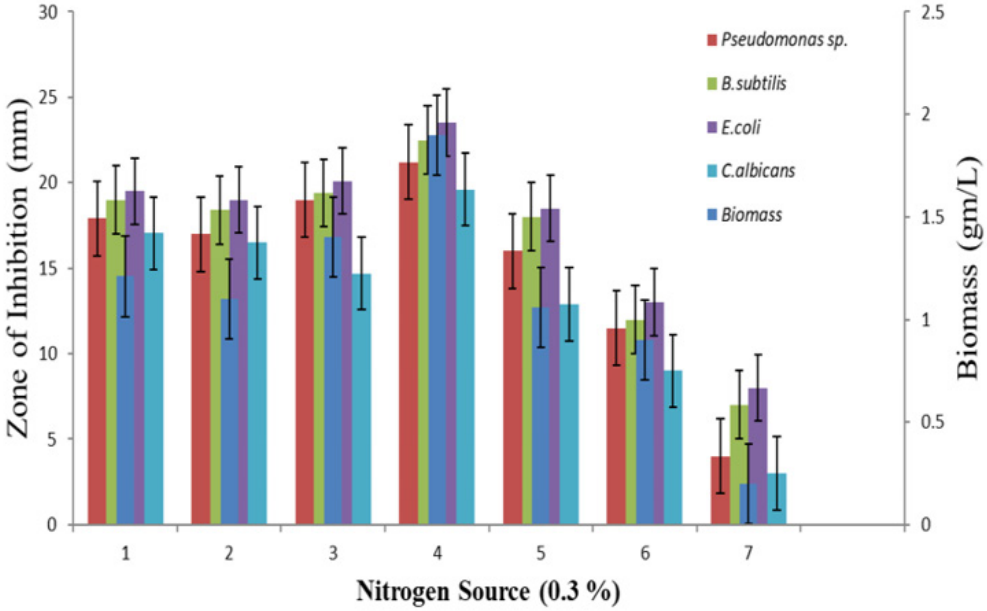

Figure 5. Effect of different nitrogen sources on antimicrobial metabolites production.

producing bioactive metabolites up to mid-level. The production of minimal bioactive metabolites was found at a 0.7% (w/v) concentration of $NaNO_3$.

3.3.4 *Effect of Different pH, Different Incubation Temperature (°C), and Different Incubation Periods for the Formation of Bioactive Metabolites and Cell Biomass Formation*

The production of bioactive metabolites and the formation of cell biomass were received in good concentration. EFB-03 found that the medium's initial pH of 7 was ideal for promoting growth and the formation of bioactive metabolites (Figure 7). The maximum zone of inhibition in a way indicates the production of maximum bioactive metabolite and cell biomass formation. (2.3 gm/L). Bioactive metabolites of pH 7 showed maximum zone of inhibition (22, 23 mm in diameter) in against *E. coli* and *B. subtilis*. Although initial pH 4, 5, 9 and 10 also supported biomass formation and bioactive metabolite production a lower

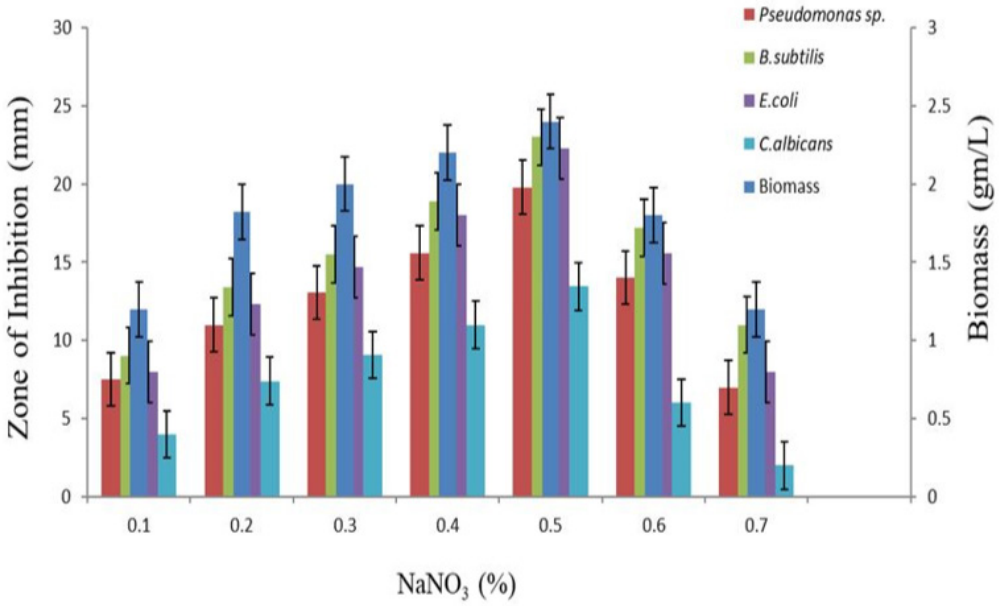

Figure 6. Effect of various concentration of $NaNO_3$ on production of antimicrobial metabolite.

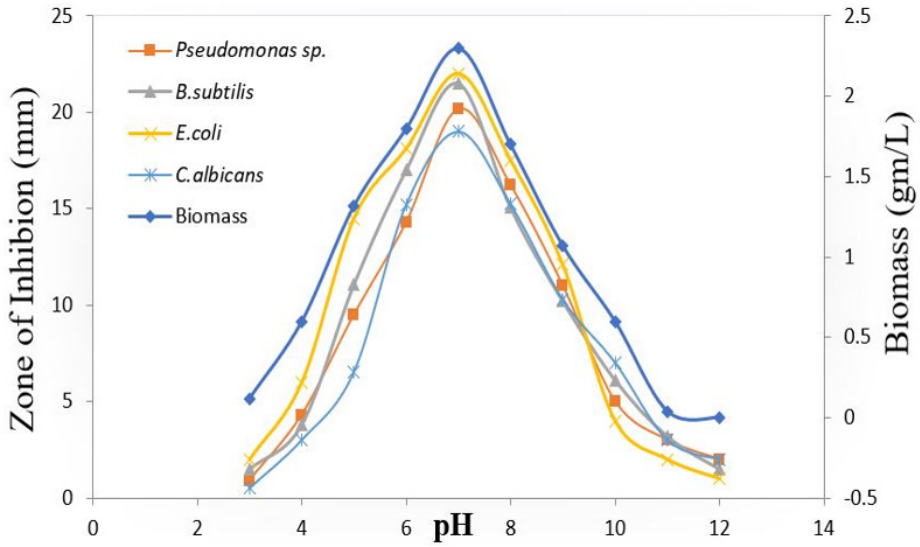

Figure 7. Effect of initial pH on antimicrobial bioactive metabolites production.

yield was observed. No concentration of cell biomass and bioactive metabolites was obtained on this pH (3, 11, and 12)[19]. It was reported by Metin Digrak[20] that the highest production of biomass was at pH 7.5. While the production and formation of bioactive metabolites and cell biomass at pH 11, and 12 was negligible. The production level of bioactive compounds was observed at different temperatures like 20, 25, 30, 35, 40, 45, 50, 55 and 60°C by EFB-03. Maximum growth of cell biomass and bioactive metabolite production by isolated EFB-03 strain were recorded on incubation temperature 35°C (±2) and it was followed by 40, and 45°C, respectively (Figure 8). The maximum zone of inhibition indicates maximum bioactive metabolite production and cell biomass formation (2.4 gm/L). Bioactive metabolites of incubation temperature 35°C (±2) are evidence for the maximum zone of inhibition (21, 22 mm in diameter) against *E. coli* and *B. subtilis*. Different amounts of bioactive metabolites and cell biomass were obtained based on different incubation periods. The maximum production of bioactive metabolites and cell biomass formation was achieved on the third day (Figure 9).

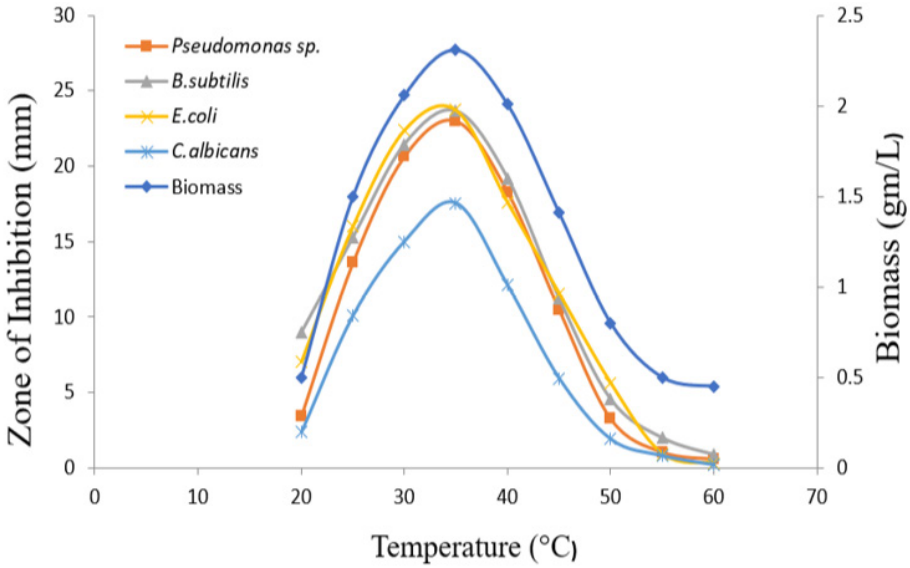

Figure 8. Effect of different incubation temperature on production of antimicrobial.

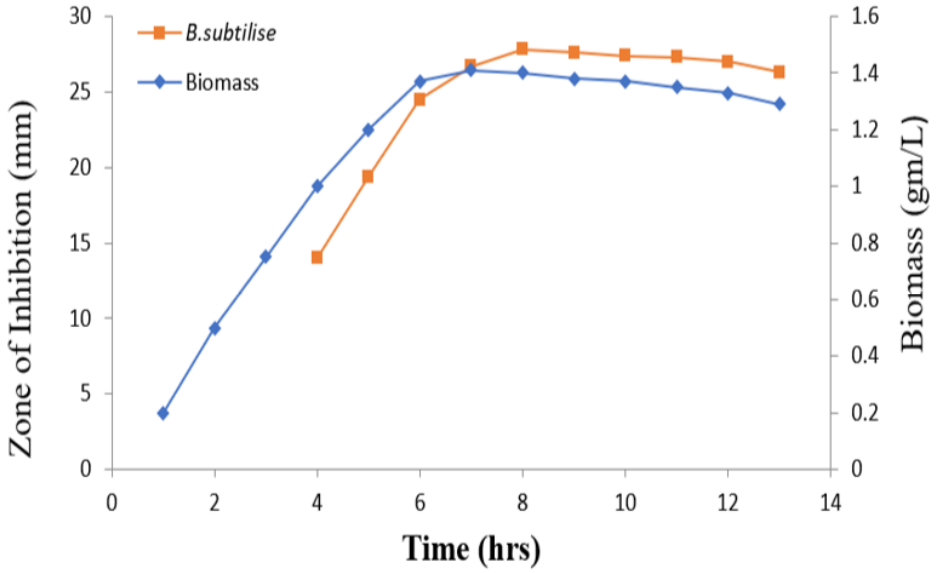

Figure 9. Effect of different incubation period on antimicrobial metabolites production.

The bioactive metabolites of the third day indicated a maximum zone of inhibition (means maximum production of bioactive metabolites and cell biomass formation) towards *B. subtilis*.

3.4 Response Surface Methodology

The response surface interpreting the quadratic effect of pH and temperature on bioactive metabolite production and cell biomass formed by endophytes bacteria (EFB-03) is shown in Figure 10- A, B, C, D, E and F. In the case of bioactive metabolite production and cell biomass formation from EFB-3, all the parameters nitrogen and carbon concentration exerted the most significant effect. The cell biomass model showed F-value conforming to 3.023 demonstrating the significance of the model with a p-value of <0.022 Table 2. And bioactive metabolite production from endophytes bacteria (EFB-3), the parameters nitrogen and carbon concentration exerted the most significant effect. These three sets of experiments yielded an average biomass production of 3.18gm/L at pH 6.89 temperature 29.49°C carbon concentration of 2.298

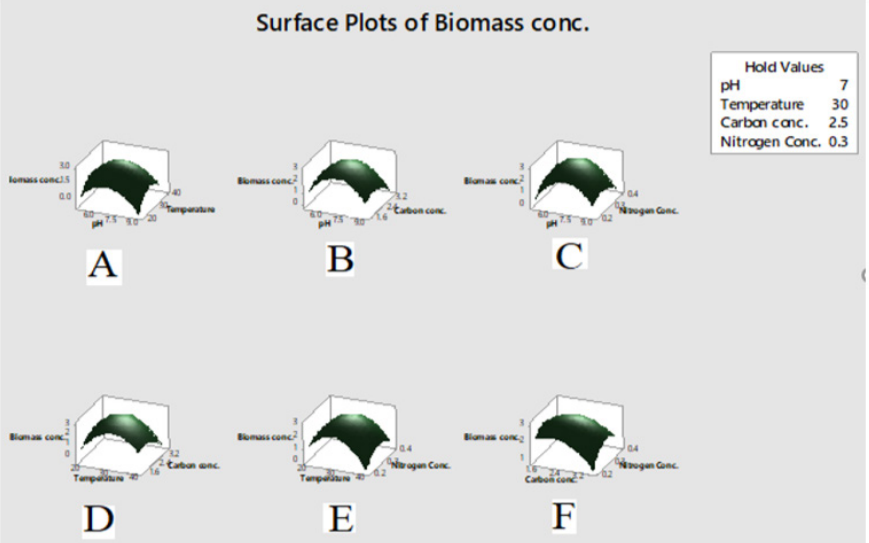

Figure 10. Response surface plots (3-dimensional view) showing interactive effects of selective variables on cell biomass formation. **A.** Effect of pH and temperature on the Cell Biomass formation using endophytes bacteria (EFB-03). **B.** Effect of pH and carbon concentration on the Cell Biomass formation using endophytes bacteria (EFB-03). **C.** Effect of pH and nitrogen concentration on the Cell Biomass formation using endophytes bacteria (EFB-03). **D.** Effect of Temperature and carbon concentration on the Cell Biomass formation using endophytes bacteria (EFB-03). **E.** Effect of Temperature and nitrogen concentration on the Cell Biomass formation using endophytes bacteria (EFB-03). **F.** Effect of carbon and nitrogen concentration on the Cell Biomass formation using endophytes bacteria (EFB-03).

Table 2. ANOVA for the Quadratic response surface model derived from the experimental designs

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	3.023	0.140	21.57	0.000	
Blocks					
1	0.1695	0.0657	2.58	0.022	1.00
pH	-0.1067	0.0692	-1.54	0.46	1.00
Temperature	-0.1192	0.0692	-1.72	0.107	1.00
Carbon conc.	-0.1642	0.0692	-2.37	0.033	1.00
Nitrogen Conc.	-0.0350	0.0692	-0.51	0.021	1.00
pH*pH	-0.4375	0.0648	-6.75	0.000	1.05
Temperature*Temperature	-0.4425	0.0648	-6.83	0.000	1.05
Carbon conc.*Carbon conc.	-0.1862	0.0648	-2.88	0.012	1.05
Nitrogen Conc.*Nitrogen Conc.	-0.2087	0.0648	-3.22	0.006	1.05
pH*Temperature	0.0662	0.0848	0.78	0.448	1.00
pH*Carbon conc.	-0.0475	0.0848	-0.56	0.584	1.00
pH*Nitrogen Conc.	-0.0812	0.0848	-0.96	0.354	1.00
Temperature*Carbon conc.	-0.1413	0.0848	-1.67	0.118	1.00
Temperature*Nitrogen Conc.	0.0750	0.0848	0.88	0.391	1.00
Carbon conc.*Nitrogen Conc.	0.0812	0.0848	0.96	0.354	1.00

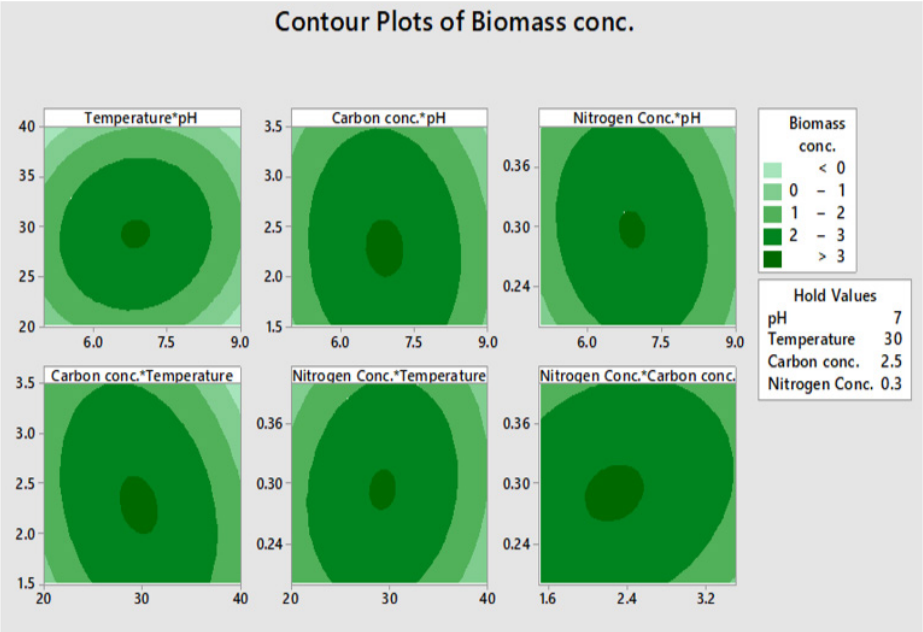

Figure 11. Contour plots of cell biomass formation of endophyte bacterial isolate.

gm/L and nitrogen concentration of 0.29 gm/L after 3 days of incubation. The lack of fit test values from the sequential model is appropriate for the quadratic model, illustrating two significant effects of fit. Adjusted R^2 and Predicted R^2 for this quadratic model came out as the finest model. Finally, the complete quadratic polynomial model in terms of the obtained normative factors is Biomass concentration = -45.42 + 6.35 pH + 0.997 Temperature + 4.78 Carbon conc. + 43.7 Nitrogen Conc. - 0.4375 pH*pH - 0.01770 Temperature* Temperature- 0.745 Carbon conc.* Carbon conc. - 83.5 Nitrogen Conc.* Nitrogen Conc.+ 0.0133 pH* Temperature - 0.095 pH*Carbon conc.- 1.63 pH*Nitrogen Conc.- 0.0565 Temperature* Carbon conc. + 0.300 Temperature* Nitrogen Conc. + 3.25 Carbon conc.* Nitrogen Conc. The model p-value of (<0.0001) for the ANOVA analysis, coefficient of determination (R^2 = 3.18) and adjusted coefficient of determination (adjusted R^2 = 3.07) (Figure 11).

4. Conclusion

Products are made naturally from endophytes, which are bacteria with a very low molecular mass. These antimicrobial extracts also showed antifungal and antibacterial properties and our observations and research indicate that endophytic bacteria isolated from *Adhatoda beddomei* have the potential to produce various antimicrobial drugs as many new bioactive metabolites have been discovered through this research work. The quadratic polynomial model is very important and can be used to show how responses relate to one another and the significant variables as shown on the supplemental and to achieve these goals, we have made various efforts for adaptation to culture environments. These enhance yields to some range, but generally not as satisfactory as we expected.

5. Future Perspectives

The future perspectives of optimization of bioactive metabolites are focused on improving efficacy, and minimizing side effects, novel antifungal agents (new classes of drugs, combination therapies), enhanced drug delivery systems, and immunotherapy and vaccines.

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