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RESEARCH ARTICLE

Statistical Optimization for the Production of Antimicrobial Compounds Produced by Mangrove Endophytic *Aspergillus sp.*

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Abstract

Objectives: The purpose of this study was to investigate the antibacterial properties of an endophytic fungus, *Aspergillus sp.*, isolated from the roots of the *Avicennia marina* belonging to an unexplored location in Kutch, Gujarat, India. The study aimed to optimize various factors to enhance the production of antimicrobial compounds. **Methodology:** Antimicrobial activity was performed using the agar-well diffusion method, and the results were compared with the standard antibiotic streptomycin as a positive control and DMSO as a negative control. The classical one-factor-at-a-time (OFT) approach was used to optimize various factors such as temperature, pH, culture media, carbon and nitrogen sources, and solvents to enhance antimicrobial production. Response Surface Methodology (RSM) was used to statistically optimize the production of antimicrobial compounds. **Findings:** The investigation revealed that the endophyte exhibited remarkable inhibitory action against sensitive strains of *Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa, Bacillus cereus, and Staphylococcus aureus*. The most suitable conditions for the production of antimicrobials were; an inoculum size of $1*10^5$ in potato dextrose medium at a temperature of 25°C and pH 6, with an incubation period of 12 days. Starch and ammonium chloride acted as the best carbon and nitrogen sources, respectively. The use of RSM resulted in a 1.46-fold increase in the antimicrobial activity against *Pseudomonas sp.*, with an increase in the zone of inhibition from 18.36mm to 26.8mm. **Novelty:** The study highlights the unique antimicrobial potential of an unexplored endophyte derived from *Avicennia marina* roots in Kutch, Gujarat, India. RSM played a crucial role in detecting optimum conditions for the production of antimicrobial compounds, resulting in a significant increase in activity. Its application emphasizes the importance of advanced statistical techniques in improving our understanding of complex biological systems.

Keywords: *Avicennia marina*; Antimicrobial; Box-Behnken design; Endophytic Fungi; Mangroves

1 Introduction

For centuries, natural medications derived from plants have been utilized to treat a variety of serious illnesses. The active isolation and extraction of cytotoxic podophyllotoxins, such as vinca alkaloids such as vincristine and vinblastine, began in the 1950s[1].The production of pharmaceuticals from medicinal plants or trees, on the other hand, poses major challenges. For instance, approximately 38,000 yew trees need to be harvested to isolate 25 kg of the anti-cancer drug taxol, enough to treat 12,000 patients[2]. This practice poses a serious threat to the environment as it leads to the endangerment and extinction of specific plant species. Microorganisms, particularly fungi, have emerged as a new source for the manufacturing of therapeutics[3]. These fungi have unrivalled potential for repeatability, efficacy, and sustainability, as well as the development of viable medication choices. Fungi, among other microbes, have been discovered to create a wide spectrum of antimicrobials with significant antibiotic and antioxidant properties[4]. This distinct feature of fungus has the potential to lead to a variety of medicinal uses in favourable or stressful situations.

Researchers have found that fungi are a good source of secondary metabolites with medical properties like antibacterial, antioxidant, immunomodulator, and immunosuppressant, making them better than bacteria and actinomycetes[5]. Fungi manufacture these metabolites in a variety of circumstances, including terrestrial, marine, and mangrove ecosystems, where they occur as saprophytes, epiphytes, and endophytes. Endophytic fungi, in particular, have been demonstrated to play an important role in the production of secondary metabolites, owing to their symbiotic connection with their host, in which they imitate the same metabolism as their host[6]. Fungal endophytes have proven to be a treasure trove of bioactive metabolites with therapeutic potential, like antioxidant, anti-cancer, antimicrobial, and anti-inflammatory properties[7–9]. *Avicennia marina* is one such incredible plant with extraordinary medicinal properties[10,11]. Due to these therapeutic properties of the plant, it was considered a promising source for harbouring endophytes with effective antimicrobial potential. However, most studies have concentrated on well-known and extensively studied locations, which may not represent the full diversity of endophytes with antimicrobial potential[12]. Additionally, while various optimization strategies have been employed to enhance antimicrobial production, there is a need for more efficient and effective approaches. As a result, this study aims to fill these discrepancies in knowledge by investigating an untapped area in Kutch, Gujarat, India, for the isolation of endophytic fungi with antibacterial potential. The study employs a unique approach to optimization through the implementation of various factors, such as temperature, pH, culture media, different carbon and nitrogen sources, and various solvents. The study intends to use this technique to determine the best circumstances for increased antibacterial production. Furthermore, the goal of this study is to contribute to the search for novel antimicrobial agents as well as provide a more efficient and effective strategy to optimize medium parameters for future investigations. Interestingly, there is no published work on the antibacterial potential of endophytic fungi belonging to the mangrove ecosystem of Kutch, Gujarat.

2 Methodology

2.1 Identification of fungi

The *Aspergillus sp.*, KR-1 10(14), used in the current study, was isolated from the root of *Avicennia marina* derived from the Kutch region of Gujarat, India (23°01'58.6" N, 70°09'27.3" E). Molecular identification of the fungi was conducted, and the resulting ITS sequences were submitted to NCBI GenBank under the accession number MW447519[13].

2.2 Test Cultures

Sensitive bacteria used in the experiments included *Pseudomonas aeruginosa* (MTCC 424), *Bacillus cereus* (MTCC 430), *Escherichia coli* (MTCC 443), *Salmonella enterica typhimurium* (MTCC 98), *Shigella flexneri* (MTCC 1457), *Klebsiella pneumoniae* (MTCC 3384), and *Staphylococcus aureus* (MTCC 737), procured from the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India.

2.3 Screening of antimicrobial activity from fungal extract

Four *Aspergillus sp.* mycelial discs (8mm diameter), grown on Czapek dox agar plates, were inoculated in Czapek dox broth (sucrose 30 g/l, sodium nitrate 2 g/l, dipotassium phosphate 1 g/l, magnesium sulfate 0.5 g/l, potassium chloride 0.5 g/l, and ferrous sulfate 0.01 g/l) followed by incubation at 25°C for 21 days under static conditions. The broth was filtered using Whatmann filter paper no. 1, and the resultant filtrate was subjected to antimicrobial analysis by agar-well diffusion assay[13]. Streptomycin sulphate and DMSO were used as positive and negative controls, respectively.

2.4 Optimization by the one factor at a time method

A conventional OFT method was adopted to optimize the production of bioactive compounds. Several physicochemical parameters, such as pH, temperature, inoculum density, incubation period, various culture media, carbon and nitrogen sources, as well as the variety of solvents used for extraction, were optimized.

2.4.1 Effects of various cultural media

For the selection of ideal culture media, *Aspergillus sp.* was cultured in five different liquid culture media including Czapek Dox broth, potato dextrose broth, Sabouraud dextrose broth, yeast extract glucose broth, and malt extract broth. About 200 ml of medium were inoculated with an 8-mm disc of *Aspergillus sp.*, followed by incubation at 25°C for 21 days. The filtrate from each broth was evaluated for antimicrobial activity. For the subsequent optimization tests, a medium that promoted growth and maximum secondary metabolite production was selected.

2.4.2 Effect of inoculum size

The inoculum size of *Aspergillus sp.* for optimal antimicrobial metabolite production was evaluated by inoculating different concentrations of homogenous spore suspension in potato dextrose broth. Microscopical enumeration and cell counting in Neubauer's chamber served to determine the cell's density. Various inoculum densities were developed, such as $1*10^3$, $1*10^4$, $1*10^5$, and $1*10^6$ spores/ml. Each 500-ml conical flask containing 200 ml of potato dextrose broth was seeded with 1% of the respective inoculum density. The flasks were incubated at 25°C for 21 days, and the resulting broth was assayed for antimicrobial activity.

2.4.3 Effect of pH, temperature, and incubation period

An effect of pH on the antibacterial potential of the endophyte was observed through the growth of fungi in potato dextrose broth with various pH ranging from 3–9 at 25°C for 21 days. Whereas, the optimal temperature was studied by growing the fungus at diverse temperatures (15°C, 20°C, 25°C, 30°C, 35°C, and 40°C) under static conditions for 21 days. Similarly, the fungus was grown at 25°C for 30 days to test the effect of the incubation period. Antimicrobial activity was measured every 3 days for up to 30 days of incubation.

2.4.4 Effect of Carbon and Nitrogen Sources

Carbon sources like starch, dextrose, maltose, xylose, fructose, carboxy methyl cellulose, and nitrogen sources like malt extract, yeast extract, urea, ammonium chloride, potassium nitrate, ammonium nitrate, and ammonium sulphate were seeded separately into the media at 10 g/l (w/v).

2.4.5 Efficacy of different solvents for the extraction of antimicrobial metabolites

The efficiency of different solvents was determined by utilizing different solvents such as hexane, ethyl acetate, acetone, ethanol, and methanol.

2.5 Optimization Using Response Surface Methodology

The Box-Behnken Design (BBD) of RSM was utilized for the enhancement of antibacterial compound synthesis from *Aspergillus sp.* in this study. According to the outcome of one-factor-at-a-time (OFT) tests, the three crucial physical parameters chosen as independent variables are temperature (°C), pH, and incubation period (days). The experiment was conducted at three different levels: -1 (low), 0 (middle), and +1 (high) using a Box-Behnken model. To understand the experimental error, the model of 15 experimental runs with a centre point was repeated three times. The experimental design was developed using Minitab 19. The minimum and maximal levels of the variables set in the culture medium were temperature (15–35 °C), pH (4–8), and incubation period (6–18 days), as shown in Table 1. All the flasks were inoculated with a uniform fungal inoculum of 1%. Following subsequent incubation, 10 ml of aliquots were taken from each flask at the respective time intervals, and the crude extract containing secondary metabolites was assessed for antimicrobial activity.

Table 1. Independent variables used for Box-Behnken design

Variables	Levels		
	-1	0	+1
pH	4	6	8
Temperature	15	25	35
Incubation Period	6	12	18

2.6 Analysis of Data

The antibacterial activity of *Aspergillus sp.* was the dependent variable. A quadratic polynomial equation was implemented for the analysis of the response.

$$Y = \beta_0 + \sum_{\text{Linear}} \beta_i X_i + \sum_{\text{Squared}} \beta_{ii} X_{ii}^2 + \sum_{\text{Interaction}} \beta_{ij} X_i X_j$$

Where,Xi and Xj are independent variables, Y is a dependent variable (or predicted response), i is the linear coefficient, ii is the quadratic coefficient, and ij is the interaction coefficient. Xi and Xj are the two independent variables, and 0 is a constant term.

2.7 Statistical Analysis

Minitab 19 was utilized to conduct the regression analysis as well as the experimental design of the data. The statistical analysis of the model was accomplished to evaluate the ANOVA (Analysis of Variance). The determination of the coefficient R^2 served to assess the quality of the polynomial equation. While the significance of the regression coefficient was determined by a t-test. While the statistical significance was tested by the F test. While fitting the models, great care was taken to ensure that there were no interference issues. A model that is close to 1 is good. The number of antimicrobials produced under optimal conditions was represented using three-dimensional graphs.

3 Results and Discussion

3.1 Identification of Fungi

The presence of endophytic microorganisms within host plants can result in the manifestation of extraordinary features such as stress tolerance and the production of compounds that can combat invading infections[14]. Particularly for the production of valuable antibacterial compounds, medicinal plants can be excellent sources of unique endophytic microorganisms[15]. *Avicennia marina* is one such exceptional plant with outstanding therapeutic capabilities that was investigated to detect the presence of an endophytic fungus. A selected fungal isolate, KR-1 10 (14), was identified using molecular methods, and its genomic DNA was extracted and amplified. The isolate was identified as *Aspergillus sp.* upon sequencing, and its genomic sequence was submitted to NCBI Genbank under the accession number MW447519.

3.2 Screening of antibacterial activity

A crude extract of *Aspergillus sp.* (9.2g dissolved in DMSO and filled 200μl in each well) effectively inhibited the growth of all five test cultures, with the zone of inhibition ranging from 17.7 mm to 21.3 mm. It exhibited strong antimicrobial activity against all five test cultures. An *Aspergillus* extract showed remarkably high antimicrobial activity against *Staphylococcus aureus*, a leading threat of antimicrobial drug resistance as well as a cause of skin infections [16]. *Aspergillus* has proven to be an endophyte exhibiting a variety of bioactivities in numerous investigations [17,18].

3.3 Optimization of by one factor at a time method

3.3.1 Effect of culture media

This study evaluated the efficiency of five different liquid culture media in stimulating growth and bioactive metabolite synthesis in a homogenous suspension of fungus. The results revealed that potato dextrose broth was the best medium for stimulating growth and the synthesis of bioactive metabolites in the investigated fungus species (Figure 1). The results corroborate prior studies on the endophytic fungus *Aspergillus tamarii*, indicating a potential harmony in the preferences of diverse fungal species for this particular culture medium [19]. The findings were consistent enough to imply that this medium might be used as a culture medium for a wide variety of fungi. This medium can even promote the development of *Penicillium sp*., which produces a significant number of antimicrobials [20].

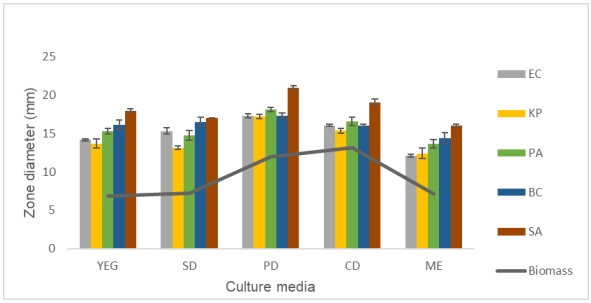

Fig 1. Effect of different culture media on antimicrobial activity by *Aspergillus sp. EC, Escherichia coli, KP, Klebsiella pneumoniae, PA, Pseudomonas aeruginosa, BC, Bacillus cereus, SA, Staphylococcus aureus*. Values are given as mean ± SE

3.3.2 Effect of inoculum size

A specific spore density can have a substantial impact on secondary metabolite synthesis and may have practical implications for improving fungal cultivation processes. Thus, in this study, the optimum inoculum density was determined employing spore densities ranging from $1*10^3$ to $1*10^6$ spores/ml for effective secondary metabolite synthesis. By inoculating potato dextrose broth with different spore densities, it was revealed that an inoculum density of $1*10^5$ spores/ml resulted in the maximum production of antibacterial compounds (Figure 2). Hence, this inoculum density was subsequently used to optimize other physiochemical parameters.

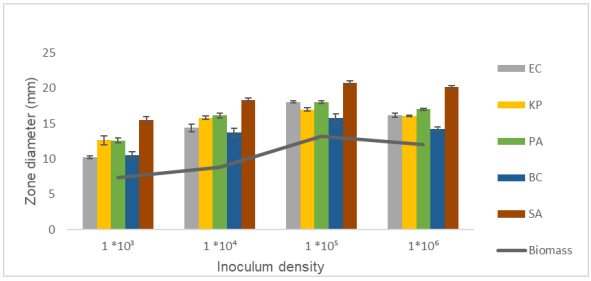

Fig 2. Effect of inoculum size on antimicrobial activity by *Aspergillus sp. EC, Escherichia coli, KP, Klebsiella pneumoniae, PA, Pseudomonas aeruginosa, BC, Bacillus cereus, SA, Staphylococcus aureus*. Values are given as mean ± SE

3.3.3 Effect of pH

To study the effect of pH on the antibacterial potential of fungi, pH was determined by inoculating fungi in potato dextrose broth varying in pH (3–9). Results showed that as the pH of the medium shifted from acidic to alkaline, there was a gradual increase in antimicrobial activity. *Aspergillus sp.* exhibited the maximum antibacterial activity at pH 6, and *Staphylococcus aureus, Escherichia coli, and Pseudomonas aeruginosa* were most susceptible to the antimicrobial activity of the crude extract (Figure 3). In contrast, *Penicillium sp.* required a pH of 5 for efficient bioactive metabolite synthesis[21]. These results suggest that different fungal species may exhibit varying optimal pH levels for significant bioactive compound production.

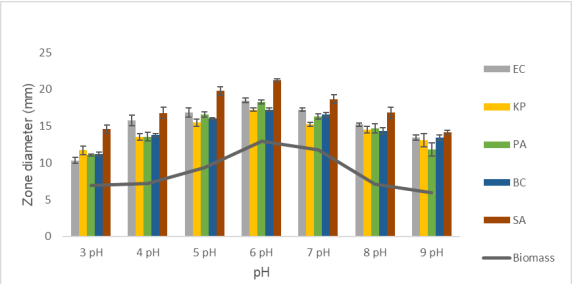

Fig 3. Effect of pH on antimicrobial activity by *Aspergillus sp. EC, Escherichia coli, KP, Klebsiella pneumoniae, PA, Pseudomonas aeruginosa, BC, Bacillus cereus, SA, Staphylococcus aureus*. Values are given as mean ± SE

3.3.4 Effect of Temperature

Maintaining a precise temperature is crucial for the growth and proliferation of endophytic fungi and the production of their bioactive metabolites. In this study, fungi displayed optimum bioactivity at 25°C (Figure 4). A significant difference in antimicrobial activity was observed with variations in temperature from 15 to 40°C. Similar results were observed in the *Moringa oleifera* endophytic fungus *Chaetomium globosum*, where there was a drastic decrease in biomass production and antibacterial activity after 30°C[22].

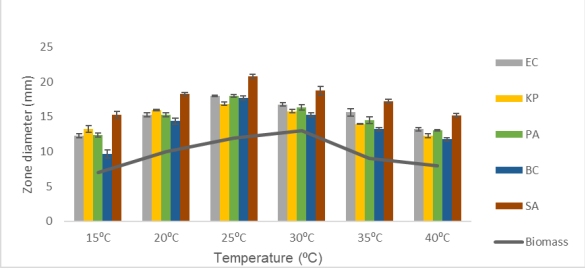

Fig 4. Effect of temperature on antimicrobial activity by *Aspergillus sp. EC, Escherichia coli, KP, Klebsiella pneumoniae, PA, Pseudomonas aeruginosa, BC, Bacillus cereus, SA, Staphylococcus aureus*. Values are given as mean ± SE

3.3.5 Effect of Incubation period

An incubation period is the most crucial factor for the enhancement of antimicrobials; as the period increases, it leads to a decrease in antimicrobial activity. The optimum antibacterial potential was displayed on day 12, whereas the optimum biomass was derived on the 15th day. A slight decrease in antibacterial activity was determined after the 15th day, as displayed in Figure 5. The activity remained stable until the 18th day, followed by a gradual decrease up to the 30th day.

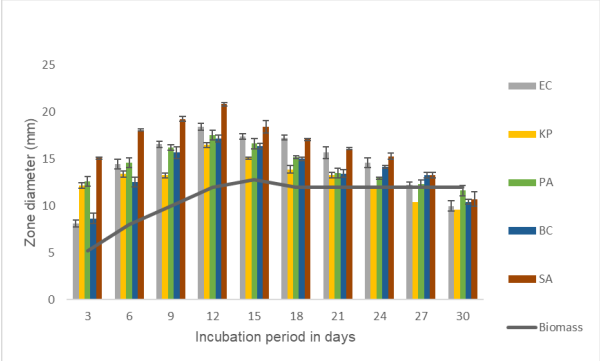

Fig 5. Effect of incubation period on antimicrobial activity by *Aspergillus sp. EC, Escherichia coli, KP, Klebsiella pneumoniae, PA, Pseudomonas aeruginosa, BC, Bacillus cereus, SA, Staphylococcus aureus*. Values are given as mean ± SE

3.3.6 Effect of Carbon and Nitrogen Source

Starch, dextrose, maltose, xylose, fructose, carboxy methyl cellulose, and sucrose were employed as the carbon sources. Starch supported the optimum production of antibacterial compounds, followed by sucrose and dextrose (Figure 6). Ammonium chloride supported the growth and production of the antimicrobial compound optimally, followed by ammonium sulphate (Figure 7).

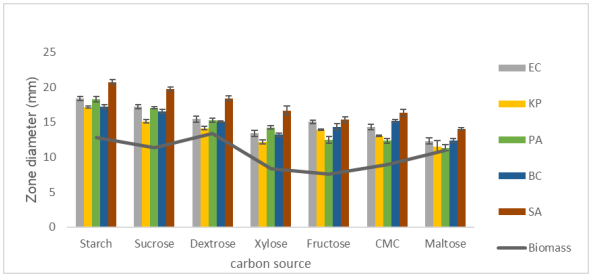

Fig 6. Effect of carbon source on antimicrobial activity by *Aspergillus sp. EC, Escherichia coli, KP, Klebsiella pneumoniae, PA, Pseudomonas aeruginosa, BC, Bacillus cereus, SA, Staphylococcus aureus*. Values are given as mean ± SE

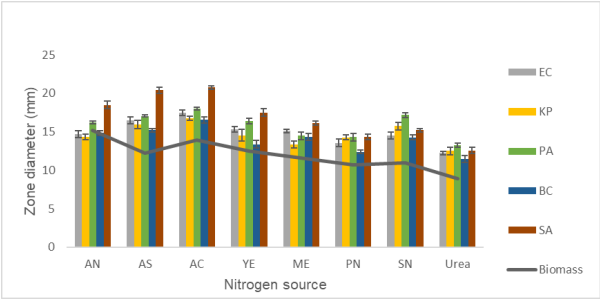

Fig 7. Effect of nitrogen source on antimicrobial activity by *Aspergillussp. EC, Escherichia coli, KP, Klebsiella pneumoniae, PA, Pseudomonasaeruginosa, BC, Bacillus cereus, SA, Staphylococcus aureus.*Values are given as mean ± SE

3.3.7 Efficacy of different solvent for the extraction of antimicrobial metabolite

The best suitable solvent for maximal antibacterial compound production was determined by using solvents such as hexane, ethyl acetate, acetone, ethanol, and methanol. Ethyl acetate and acetone were equally effective for the extraction of antimicrobial compounds.

3.4 Optimization using response surface methodology (RSM)

Three significant parameters including temperature, pH, and incubation period, were selected to optimize secondary metabolite production. According to the classical method, the optimum temperature to obtain maximal antibacterial activity and biomass production was 25°C. The temperatures below 20°C and above 30°C did not support efficient production of bioactive compounds, so a temperature range of 15–35 °C was chosen. While optimum antibacterial production was achieved on the 12^{th} day of incubation, after the 18^{th} day, there was a drastic decrease in antimicrobial activity, so an incubation period range of 6 to 18 days was chosen. The optimum antimicrobial activity was determined at pH 6. With the increase in pH above 7, there was a decrease in antibacterial activity. So, a pH range of 4–8 was chosen to further optimize the study.

A three-level Box-Behnken design optimized the effect of independent variables. About 15 experimental runs with three replicates at the centre point were performed. P-values and analysis of variance (ANOVA) were used for the determination of the model's significance. Regression was statistically significant ($P< 0.05$) with a 95% confidence level for all organisms except *Pseudomonas aeruginosa*. A coefficient of determination, R^2, was observed as 94.75%, 97.04%, 96.86%, 94.87%, and 93.51% for *E. coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Bacillus cereus, and Staphylococcus aureus*, respectively.

In Figure 8, the predicted model is displayed in the form of three-dimensional (3D) response surface graphs with one variable set to zero or coded, and the impacts of two variables are seen. The optimal values obtained for variables were pH, 6; incubation period, 12 days and temperature, 25°C using the response surface 3D plots. To determine which parameters, have a statistically significant influence on antimicrobial activity, Pareto charts were examined. The significance of each variable and its interaction was investigated using the reference lines on the charts. At α=0.05, the charts produced varying results. It included pH (A), temperature (B), and incubation period (C). According to the RSM results, all three factors significantly influenced the antimicrobial activity of *Aspergillus sp.,* with the incubation period having the highest effect, followed by pH and temperature, which had the least effect.

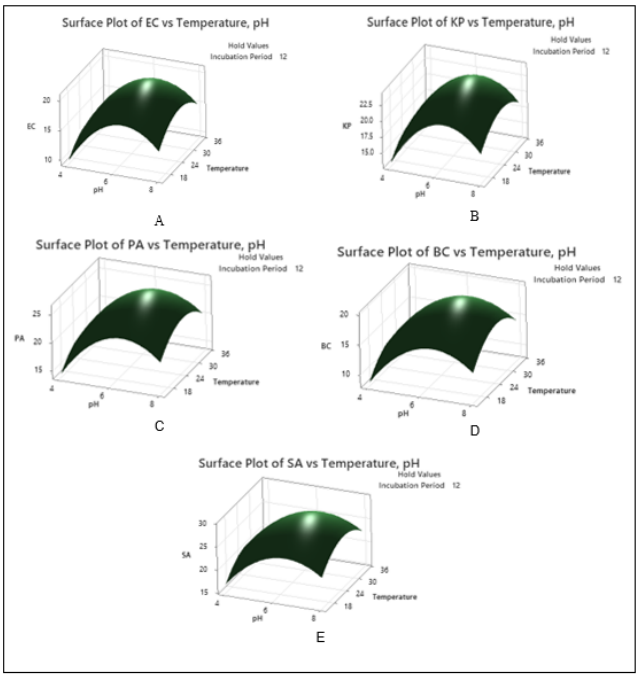

Fig 8. Response surface plots of A- EC, *Escherichia coli*, B-KP, *Klebsiella pneumoniae*, C-PA, *Pseudomonas aeruginosa*, D-BC, *Bacillus cereus*, D- SA, *Staphylococcus aureus*

3.5 Validation of Results

The statistical results were validated with the use of an optimized medium to evaluate the precision and suitability of a model. An output revealed that the experimental values coincided with a predicted value. Based on the results of the response surface methodology, pH, temperature, and incubation period were confirmed as significant variables that aided in the production

of bioactive metabolites by *Aspergillus sp.* According to the predictive analysis in BBD design, the bioactivity response for *Staphylococcus aureus* was 28.4 mm and the actual experimental response achieved was 28.0 mm, which indicated that there is adequate symmetry between the experimental and predicted values. A 1.46-fold increase in bioactive compound production was obtained through RSM analysis of *Aspergillus sp.* Similarly, the study on the endophytic fungus *Arcopilus aureus* displayed a 1.49-fold rise in bioactive metabolite synthesis [23]. Hence, both, the classical optimization method and the statistical method were efficient to increase the bioactive metabolite synthesis by the selected fungi. According to the predictive analysis in BBD design, the bioactivity response for Staphylococcus aureus was 28.13 mm and the actual experimental response achieved was 28.0 mm, which indicates that there is adequate symmetry between the experimental and predicted values.

Table 2. Comparative antimicrobial potential of *Aspergillus sp.* and other endophytic fungi derived from the Mangrove - *Avicennia sp.*

Sensitive Test Organism	Antimicrobial activity of *Aspergillus sp* . studied	Antimicrobial activity of previously reported endophytic fungi	
	(Zone of inhibition)	(Zone of inhibition)	Endophytic Fungi
Staphylococcus aureus	28.4mm	21.00mm [24]	*Excoecaria Agallocha*
Escherichia coli	20.6mm	23.00mm [24]	*Excoecaria Agallocha*
Pseudomonas aeruginosa	26.8mm	27.33mm [25]	*Penicillium rubens*
Bacillus sp.	19.9mm	27.02mm [25]	*Penicillium rubens*
Klebsiella pneumoniae	24.00mm	01.80mm [26]	*Aspergillus tamari*

Table 2 depicts the comparative antimicrobial potential of various endophytic fungi derived from *Avicennia sp. and Aspergillus sp.* used in the present study. With reference to its antibacterial efficacy, the zone of inhibition produced by *Aspergillus sp.* against *Staphylococcus aureus* was much larger (28.4mm) than the previously reported activity (21.00mm). A narrower zone of inhibition (20.6mm) was seen against *Escherichia coli* than the reported activity (23.00mm), indicating a limited inhibitory effect against this strain. Notably, *Aspergillus sp.* showed significantly higher inhibitory activity against *Klebsiella pneumoniae* (24.00mm) than reported (01.80mm), demonstrating its potential to target multidrug-resistant bacteria. Thus, it is evident from our results that *Aspergillus sp.* has the potential to produce broad-spectrum antimicrobial compounds with varied potencies against different bacterial strains.

4 Conclusion

Gujarat is at the forefront of the growth of the pharmaceutical industry in India. Accounting for nearly 42 percent of India's pharmaceutical turnover, 22 percent of its drug exports, and 20 percent of its chemical output, Gujarat's pharmaceutical industry has evolved into an innovation-driven, knowledge-focused industry. However, there is an alarming scarcity of new antibiotics in the pipelines of the pharmaceutical industry. A serious concern for pharma companies worldwide is the declining Research and Development (R&D) productivity, with a reduced number of blockbuster drugs entering the market annually. The search for novel resources amidst the already explored ones leads us to investigate endophytic fungi in the pristine environments of Kutch (Gujarat). In addition, with the alarming rise of antibiotic-resistant bacteria, the utilization of natural compounds produced by the fungus as a potential alternative to standard antibiotics is critical. Our investigations emphasize the capability of fungal endophytes, obtained from *Avicennia marina* from the coastal line of Gujarat, as a plentiful reservoir of bioactive substances possessing noteworthy antimicrobial characteristics. Further improvements in optimizing the growth parameters and biochemical pathways in these organisms will surely turn out to be a valuable addition to the research focusing on the bulk production of antimicrobial compounds from endophytic fungi, where such data are scarce.

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