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Optimization using response surface methodology and analysis of antimicrobial activity of pink pigmented *Fusarium foetens* CBS 110286

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ABSTRACT

Fusarium is a diverse genus known for its hyaline, cotton-like colonies, with many species acting as pathogens in plants, animals, and humans. However, specific species such as *Fusarium oxysporum*, *Fusarium moniliforme* (now *Gibberella fujikuroi*), and *Fusarium solani* produce carotenoid pigments valuable to the food, textile, and cosmetic industries. This study reports, probably for the first time, the isolation of pink-pigmented *Fusarium foetens* CBS 110286 from Punjab, India, optimizes its pigment production via response surface methodology, and evaluates its antimicrobial activity. The Design-Expert® software (version 13, Stat-Ease, USA) was used to ascertain the effects of five independent variables: temperature (25°C–45°C), incubation time (7 days), peptone (0.1–0.3 g/l), fructose (0.1–0.3 g/l), and initial pH (4–8) were analyzed. In addition, high performance liquid chromatography and gas chromatography mass spectrophotometer characterization was carried out to identify and quantify the major metabolites. The optimized conditions yielded significant pigment production, exhibiting a broad-spectrum effect in peptone and fructose, as well as a zone of inhibition (11 mm) against the Gram-positive *bacterium Staphylococcus aureus*, and approximately 8 mm against *Escherichia coli*. This result may have potential applications in the textile, pharmaceutical, and biotechnological industries upon complete characterization.

INTRODUCTION

Recent studies have identified particular pigmented *Fusarium* species, such as *Fusarium fujikuroi*, that produce a purple color [1]. Earlier, the species were a pathogenic, destructive, and complex genus worldwide, causing diseases in plants, animals, and humans that colonize various host plants and crops, resulting in stem, crown, and root rot, as well as wilt, leading to considerable losses. *Fusarium* is generally recognized as a diverse genus that typically forms colorless, cotton-like, and hyaline colonies [2]. For the first time, this study identifies *Fusarium foetens* CBS 110286 as a producer of pink pigment, highlighting its unique and previously unexplored capability in pigment biosynthesis. This strain was isolated from the Jalandhar district of Punjab, a region where pigmented forms of this species have not been previously documented.

However, a comparative study of *F. foetens* with other existing pigmented *Fusarium species* highlighted their ecological roles, pathogenicity, metabolic pathways, and potential applications, as well as given the diversity of some *Fusarium species*, such as *Fusarium oxysporum, Fusarium moniliforme* (now *Gibberella fujikuroi*), and *Fusarium solani*, producing beneficial pigments of carotenoids for usage in textile, food, pharmaceutics, and cosmetics in biotechnological processing [3]. The species are well-documented producers of various secondary metabolites [4]; some exhibit pigmentation due to their diverse biological activities, including antimicrobial, antioxidant, and anticancer properties, as well as those with economic and scientific applications against plant pathogenic fungi [5,6]. However, various environmental and nutritional

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factors influence the production and optimization of the pigment yield, necessitating a systematic approach [7].

The use of response surface methodology (RSM) as a powerful statistical tool model to analyze and process multiple variables and influence a desired pigment yield response is useful in optimizing biological processes, such as microbial metabolite production, through interactions between variables that are complex and non-linear, as well as determining the optimal conditions for maximizing pigment yield, that enhance the antimicrobial potential of the extract [8,9]. Recent studies have demonstrated the antimicrobial potential of fungal pigments, attributed to their diverse bioactive secondary metabolites [10]. Geweely [11] also reported significant antibacterial activity of these pigments against four human pathogens, including Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*) and Gram-positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis*). However, pigmented *Fusarium* species, including *Fusarium javanicum*, *Fusarium martii*, and *F. solani*, have long been recognized for their pigment production and associated antimicrobial properties [12,13]. The rise of antibiotics and multidrug-resistant microbes has intensified the search for novel bioactive compounds. Certain fungi are commercially utilized not only for antibiotic production but also as sources of natural colorants such as carotenoids and anthocyanins. Key fungal pigments, such as canthaxanthin, astaxanthin, prodigiosin, phycocyanin, violacein, riboflavin, β-carotene, melanin, and lycopene, are widely used in the food and pharmaceutical industries [14]. The research findings aimed to optimize the production of novel pink-pigmented *F. foetens* CBS 110286 with GenBank accession number PQ878325 using RSM and to evaluate the antimicrobial activity against *S. aureus*.

MATERIALS AND METHODS

Culture preparation

The culture isolation method was selected for its efficiency in supporting optimal fungal growth and pigment synthesis under controlled conditions in this study, following the protocol outlined by Sonkar *et al.* [15]. Likewise, the use of a nutrient-rich Potato Dextrose Agar (PDA)medium, along with regulated parameters such as pH and temperature, enhances the production of pigmented secondary metabolites, as well as the optimization of fungal pigments [16]. The strain *F. foetens* CBS 110286 strain was isolated from soil in Jalandhar Cantt. Railway station by serial dilution using the standard spread plate technique [17]. PDA was used for isolation, with the following composition: potato infusion (40 ml), dextrose (2.0 g), agar (1.5–2.0 g), distilled water (100 ml), and pH 6.0, as outlined by Gomaa *et al.* [18]. The solidified culture plate was incubated at 28°C ± 2°C for 7 days in a rotary shaker incubator. Pigmented fungal colonies were observed, and visual identification was also conducted based on the color assessment of the produced pigments, as shown in Figure 1 [19,20].

Identification of pigmented JRSSS strain

The present isolated pink-pigmented *F. foetens* CBS 110286 strain was isolated from the Jalandhar district of Punjab, a region where pigmented forms of this species have not been previously documented. For identification, morphological features of the pigmented colonies were first observed based on the color assessment of the produced pigments, followed by molecular characterization using internal transcribed spacer region sequencing [21]. The combined approach used ensures accurate species identification and aligns with recent findings [16].

Pigment production by JRSSS strain

A 5 mm borer was used to cut the pink pigmented fungus colony, and it was immersed in the grown 100 ml sterile potato dextrose broth, pH 6. The culture was incubated at 28°C ± 2°C for 7 days and set at 150 rpm in a rotary shaker incubator. After incubation, fungal pellets were collected using Whatman filter paper. For pigment extraction, the pellets were treated with a solvent mixture containing methanol and 90% ethanol (10 ml each). The mixture was then centrifuged at 10,000 rpm for 15 minutes to separate the fungal biomass [22]. The pellets were dried in a hot air oven at 40°C, while the supernatant was analyzed using a UV–Visible spectrophotometer (UV-1800, Shimadzu Corporation, Tokyo, Japan) at wavelengths of 200–800 nm [23]. All experiments were duplicated, and pigment yield was calculated [24].

Assessment of pigment yield and dry biomass calculation

Assessment methods for pigment yield are calculated using extinction coefficients in this research, as outlined in the protocol described in [13]. Three milliliter of 7-day incubated broth culture was withdrawn from the conical flask containing 100 ml potato dextrose agar (PDB) broth to measure the optical density (OD) of the sample throughout the cultivation period. The sample was centrifuged at 10,000 rpm for 15 minutes to separate the supernatant from the pellet. The color supernatant was quantified using a UV-vis spectrophotometer at 540 nm; the values were converted into g/l equivalents [13]. The concentrations of the pink pigments were calculated using the formula below:

$$\text{Pigment yield (OD/g)} = \frac{\text{Optical density (OD)} \times \text{Dilution factor} \times \text{Total volume}}{\text{Dry weight of the pigment}}$$

The OD of the fungal biomass was assessed using a UV-Vis spect. (UV-1800, Shimadzu Corporation, Tokyo, Japan). The measurements within the linear detection range of the extract

Figure 1. Growth morphology and pigment production of JRSSS fungal isolate on PDA solid medium.

Table 1. Central composite design with observed response and predicted value over process parameters in *Fusarium foetens CBS* 110286, using four independent variables.

Standard	Run	A	B	C	D	Actual response	Predicted
		pH	Temperature	Fructose	Peptone		
12	1	8	45	0.1	0.3	0.245	0.2034
19	2	6	25	0.2	0.2	0.508	0.4564
9	3	4	25	0.1	0.3	0.492	0.4707
8	4	8	45	0.3	0.1	0.192	0.2181
1	5	4	25	0.1	0.1	0.461	0.4652
22	6	6	35	0.3	0.2	0.723	0.6099
28	7	6	35	0.2	0.2	0.702	0.5600
16	8	8	45	0.3	0.3	0.272	0.2779
23	9	6	35	0.2	0.1	0.522	0.4813
29	10	6	35	0.2	0.2	0.465	0.5600
14	11	8	25	0.3	0.3	0.523	0.5419
17	12	4	35	0.2	0.2	0.723	0.6295
6	13	8	25	0.3	0.1	0.477	0.4508
13	14	4	25	0.3	0.3	0.521	0.5493
25	15	6	35	0.2	0.2	0.487	0.5600
10	16	8	25	0.1	0.3	0.437	0.4315
2	17	8	25	0.1	0.1	0.398	0.4048
20	18	6	45	0.2	0.2	0.234	0.2259
27	19	6	35	0.2	0.2	0.467	0.5600
5	20	4	25	0.3	0.1	0.433	0.4795
26	21	6	35	0.2	0.2	0.532	0.5600
15	22	4	45	0.3	0.3	0.287	0.2851
18	23	8	35	0.2	0.2	0.562	0.5957
4	24	8	45	0.1	0.1	0.226	0.2078
11	25	4	45	0.1	0.3	0.206	0.2423
7	26	4	45	0.3	0.1	0.231	0.2465
3	27	4	45	0.1	0.1	0.282	0.2680
24	28	6	35	0.2	0.3	0.533	0.5140
21	29	6	35	0.1	0.2	0.512	0.5654
30	30	6	35	0.2	0.2	0.528	0.5600

The boldness in the predicted values 0.6295 in Run 12 signifies a notably high pigment yield under conditions of low pH (4.0) and moderate temperature (35 °C) with stable fructose and peptone levels (0.2 g/100 ml each). This highlights the strain's acidophilic nature and the effectiveness of these parameters in enhancing pigment production. The close match between actual and predicted values indicates high model accuracy, confirming the significance of this combination for optimized yield in JRSSS strain.

were diluted at 1950:50 µl ratio using autoclaved PDB and the crude pigmented sample, respectively [13]. The appropriate dilution factor was used to assay the final OD, and all readings were recorded in duplicate. The 10 ml of the crude extract sample was filtered using Whatman filter paper (0.45 µm pore size), rinsed with distilled water, and dried at 40°C in a hot air oven [25,26].

Experimental design for optimization

RSM was utilized for optimizing the input variables that affect the pigment yield during production. The run table was created, the experiments were performedas per that in Table 1. Four independent variables were used to create the experimental design with Design Expert V 13.0. In this study, central composite design under RSM was employed to optimize the production of pink pigment by the JRSSS strain and to evaluate the interactions among selected parameters [27,28]. The values of the four factors involved in the study are pH, temperature, peptone concentration,

Table 2. Nutrient levels parameters used for central composite design parameters.

Factors	Units	Low	High	Centre	−α value	+α value
Temperature	˚C	25	45	35	11.22	58.78
pH	-	4	8	6	1.24	10.76
Peptone	g/l	0.1	0.3	0.2	−0.038	0.438
Fructose	g/l	0.1	0.3	0.2	−0.038	0.438

and fructose concentration. The range of values of these variables in the study is given in Table 2. The run table was created the experiments were performed as per that. The outcomes were recorded and fed to the same software for analysis [24,29]. All experiments were in triplicate and pigment.

Statistical analysis

Experimental validation was performed to determine the optimal conditions for higher pigment production. Design-Expert Software was used for the analysis of experimental data. Analysis of variance (ANOVA) was used as an analysis tool, and the *F*-test was used to analyze the effect of independent variables, which were identified by *p*-value < 0.0002 [30]. The correlation coefficients (R^2) and R^2 (adj.) were used to evaluate the fitness of the second-order polynomial equation. However, three-dimensional (3D) surface plots demonstrated the interaction between the coded variables and the responses, and each experimental result was presented as the mean ± SD.

RESULTS AND DISCUSSION

The statistical significance of the model equation in this study was assessed using the *F*-test within the context of ANOVA. The obtained ANOVA results are shown in Tables 3 and 4. Moreover, multiple regression analyses on the RSM were employed to identify the optimal levels of each factor for maximizing pink pigment and biomass production. A 3D surface plot was generated to illustrate the effects of the four factors. This design facilitates the understanding of the individual and interactive effects of the variables. The plots provided predictions of response values, consistent with the findings of [27,31].

Table 3. Model adequacy and statistical validation.

SD	0.0750	**R^2**	0.8743
Mean	0.4394	**Adjusted R^2**	0.7570
C.V. %	17.07	**Predicted R^2**	0.6314
		Adeq precision	8.0367

ANOVA and regression analysis for Quadratic model.

Statistical data analysis

Stat-Ease, Inc., Design-Expert® Software Version 13 (Minneapolis, MN) was used for all the statistical analysis conducted in this research, including experiments design, data analysis, regression coefficients calculation, and response surface graph, as well as ANOVA, to the statistical significance of the RSM experimental results as shown in Tables 3 and 4, respectively [29].

The ANOVA results confirm the quadratic model's suitability for optimizing pink pigment production by *F. foetens* CBS 110286 [32]. The model demonstrated statistical significance with an *F*-value of 7.45 and a *p*-value less than 0.0002, indicating a minimal likelihood that these findings are due to random variation. Key statistical parameters R^2 (0.8743), adjusted R^2 (0.7570), and predicted R^2 (0.6314) suggest a strong model fit and reliable predictive capability [27]. An adequate precision value of 8.04, exceeding 4.0, confirms a robust signal-to-noise ratio and validates the model's effectiveness. These findings align with previous studies that have been successfully applied. The results of this study align with earlier findings on pigment production

Table 4. ANOVA and regression coefficients of the Quadratic model for pink pigment production by JRSSS.

Source	Sum of squares	df	Mean square	*F*-value	*p*-value	
Model	0.5869	14	0.0419	7.45	0.0002	Significant
A-pH	0.0051	1	0.0051	0.9130	0.3545	
B-Temperature	0.2392	1	0.2392	42.54	<0.0001	
C-Fructose	0.0089	1	0.0089	1.58	0.2279	
D-Peptone	0.0048	1	0.0048	0.8539	0.3701	
AB	6.250E−08	1	6.250E−08	0.0000	0.9974	
AC	0.0010	1	0.0010	0.1793	0.6780	
AD	0.0005	1	0.0005	0.0803	0.7808	
BC	0.0013	1	0.0013	0.2273	0.6404	
BD	0.0010	1	0.0010	0.1737	0.6828	
CD	0.0041	1	0.0041	0.7341	0.4050	
A^2	0.0072	1	0.0072	1.27	0.2766	
B^2	0.1242	1	0.1242	22.08	0.0003	
C^2	0.0020	1	0.0020	0.3509	0.5624	
D^2	0.0101	1	0.0101	1.79	0.2004	
Residual	0.0844	15	0.0056			
Lack of fit	0.0447	10	0.0045	0.5641	0.7938	Not significant
Pure error	0.0396	5	0.0079			
Cor total	0.6712	29				

in the *Fusarium verticillioides* species. However, a strong correlation and a well-fitting model were indicated by the model correlation coefficient (R^2) of 0.8954, using RSM for the optimization process [33], which closely matches our finding of 0.8743. The adjusted R^2 value of 0.7570 further supports the model's reliability, which is consistent with the recent findings of Mwaheb *et al*. [13], highlighting the importance of optimizing environmental and nutritional conditions for enhanced pigment biosynthesis in *Fusarium*. Although the model showed a high R^2 (0.8743), the lower predicted R^2 (0.6321) suggests moderate limitations in predicting new data. Still, it provides a strong basis for further experiments and optimization of pigment production, as similar models have been effectively used in previous studies [32,34]. . To enhance predictive reliability, the model can be refined by removing non-significant terms.

RSM-based statistical approach for process variable optimization

The developed model of 3D response graphs was plotted to visualize the interaction between factors and the variation in response surface curves for the production of pink-pigmented *F. foetens*.

Figure 2 presents 3D response surface plots illustrating the interactive effects of pH, temperature, fructose, and peptone on the response variable (R1), modeled using RSM [27]. The pH–temperature interaction (AB) exhibits a moderate curvature, with a peak response at pH levels of 6.0–7.0 and temperatures of 30°C–35°C. The pH–fructose interaction (AC) reveals a flat surface, indicating minimal effect within the tested range. The pH–fructose interaction (AC) reveals a flat surface, indicating minimal effect within the tested range as depicted in Figure 2 The pH–peptone interaction (AD) exhibits a gentle curvature, with an optimal response at a pH of 5.0 and peptone levels of 0.15–0.2 g/l. Overall, temperature and peptone emerged as the most influential variables under the experimental conditions [31].

Response surface plots illustrate the interactive effects of key variables on the response (R1), as shown in Figure 3. In plot (BC), increasing temperature enhances R1 at moderate fructose levels. Plot (BD) shows R1 rising with temperature and peptone but plateauing beyond optimal conditions. Plot (CD)

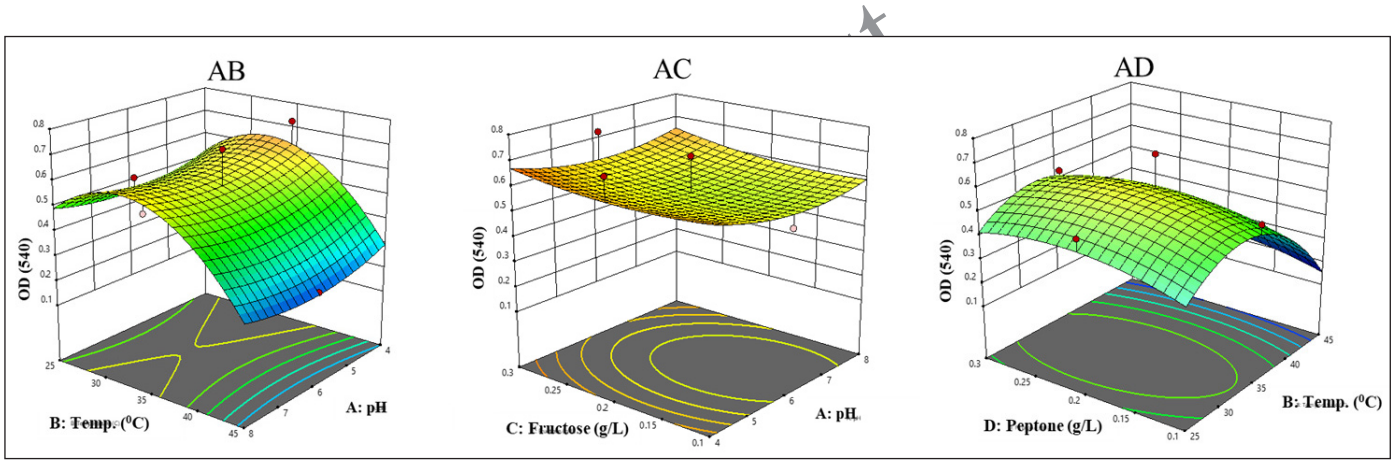

Figure 2. 3D response surface plots showing the interactive effects of temperature and pH (AB) fructose concentration and pH, (AC) peptone concentration and pH (AD) on the response variable (R1).

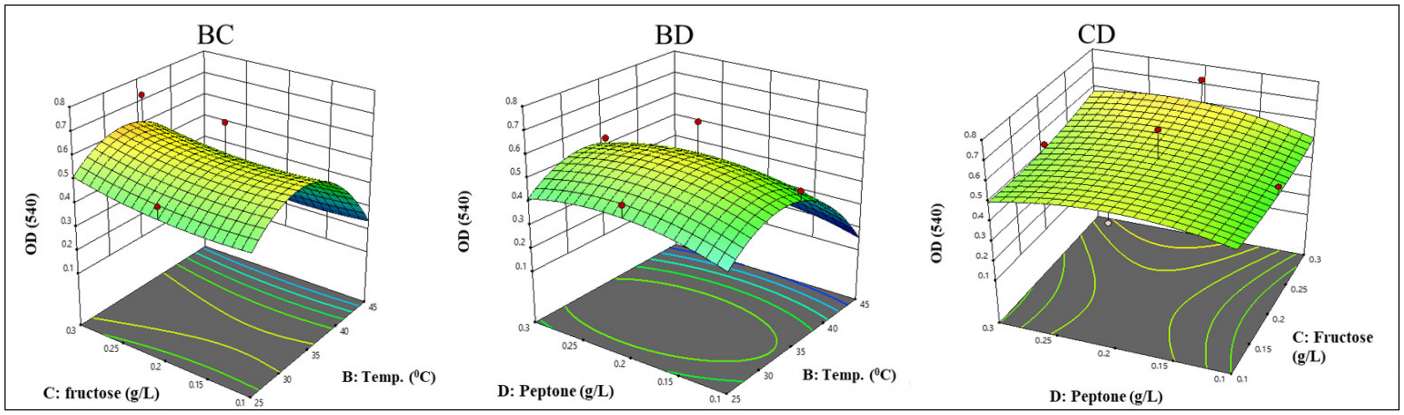

Figure 3. 3D surface plots showing the interactive factors effects on JRSSS pigment yield; temperature and fructose effect. (BC) Temperature and peptone effect. (BD) Temperature and peptone effect (CD).

reveals a synergistic effect between temperature and incubation time, with peak response at mid-range levels [24].

The standard probability plot (Fig. 4) shows that most residuals lie close to the red diagonal line, indicating approximate normality of errors—supporting ANOVA and regression assumptions [18]. Minor deviations at the tails are acceptable in experimental studies. No points exceed ±3, suggesting no significant violations of normality or systematic bias.

Model validation

Model validation was performed through shake flask experiments under optimal conditions predicted by the RSM model, following the approach of Sen *et al.* [14]. The predicted maximum response occurred at 32°C, pH 4, 0.3 g/l fructose, and 0.22 g/l peptone. Experimental validation yielded an OD_{540} of 0.603, which closely matched the predicted value of 0.689, confirming the model's reliability for the JRSSS strain under these conditions and using RSM, Design-Expert® software compared actual and predicted values to determine 32°C, pH 4, 0.3 g/l fructose, and 0.22 g/l peptone as optimal conditions for pink pigment production by the JRSSS strain. These parameters support enzymatic activity in acidic and moderate-temperature environments while providing a balanced carbon and nitrogen source for efficient biosynthesis [15]. Notably, there have been no prior reports of *F. foetens* CBS 110286 isolated in the Jalandhar region. This study is the first to report the isolation and ability of this organism to produce a pink pigment.

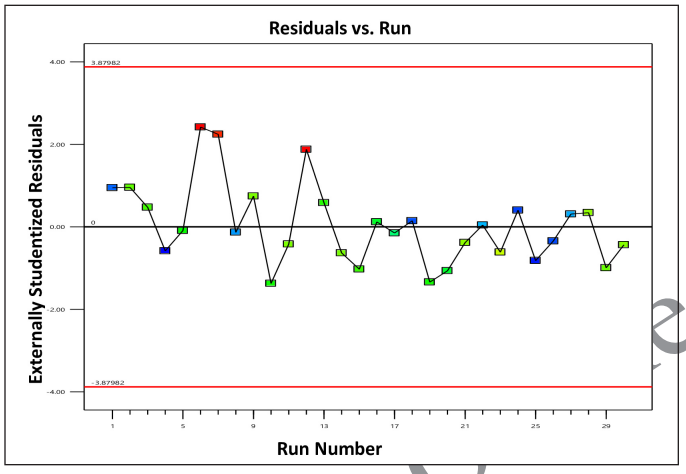

Figure 4. Illustrating the adequacy of the model fit and the normal distribution of residuals versus run plots.

Characterization of JRSSS pigments using high performance liquid chromatography

HPLC-PDA analysis of the JRSSS pigment extract revealed wavelength-dependent chromatographic profiles. At 390 nm, four peaks were observed, with the dominant compound eluting at 6.507 minutes (85.06%), indicative of UV-active metabolites, while at 470 nm, two peaks were detected, the major at 3.573 minutes (64.16%), with enhanced separation efficiency (theoretical plates: 52,301; tailing factor: 0.706). However, superior resolution was achieved at 500 nm, where the major peak accounted for 87.90% and demonstrated excellent chromatographic performance (theoretical plates: 50,139; tailing factor: 0.671) shown in Figure 5. These findings align with reports on *Monascus* and *Talaromyces* pigments showing optimal detection at 470–500 nm [35,36], highlighting 500 nm as optimal for strain quantification and prospective industrial applications.

Characterization of JRSSS pigments by Gas chromatography mass spectrophotometer (GC-MS)

GC-MS analysis of the JRSSS pigment extract revealed a complex metabolite profile comprising fatty acid esters (Hexadecanoic acid, methyl ester, 10.72%), bioactives (lidocaine, 8.09%; benzyldiethyl-(2,6-xylylcarbamoylmethyl)-

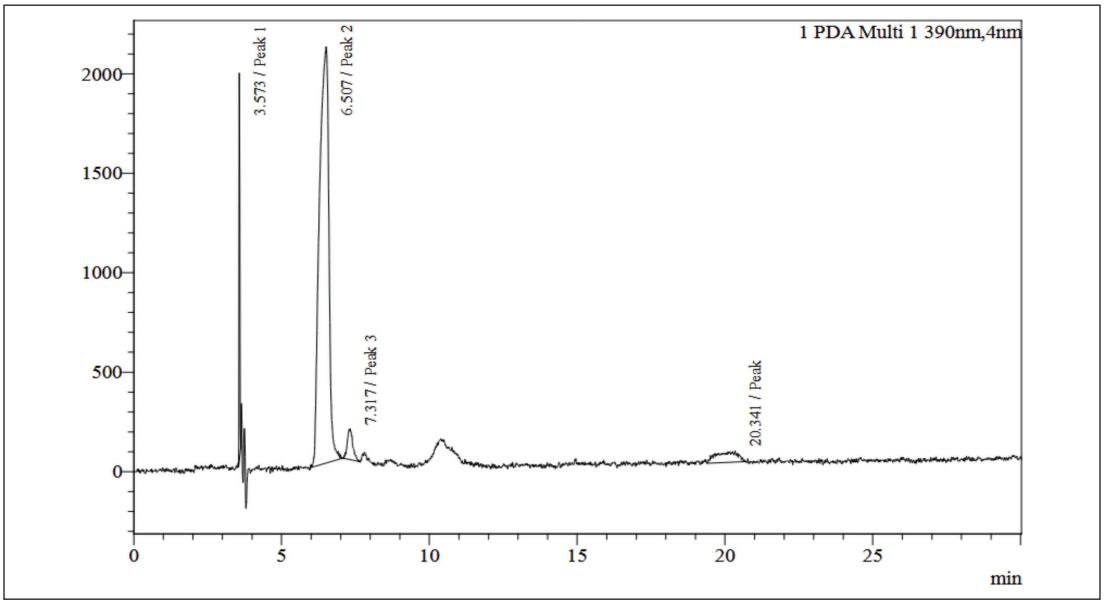

Figure 5. Illustration of chromatograph photodiode array (PDA) detection of analytes at 390 nm.

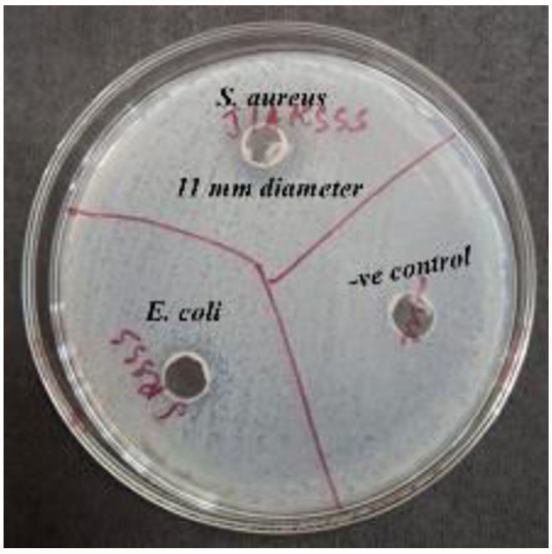

Figure 6. Antibacterial activity of pigmented fungal extract JRSSS against *S. aureus* and *E. coli*.

ammonium benzoate, 8.47%), hydrocarbons (eicosane, nonadecane), and siloxanes (Disiloxane, hexaethyl-, 16.68%) shown in Figure S1. These metabolites, known for their antioxidant, antimicrobial, and emulsifying activities, support previous findings on fungal pigment bioactivities [37]. Such chemical diversity highlights the potential of JRSSS pigment as a multifunctional natural colorant for textile, pharmaceutical, cosmetic, and food applications, upon further characterization.

Antimicrobial activity assay

The antibacterial activity of the JRSSS fungal pigment was evaluated using the agar well diffusion method on Mueller–Hinton Agar, following the protocol of Narendrababu and Shishupala [25]. A 20 µl suspension of *S. aureus* and *E. coli* was uniformly spread across the agar surface, and 8 mm wells were aseptically prepared as described by Balouiri *et al.* [2] with slight modifications. Subsequently, 50 µl aliquots of the JRSSS fungal extract were loaded into each well using a micropipette [38]. Vancomycin and Ciprofloxacin (50 µg/ml) served as +ve controls for *S. aureus* and *E. coli*, respectively, while two-fold serial dilutions (50–0.05 µg/ml) were prepared for determining the minimum inhibitory concentration (MIC). MIC values were calculated based on the lowest concentration of the pigment and standard antibiotics that completely inhibited visible bacterial growth after 24 hour of incubation at 37°C. The pigment exhibited inhibition zones of 11 mm against *S. aureus* and 8 mm against *E. coli*, as depicted in Figure 6, with a corresponding MIC of 25 and 50 µg/ml, respectively. In comparison, Vancomycin and Ciprofloxacin showed lower MICs of 1.25 and 0.625 µg/ml, reflecting their higher potency. This activity aligns with bikaverin from *F. oxysporum* (10–12 mm) [39] and exceeds Monascus pigments (~8 mm) [40], but is slightly lower than azaphilones from *Talaromyces* spp. (14 mm) [41], likely due to differences in pigment structure and bacterial cell wall permeability. The study identifies *F. foetens* as a novel producer of a pink pigment with promising biotechnological potential. Previous studies on *Fusarium* spp. pigments highlight antimicrobial, antioxidant, and anti-inflammatory activities, supporting their applicability in food, pharmaceutical, and cosmetic industries [42,43]. These findings underscore the potential of *F. foetens* pigments for industrial applications. Natural pigments are increasingly recognized in food, pharmaceutical, cosmetic, and biotechnological sectors due to their diverse bioactivities [44]. Microbial pigments exhibit antibacterial effects by disrupting cell membranes, inhibiting nucleic acid and protein synthesis, generating reactive oxygen species, and chelating essential metal ions. In addition, carotenoids and related metabolites may enhance antibacterial efficacy by altering membrane integrity and inducing oxidative stress [11,45].

CONCLUSION

This study reports, probably for the first time, the isolation of pink-pigmented *F. foetens* CBS 110286 from soil at Jalandhar Cantt railway station, Punjab, India. Pigment production was optimized using RSM, with significant enhancement observed at optimized levels of peptone and fructose. Statistical analysis validated the model's effectiveness. The crude extract demonstrated notable antimicrobial activity against *S. aureus*, indicating its potential applications in textile, pharmaceutical, and food industries upon complete characterization.

Further research will focus on advanced **chemical and structural characterization** of the pigments using spectroscopic and chromatographic methods to improve purity and stability. In addition**,** *in vivo* **studies** are essential to evaluate safety, pharmacokinetics, and antimicrobial efficacy in animal models and should be employed. Similarly, the development of **formulated products** for antimicrobial, food, and cosmetic applications should be explored, as well as optimizing **bioproduction processes**, which will be key for scalable commercial applications.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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CONFLICT OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL STATEMENT

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed during this study are presented in this article.

PUBLISHER'S NOTE

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USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that they did not utilize any artificial intelligence (AI) tools for writing or editing the submitted manuscript, and that none of the images were altered using AI.

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