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Bio-induced overproduction of heterocycloanthracin-like bacteriocin in *Lysinibacillus macroides* by *Aspergillus austroafricanus*: optimization of medium conditions and evaluation of potential applications

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Abstract

Background Plants and microorganisms are at the forefront of natural exploitable bioresources for the discovery of novel bioactive compounds (BACs) to provide solutions to food and agricultural challenges. The present study aimed to produce a novel biotechnologically-relevant BAC from a mangrove sediment bacterium under optimized bioprocess medium conditions. The BAC-producing bacteria were isolated via the crowded plate technique, and medium optimization was performed via sequential statistics of response surface methodology (RSM). The RSM model predictions were optimized, validated, and scaled up in a 5-L bioreactor via submerged batch fermentation. The BAC was extracted with ethyl acetate, purified via silica gel column chromatography, and identified via semipreparative high-performance liquid chromatography using bioactive standards with known retention times. The biocontrol, antioxidant and biopreservation potential of the BAC were evaluated via standard methods.

Results The results revealed that strain GKRMS-A9 produced the largest inhibition zone diameter (ZND) of 17 mm against the susceptible mould. The bacterium and its susceptible mould were identified as *Lysinibacillus macroides* and *Aspergillus austroafricanus* strains, respectively. Bioprocess medium optimization produced 9.6 g L^{-1} of the BAC with a ZND of 47.1 mm using 44.84% [v v^{-1}] rice processing effluent, 8.58 gL^{-1} casamino acid, 1.39 g L^{-1} $MgSO_4.7H_2O$, 2.78 g L^{-1} $CaCl_2.2H_2O$, 16.94% [v v^{-1}] inoculum volume, and 10.45 g L^{-1} Na_2HPO_4/NaH_2PO_4. The BAC concentration increased 48.7-fold in response to biological induction with susceptible mould. Silica gel chromatography revealed 9 bioactive fractions in the ethyl acetate extract, with fraction C (retention time of 9.02 min) eliciting the largest mean

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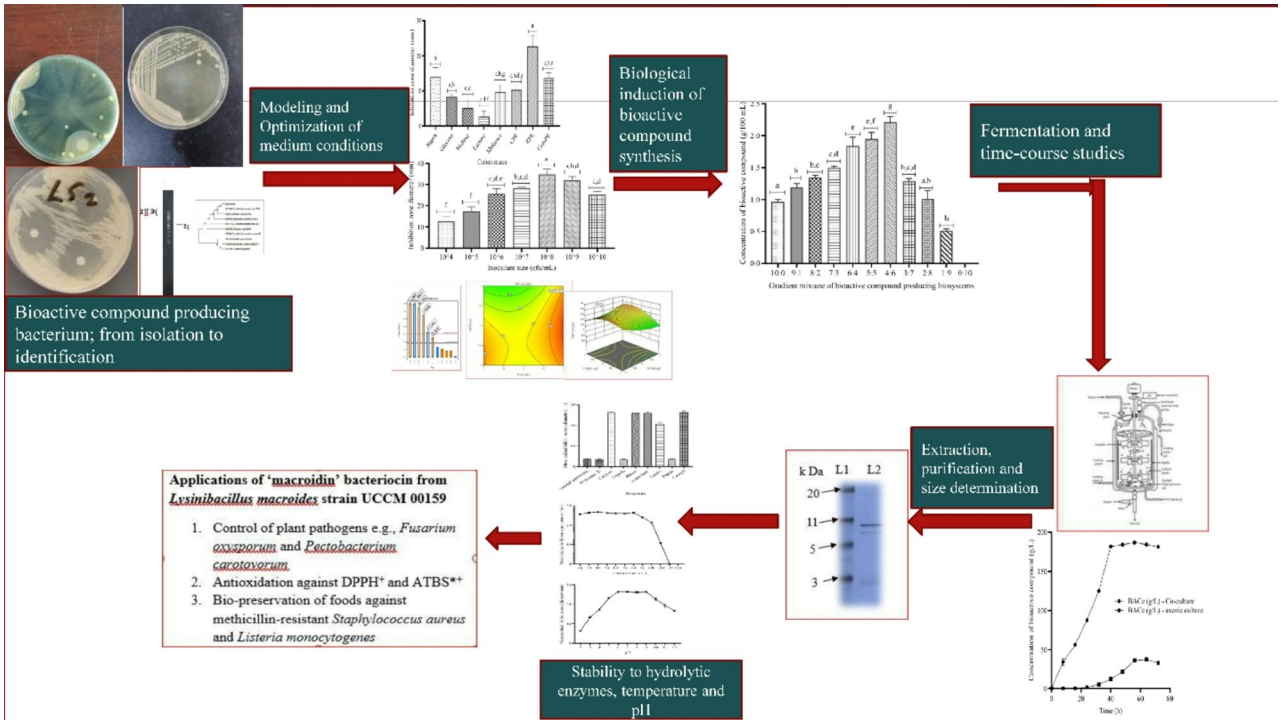
ZND of 38.1 ± 1.7 mm against *Aspergillus austroafricanus*. Fraction C was identified as a heterocycloanthracin-like class II bacteriocin with a molecular weight of 10.5 kDa.

Conclusion The bacteriocin 'macroidin' is stable over a wide range of pH values and temperatures and has significant antimicrobial activity against Gram-positive food-borne and phytopathogenic strains of bacteria and moulds. Its antioxidant activities against DPPH and ABTS^{*+} radicals are comparable to those of ascorbic acid, making this biomolecule a promising agent for biopreservation and phytopathogen control applications in the food and agricultural sectors.

Graphical abstract



Keywords Heterocycloanthracin-like bacteriocin, *Lysinibacillus macroides*, Coculture system, Antioxidation, Phytopathogen control, Biopreservation

Introduction

Microbial secondary metabolites are more commonly called specialized metabolites and possess unusual chemical structures [1]. They may be bioactive lipids, proteins or carbohydrates and have demonstrated significant effects on health, nutrition, agriculture, the environment and national economies over the years [2]. They are classified as low-molecular-weight products of secondary metabolism in microorganisms and plants that may not contribute to primary or cellular metabolism in biological systems. Many of these compounds are already on the market, such as antimicrobials, antioxidants, antitumour agents, insecticides, vasorelaxants, vasocontractants, diuretics, laxatives, colorants, and plant and animal growth promoters [3]. All biological systems produce one form of bioactive compound or another; however, approximately 53% of FDA-approved natural product-based drugs originate from microorganisms [4].

The synthesis of microbial secondary metabolites is regulated by growth conditions, including nutritional and environmental conditions, feedback control and enzyme induction and activation [5]. Environmental cues, especially competition for nutrients and space, are primary inducers of silent biosynthesis-related gene clusters, which are responsible for BAC production [6]. The application of metabolomics to industrial microorganisms offers new opportunities for better understanding, strain discovery, characterization and improvement of secondary metabolite production [7]. Thus, great potential exists for the development of novel compounds for the pharmaceutical, nutraceutical, dyeing, agricultural or food industries [8].

The surge in antimicrobial-resistant infections and the concurrent increase in multidrug-resistant organisms have jeopardized the healthcare system and threatened public health. Annually, thousands of lives are lost due to resistant infections, and without robust systems, the world will soon experience more than 10 million deaths annually [9]. Currently, increasing antimicrobial resistance has resulted in the efficacy of antimicrobials being of questionable utility. Therefore, the search for alternative antimicrobial agents has become necessary for therapy, agriculture and food preservation [8]. Some microbial metabolites serve as therapeutic leads in rational drug design studies [3].

The food industry needs antioxidant compounds, biological preservatives and colorants in food and nutraceuticals [10]. The larger agricultural sector will benefit from new plant growth-promoting hormones, biofertilizers and biocontrol agents for crop development and improvement. The global need for self-discovery, actualization and independence through local-content-driven industrialization for global assertiveness, which is the overall aim of the United Nations' 2030 Sustainable Development Goals, may be realized through studies such as this. For all these, the demand-supply curve has long since been skewed towards demand, implying a dearth of metabolites for applications in the respective areas.

The applications in sight are not feasible when product availability is not guaranteed. Strain improvement via different mutational and/or genetic manipulations has been reported to increase yield [11]. Media and environmental conditions are being optimized to elucidate the nutritional and environmental cues that drive sustained secretion by regulating the metabolic turn-on/turn-off system of the producing microorganisms. Response surface methodology (RSM) has emerged as an efficient technique to solve the industrial yield development problems associated with microbial metabolite production. The methodology involves a series of statistically designed steps that suggest, screen, identify and optimize levels of media, environmental and operational parameters that contribute significantly to increasing the yield of the bioactive compound [12]. The objective of RSM is to save time and cost, and approaches yield improvements from range-finding tests that gather information about the parameters that matter to a bioprocess via the one-factor-at-a-time (OFAT) approach. It then screens a set of suggested influencing parameters for a few significant ones via Placket–Burman design (PBD). Finally, the levels of the significant factors are optimized via central composite or Box–Behnken design of experiments (DoE) and then validated in real time.

The present study describes the rare isolation of a naturally high-yielding bioactive metabolite-producing bacterium from freshwater mangrove sediment of the Great Kwa River, Nigeria, and the bioprocess engineering effort made to optimize medium conditions for enhanced production of the bioactive compound. Evaluations of BAC applicability and functionality in the agricultural and food industries are also reported.

Materials and methods

Sampling and isolation of bioactive compound-producing strains

Five sediment samples were collected from the Great Kwa River, Nigeria, along coordinates 4.781903°N and 8.398018°E. Tenfold sequential dilutions from 10^{-1} to 10^{-3} were performed via the soil serial dilution plate method [13], and a 0.1 mL diluted aliquot of each sample was pipetted onto actinomycete agar plates with the following ingredients (g L^{-1}): sodium caseinate 2.0, L-asparagine 0.1, sodium propionate 4.0, K_2HPO_4 0.5, $MgSO_4$ 0.1, $FeSO_4$ 0.001, and agar 15, pH 8.0 (± 0.2) [14], following the spread plate technique for the selective isolation and cultivation of individual actinomycete colonies. The plates were incubated at room temperature (28 ± 2 °C) for 48–72 h. Colonies showing zones of clearance and the inhibited strains were isolated as BAC-producing bacteria and primary susceptible microorganisms (PSMs), respectively.

Secondary screening for bioactive compound production in shake flasks

The isolated colonies were further purified by subculturing via the quadrant-streak plate technique. Luria-Bertani (LB) broth cultures of purified bacteria were prepared in 100 mL Erlenmeyer flasks. The flasks were incubated at room temperature for 72 h, the culture broth was subsequently centrifuged at 3,075 × *g* for 10 min, and the cell-free culture supernatant was cold sterilized via the microfiltration membrane technique (0.22 µm; Millipore, USA). The sterile supernatants were used as crude BAC extracts against pure cultures of their corresponding PSMs via a disk diffusion assay. Only sterile culture supernatants that retained antimicrobial activity against their PSM were selected for further studies. The pH of the sterile supernatant was adjusted to 7 to eliminate organic acids, and another portion was treated with catalase to eliminate hydrogen peroxide. Isolates that retained antimicrobial activity in their culture supernatants against their PSM after these treatments were selected for further studies.

Characterization of selected bioactive compound-producing bacterium and its susceptible microorganism

The identification of selected bacterium was performed by examining its cultural, morphological and biochemical characteristics and via 16 S rRNA partial-gene

sequencing techniques. Molecular characterization was performed via DNA isolation, quantification, amplification, purification and sequencing of the 16 S rRNA gene via the following universal bacterial primers: forward, 27 F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and reverse, 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′) [15]. The PSM was characterized by morphological characteristics and molecular methods involving internal transcribed spacer (ITS) region sequencing via the following universal fungal primers: forward: ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and reverse: ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) [16]. The obtained sequences were compared with those in the National Centre for Biotechnology Information (NCBI) via the basic local alignment search tool (BLAST), and the closest relative was obtained via the neighbour-joining method in molecular evolutionary and genetic analysis (MEGA) software version 11. Purified peptidoglycan preparations were obtained by cell disruption by shaking with glass beads and subsequent trypsin digestion. The cell wall was digested with 6 M HCl at 120 °C for 16 h, and the amino acids and peptides in the hydrolysates were analysed via two-dimensional ascending thin layer chromatography via a solvent system [17]. The molar ratios of the amino acids were determined from N-heptafluorobutyryl amino acid isobutyl esters via gas chromatography-mass spectrometry (GC–MS).

Response surface modelling and optimization of bioprocess variables

One-factor-at-a-time (OFAT) variable selection.

Carbon sources, including soluble starch, glucose, maltose, lactose, molasses (1% w v^{-1}), cassava processing effluent, rice processing effluent, and corn processing effluent (1% v v^{-1}), were screened for improved BAC production in 250 mL Erlenmeyer flasks. The minimal medium modified from Bharti et al. [18] contained (in g L^{-1}) NH_4Cl (1.4), KH_2PO_4 (2.0), $MgSO_4.7H_2O$ (0.3), $CaCl_2$ (0.3), $FeSO_4.7H_2O$ (0.005), $ZnSO_4.7H_2O$ (0.0014), $CoCl_2.6H_2O$ (0.002), and $MnSO_4.5H_2O$ (0.0016). Nitrogen sources such as casamino acid, asparagine, peptone, yeast extract, KNO_3, NH_4Cl, NH_4NO_3 and urea were also screened (1% w v^{-1}). Phosphorous combinations, including K_2HPO_4/KH_2PO_4, Na_2HPO_4/NaH_2PO_4, K_2HPO_4/NaH_2PO_4, and Na_2HPO_4/KH_2PO_4, were also screened and added at 1% (w v^{-1}) to minimal medium supplemented with selected carbon and nitrogen sources. The inoculum size, prepared by adjusting the McFarland standard to 0.5, was screened within the range of 10^4-10^{10} cfu mL^{-1} at 2% (v v^{-1}). In all the cases, the response variable was the inhibition zone diameter, ZND (mm). Triplicate determinations were analysed in GraphPad Prism 8 via ordinary one-way analysis of variance (one-way ANOVA), and significant means were separated by Tukey's HSD multiple comparisons test at the 5% significance level.

Plackett–Burman design screening for significant variables

A mixture of 11 nutrients, major and trace, comprising rice processing effluent, casamino acid, Na_2HPO_4/NaH_2PO_4, inoculum volume, $MgSO_4.7H_2O$, KCl, $CaCl_2.2H_2O$, $ZnCl_2$, $MnCl_2$, $NiCl_2$, and $FeSO_4.7H_2O$, was screened via Placket–Burman design (PBD). Each parameter was tested at 2 levels, high (+) and low (-), as presented in the design matrix in Table S1. A first-order model was built via ANOVA-derived coefficients of significant variables as follows:

$$y = b_0 + \sum b_i x_i + \sum b_{ij} \sum x_i x_j + \varepsilon \qquad (1)$$

where y is the response variable, ZND (mm), b_0 is the coefficient of the constant term, b_i is the coefficient of the linear term, b_{ij} is the coefficient of the interaction terms, and ε *is* the error term. Model curvature was investigated by introducing centre points in the design.

Path of steepest ascent experimentation

Using the coefficients of the significant main effects in the first-order model from the PBD, the levels of the significant factors were gradually adjusted through a series of experiments to approach the optimum region. Since one unit (coded) of steepest ascent point away from the centre is desired to maximize the response, y, the coordinates of the significant factor levels were determined by first calculating the slope of the model using the largest absolute regression coefficient, βj, in the model and denoted Δxj as step size 1 [12]. Other step sizes were then calculated as follows:

$$\Delta x_i = \frac{\beta_i}{(\beta j/\Delta xj)},\ i = 1,\ 2\ \ldots\ .ki \neq j \qquad (2)$$

The coded step sizes were finally converted to natural levels via the relationship between the coded and natural levels as follows:

$$dX_i = S_i(\rho), i = 1,\ 2\ \ldots\ .k \neq j \qquad (3)$$

where dXi is the change in the natural level of the predictor variables, Δxi is the change in the coded level of the predictor variables, S is the slope with respect to each predictor, and ρ is the natural level change (proportion) in the predictor, j, with the largest absolute coefficient, β.

Fermentation was conducted as previously described, and the mean ZND was the response variable. All the data were collected in triplicate, the results were analysed with GraphPad Prism 8, and the means were plotted as

bar graphs with standard deviations as error bars. Significant means were separated by Tukey's HSD post hoc test at the 5% significance level.

Response surface modelling

The factor levels that yielded the largest mean ZND in the PSA were considered centre points (0, 0, 0, 0, 0, 0) for response surface modelling via central composite rotatable design (CCRD) in Design Expert software version 13.0 (Stat-Ease Inc., Minneapolis, USA). The matrix of the CCRD highlighting the coded and actual levels of the 6 significant variables is presented in Table S2. The variables were coded as follows: rice processing effluent (X_1 - % v v^{-1}), casamino acid (X_2– g L^{-1}), Mg^{2+} (X_3– g L^{-1}), Ca^{2+} (X_4– g L^{-1}), inoculum volume (X_5 - % v v^{-1}) and Na_2HPO_4/NaH_2PO_4 (X_6– g L^{-1}). The design matrix included 8 centre points, 12 axial points and 32 factorial points in a total of 52 experimental runs. Two responses, the ZND (Y_1 - mm) and the concentration of BAC (Y_2– g L^{-1}), were measured. Fermentation was conducted in 500 mL Erlenmeyer flasks containing one-fifth of the reaction volume, and the flasks were incubated at 150 rpm and 30 °C for 72 h. Nonsignificant medium variables were added at the levels used in the PBD experiment.

The culture broths were centrifuged post-fermentation at 14,489 × *g* for 10 min, and the supernatant was sterilized by filtration (0.22 µm, Millipore). Preliminary evaluation of the best extraction solvent was performed via a battery of solvents at a ratio of 1:1 (v v^{-1}). The solvents used included chloroform, methanol, ethanol, ethyl acetate, petroleum ether and acetone. The extracts were evaporated to dryness via rotary evaporation at 40 °C and quantified via gravimetry [19]. The antimicrobial activity of the dried extracts was evaluated against the PSM by the disk diffusion method at 1 µg $disk^{-1}$ (8 mm), which was prepared by dissolving 1 mg mL^{-1} of extract in dimethylsulfoxide (DMSO) [20]. Three replications of the experiment were set up. The means of the ZND were compared via one-way ANOVA. The solvent that recovered the most BAC was used for all subsequent extractions.

The results were analysed via multiple regression, and models were built via the method of least squares via the general quadratic function below:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i<j=2}^{k} \beta_{ij} x_i x_j + \varepsilon \quad (4)$$

where β_0 denotes a constant coefficient, k = the k^{th} factor, β_{ii} = the quadratic effect of the i^{th} factor and β_{ij} = the effect of the interaction between the i^{th} and j^{th} factors; x_1, x_2……, x_k are the independent variables; and ε is the error arising from the computation of the response variable Y.

Multiobjective optimization of the CCRD-RSM models

The conditions predicted by both CCRD-RSM models were simultaneously optimized by the response optimizer in Design Expert, with all the input variables set to 'in range' and the importance left at 'moderate'. The response variables were set to 'maximum' with their importance at the 'highest level'. The optimal conditions were selected by the desirability function. The actual variable levels were calculated from the coded settings via the expression below:

$$Coded\ value = \frac{x - \left(\frac{h+l}{2}\right)}{\left(\frac{h-l}{2}\right)} \quad (5)$$

where x is the actual value, h is the high actual level, and l is the low actual level.

Validation of optimized bioprocess conditions

The calculated actual levels were validated via real-time fermentation in 500 mL Erlenmeyer flasks. The responses obtained were compared with those predicted by the response optimizer, and only differences ≤ 5% were accepted as reliable and adopted for fermentative production of the BAC.

Bench-scale bioreactor production of bioactive compound in optimized medium

The fermentation medium contained all the components used in the PBD screening except that rice processing effluent, casamino acid, $MgSO_4.7H_2O$, $CaCl_2.2H_2O$, and phosphates (Na_2HPO_4/NaH_2PO_4) were supplied at their validated optimized levels.

The BAC-producing bacterium was prepared by inoculating purified bacterium on freshly prepared tryptic soy agar and incubating for 24 h at room temperature. One discrete colony was transferred aseptically to LB broth in a test tube and incubated for 18 h. Five millilitres of 18-h-old LB broth culture were transferred to 15 mL of similar medium in a 100 mL Erlenmeyer flask and incubated on an orbital shaker (150 rpm) for 18 h at 30 °C. The resulting 20 mL was transferred to freshly prepared (121 °C, 15 psi, 15 min) 80 mL LB in a 500 mL Erlenmeyer flask, which was subsequently incubated on an orbital shaker (150 rpm) at 30 °C for 18 h. The 100 mL LB broth culture was harvested by centrifugation (4,025 × *g*) for 10 min and washed twice to remove the spent LB ingredients.

The susceptible mould was prepared in Czapek-Dox agar (CDA) in Roux bottles and incubated at 35 °C for 14 days. By gently inverting the bottle, the spores were dislodged from mycelia after 20 mL of cold distilled water mixed with glass beads was added [12]. The suspension was washed twice with cold water by centrifugation at 3,075 × *g* for 5 min. A concentrated spore suspension was prepared by resuspending the washed spores in 3 mL of

cold water. A test concentration of 10^7 spore-forming units per millilitre (sfu mL^{-1}) was prepared from the concentrated stock solution by spectrophotometry and plate counting.

The optimized inoculum volume (% v v^{-1}) was prepared as a gradient mixture of washed suspensions of bacterium and mould and as their axenic cultures at the following ratios: 10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, and 0:10 (% v v^{-1}). The sterile medium served as the '0' portion for the axenic cultures of the producing and primary test organisms. These preparations were used to inoculate fermentation medium containing optimized levels of significant and unoptimized levels of nonsignificant variables in 500 mL Erlenmeyer flasks. The flasks were incubated for 72 h in an orbital shaker (150 rpm) at 30 °C. The bioactive compounds were extracted as previously described, quantified via gravimetry and tested against susceptible mould. The mean ZND (mm) and BAC concentration (g L^{-1}) were compared via one-way ANOVA, and significant means were separated via Tukey's HSD multiple comparisons test at the 5% significance level.

The seed bacterial–fungal mixture with the highest concentration of BAC was used to inoculate production medium at the CCRD-RSM-validated optimized level in a 5-L bench-scale bioreactor (BioStat B, Sartorius, Germany) for 72 h. The bioreactor was operated in batch mode with a working volume of 3 L for 72 h. The pH, agitation speed and dissolved oxygen content were maintained at 7.0 ± 0.2, 150 rpm and 50%, respectively. Filter-sterilized air was allowed to flow into the headspace of the vessel at a rate of 1 L min^{-1}, which was controlled by a mass flow controller. The medium was sterilized by a single in-place sterilization technique and the bioreactor allowed to cool to nearly 30 °C before inoculation with the optimized level of seed bacterial–fungal coculture as inoculum (% v v^{-1}). At 8-h intervals, triplicate determinations of the crude BAC concentration were made from the supernatants of the fermentation broth.

Extraction and purification of the bioactive compound

The BAC was extracted as previously described and purified via silica gel chromatography equipped with a vacuum liquid chromatography column. A stepwise gradient solvent system of petroleum ether (PE)-CH_2Cl_2 (7:3, 3:7 and 0:1) was used, followed by CH_2Cl_2-methanol (99:1, 49:1, 19:1, 9:1, 4:1, 1:1, and 0:1) to obtain several fractions. Each fraction was tested for antimicrobial activity, and the fractions with antimicrobial activity were pooled and subjected to Sephadex LH-20 column (120 × 2 cm) chromatography with CH_2Cl_2-methanol (1:1) as the mobile phase. The eluted bioactive fraction was finally purified separately via a semipreparative HPLC column (Cosmosil 5C18-MS-II, 250 × 10 mm, 5 µM) with different concentrations of methanol in H_2O. The amount of protein in the fraction was quantified via the Bradford protein assay [21], carbohydrates were quantified via the di-nitro-salicylic (DNS) acid method [22], and lipids were quantified via Folch et al. [23].

Tricine sodium dodecyl sulfate–polyacrylamide gel electrophoresis to determine the molecular weight

The molecular weight of the BAC in this study was determined via tricine–sodium dodecyl sulfate-polyacrylamide gel electrophoresis (tricine SDS-PAGE) via the protocol of Schagger and von Jagow [24]. The protein ladder included proteins with molecular weights ranging from 3 to 24.5 kDa. The purified BAC was loaded twice onto a 16.5% gel and allowed to migrate, after which the gel was stained with Coomassie Brilliant Blue R-250 dye (Research Organics, Cleveland, USA).

Amino acid profiling and in silico characterization of the bioactive compound

The amino acid sequence of the purified bioactive compound was analysed with an amino acid analyser (Agilent 1100 HPLC, USA) [25]. The obtained sequence was subjected to in silico analysis via the ExPASy ProtParam tool [26] to determine the major physicochemical characteristics of the protein portion of the bioactive compound. The important physicochemical parameters of the peptide evaluated were molecular weight, charge, polarity, hydrophobicity, isoelectric point (pI), extinction coefficient, half-life, instability index, aliphatic index and grand average hydropathicity (GRAVY).

Stability of BAC to enzymes, pH and temperature

The stability of the purified BAC was evaluated for lipase, neutral proteinase, trypsin, pepsin, proteinase K, RNase, catalase and α-amylase (Sigma Aldrich, USA) at their optimum pH values. A solution of the purified BAC in DMSO (1 mg mL^{-1}) was mixed with 1 mg mL^{-1} of the above enzymes at 9:1 (vv^{-1}), incubated at 30 °C for 1 h, and held in a water bath at 70 °C for 5 min [27] before antimicrobial susceptibility testing. The BAC in DMSO was also exposed to different pH values ranging from 3 to 10, and to temperatures ranging from 20 to 121 °C before antimicrobial activity was measured. Evaluations were performed in triplicate, and the results are expressed as the mean ZND ± standard deviation. All evaluations were made against the primary susceptible microorganism (PSM).

Evaluation of antimicrobial susceptibility and minimum inhibitory concentration of BAC

Antimicrobial susceptibility testing was conducted according to the protocols established by EUCAST [28]. The minimum inhibitory concentration (MIC) of the

BAC in this study was evaluated via the broth microdilution method against its PSM, *Fusarium oxysporum* ATCC 16,322, *Aspergillus flavus* ATCC 21,882, *Saccharomyces cerevisiae* UCCM 00054, *Listeria monocytogenes* ATCC 23,074, *Staphylococcus aureus* ATCC 33,591, *Erwinia carotovora* ATCC 39,048 and *Pseudomonas stutzeri* NCTC 12,262. All strain suspensions were adjusted to McFarland Standard 0.5 (1.5×10^8 cfu mL^{-1}), from which cell or spore densities of 5×10^5 cfu mL^{-1} (or sfu mL^{-1} in the case of moulds) were prepared and used as test inoculum. The BAC was dissolved in DMSO (1 mg mL^{-1}) and diluted to 500, 250, 125, 62.5, 31.3, 15.6, 7.81, 3.91, 1.95, 0.98 and 0.49 µg mL^{-1} concentrations via two-fold dilutions in a 96-well plate. Whereas chloramphenicol served as a positive control for antibacterial activity, nystatin served as a positive control for antifungal activity. DMSO served as a negative control in both cases.

Evaluation of the in vitro antioxidant potential of the bioactive compound

The antioxidant potential was investigated by determining the free-radical scavenging potential via the protocol described by Mihooliya et al. [29]. L-ascorbic acid (Sigma–Aldrich, USA) served as a standard antioxidant. The BAC and the standard were both evaluated at concentrations of 0.49 to 500 µg mL^{-1}. The change in colour of 2,2'-diphenyl-1-picryl-hydrazylhydrate (DPPH) from deep violet to pale yellow monitored via a UV–Vis's spectrophotometer (DR6000, HACH, Loveland, CO) at a wavelength of 517 nm for 3 h was indicative of antioxidant activity. Evaluations were performed in triplicate. The antioxidant activity was calculated as follows:

$$\%\ Scavenging\ of\ DPPH = \frac{A_0 - A_1}{A_0} \times\ 100 \quad (6)$$

where A_0 is the absorbance of the blank and A_1 is the absorbance of the test sample/ascorbic acid.

Additionally, the scavenging activity of the purified BAC was also tested against the dark blue 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) radical cation ($ABTS^{*+}$), as reported by Liu et al. [30]. Briefly, a solution of decreasing concentrations (µg mL^{-1}) of ABTS* cation, with an absorbance of 0.705 ± 0.04 at 734 nm, was mixed with ABTS diluent and gently shaken. Subsequently, 10 µL of pure BAC (0.49 to 500 µg mL^{-1}) was added to the reaction mixture and allowed to stand for 6 min in the dark at room temperature. The absorbance drop was measured at a wavelength of 734 nm via a microplate reader (Thermo Scientific, USA). The scavenging rate (%) was used to evaluate the $ABTS^{*+}$ scavenging capacity of the BAC as given by the expression:

$$\%\ Scavenging\ of\ ABTS^{*+} = \frac{A_0 - A_1}{A_0} \times\ 100 \quad (7)$$

where A_0 is the absorbance of the blank and A_1 is the absorbance given by test ($ABTS^{*+}$)/control (ascorbic acid).

A ferric (Fe^{3+})-reducing antioxidant power (FRAP) assay of the BAC was also performed to determine total antioxidant activity [31]. Briefly, the FRAP reagent was first prepared by mixing 1 mL of 0.01 M TPTZ (2,4,6-tripyridyl-5-triazine) (Fluka Chemicals, Switzerland) solution prepared in 0.04 M HCl) and 1 mL of 0.02 M $FeCl_3.6H_2O$ (Sigma–Aldrich, USA) aqueous solution with 10 mL of 0.3 M acetate buffer (pH 3.6). The reagent was then incubated for 30 min at 37 °C, and 0.95 mL was added to 0.05 mL of 0.49 to 500 µg mL^{-1} BAC sample. The mixture was incubated at room temperature for 5 min, and the absorbance of the developed intense blue colour was subsequently measured at 593 nm via a UV–Vis's spectrophotometer (DR6000, HACH, Loveland, CO). Triplicate determinations were made for each concentration, and the mean absorbances were used to calculate the FRAP value via a calibration curve prepared with $FeSO_4$ (Sigma–Aldrich, USA) in the range of 5–100 nM. The antioxidant power of the BAC was expressed as millimolar equivalents of $FeSO_4$. The FRAP value was expressed as follows:

$$FRAP\ value = \frac{D}{V} \times\ F \quad (8)$$

where D is the dilution factor of the reaction mixture to fit into the calibration curve range, V is the volume of sample expressed in µL, and F is the amount of ferrous sulphate in nM.

Determination of the biopreservation potential of the antimicrobial compound

The method of Chopra et al. [32] was adopted with modifications in terms of the nature and number of test organisms, concentration of the BAC and duration of incubation. A mixture of fresh fruit juice (orange and pineapple) was filter sterilized (0.2 µm, Millipore, USA), and 99 mL of juice was inoculated separately with 1 mL of 5×10^5 cfu mL^{-1} overnight LB cultures of *Staphylococcus aureus* and *Listeria monocytogenes.* Initial counts of inoculated bacteria were recorded, followed by the addition of purified BAC at different concentrations. The fruit juice mixture was stored in a refrigerator at 4 °C for 120 d, and 5 mL samples were withdrawn every 30 d and plated on appropriate media (tryptic soy agar for bacteria and yeast-extract-peptone agar for yeast). Counts were recorded and compared with those of the control (fruit

juice without BAC) via ordinary one-way ANOVA in GraphPad Prism 8 software.

Results and discussion

Isolation and selection of the producing bacterium

Only 11 bacterial isolates from the Great Kwa River mangrove sediment (GKRMS) demonstrated antimicrobial activity, with one isolate (A9) having antimicrobial activity with an inhibition zone diameter (ZND) of 17 mm that was due neither to acidity nor to hydrogen peroxide (Fig. 1a). Figure 1b shows a pure culture of the producing bacterium on tryptic soy agar with creamy pigmentation, raised elevation, a round form and an irregular margin. Other characteristics included being Gram positive, rod shaped, catalase positive, motile, oxidase positive, Voges–Proskauer positive and indole production positive. The bacterium grew well in media without NaCl but also tolerated 3.5% salt. It grew well at temperatures between 20 and 40 °C and strongly in air. Interestingly, the bacterium did not react to the battery of reactions in the API 50 CHB test Kit but positively utilized raffinose and glucose oxidatively.

Molecular analysis of the bacterium revealed 38.18% G + C content (by HPLC), 1502 bp of the 16 S rRNA partial-gene sequence, and > 99% sequence identity with *Lysinibacillus macroides* strain PF29X; GenBank number: MK574961.1 (https://www.ncbi.nlm.nih.gov/nuccore/MK574961.1/) (Fig. 1c). The bacterium shares the A4 α-L-Lys-D-Asp peptidoglycan with type strains of *Lysinibacillus*, confirming its generic status. This peptidoglycan type, which features lysine and aspartic acid as diagnostic amino acids, is also common among *Kurthia* species, but the producing bacterium was quickly distinguished from *Kurthia* species by its ability to form subterminal endospores in 36 h [17].

A pure culture of the primary susceptible mould (PSM) grown on malt-extract yeast-extract agar at room temperature rapidly produced a mat of green conidia. The reverse of the plate appeared yellowish orange, but the mould showed spherical biseriate conidial heads with phialides (3.5 × 4.0 µm) in light microscopy after being stained with cotton blue dye in lactophenol. The PSM was identified as *Aspergillus austroafricanus* by ITS region sequencing (Fig. 1d). Mahmoud et al. [33] reported the production of mycotoxins from a strain of *Lysinibacillus macroides*, which they isolated from yellow corn and corn flakes. A strain very close to *Lysinibacillus xylanilyticus* and *L. sphaericus* was reported by Ahmad et al. [34] to produce bacteriocin with both antibacterial and antifungal activities. These findings indicate that the genus *Lysinibacillus* is not new to bioactive metabolite production. The producing bacterium and its PSM, along with their sequences, were deposited with the University of Calabar Collection of Microorganisms (UCCM) and named *Lysinibacillus macroides* strain UCCM 00159 and *Aspergillus austroafricanus* strain UCCM 00160 (https://ccinfo.wdcm.org/details?regnum=652).

One-factor at-a-time screening

Rice processing effluent (RPE) mediated the greatest inhibitory effect on the PSM, with a mean ZND of 22.67 ± 3.22 mm (Fig. 2a), indicating a 1.33-fold improvement in bioactivity. One-way analysis of variance revealed that the inhibition mediated by RPE was significantly different ($p = 0.05$, $R^2 = 0.9195$) from that mediated by other carbon sources. Rice processing effluent was selected as the best carbon source for further studies and was chemically characterized (Table S3, Supplementary material). The effluent was composed of high levels of biological and chemical oxygen demand, suggesting high levels of utilizable organic carbon to support bacterial growth [35]. The production of microbially-derived value-added metabolites from agro-industrial wastes has been heralded as a cost-effective approach toward a sustainable circular bioeconomy and environmental waste management [36].

Casamino acid was selected as the best nitrogen source (Fig. 2b), as it mediated a mean ZND of 28.33 ± 2.52 mm ($R^2 = 0.9480$, $p < 0.05$), indicating a 1.25-fold increase from the carbon source screening and a 1.67-fold increase from the baseline. It is the hydrochloric acid digest of casein containing a fantastic amino acid blend that presents ammonium ions for protein, nucleic acid and ATP biosynthesis, which drive the production of microbial products [37].

The pairing of Na_2HPO_4/NaH_2PO_4 emerged as the best source of phosphorus for the synthesis of ATP and nucleic acids in bacteria and provided the best buffering capacity to resist drastic changes in the pH of the medium (Fig. 2c). This phosphorus source led to a mean ZND of 30.67 ± 1.12 mm, which represented a 1.804-fold increase from baseline. The significant model adequacy of R^2 of 0.9247; $p < 0.00001$ selected Na_2HPO_4/NaH_2PO_4 (2:1) for further studies [38].

The inoculum size of 10^8 cfu mL^{-1} was not significantly different from that of 10^9 cfu mL^{-1}, as the ZND values were 34.67 ± 2.52 mm and 32.00 ± 1.73 cm, respectively (Fig. 2d). The careful selection of inoculum size is critical for microbial fermentation [39]. Care is taken not to use inoculum sizes that are too low or too high, both of which have tremendous reductive effects on product yield. At high inoculum sizes, a phenomenon called the inoculum effect ensues, which is mediated by quorum sensing. Low inoculum sizes contribute significantly to long fermentation cycles and low productivity and may increase the likelihood of contamination [40]. The optimal inoculum size for fermentation is a function of the

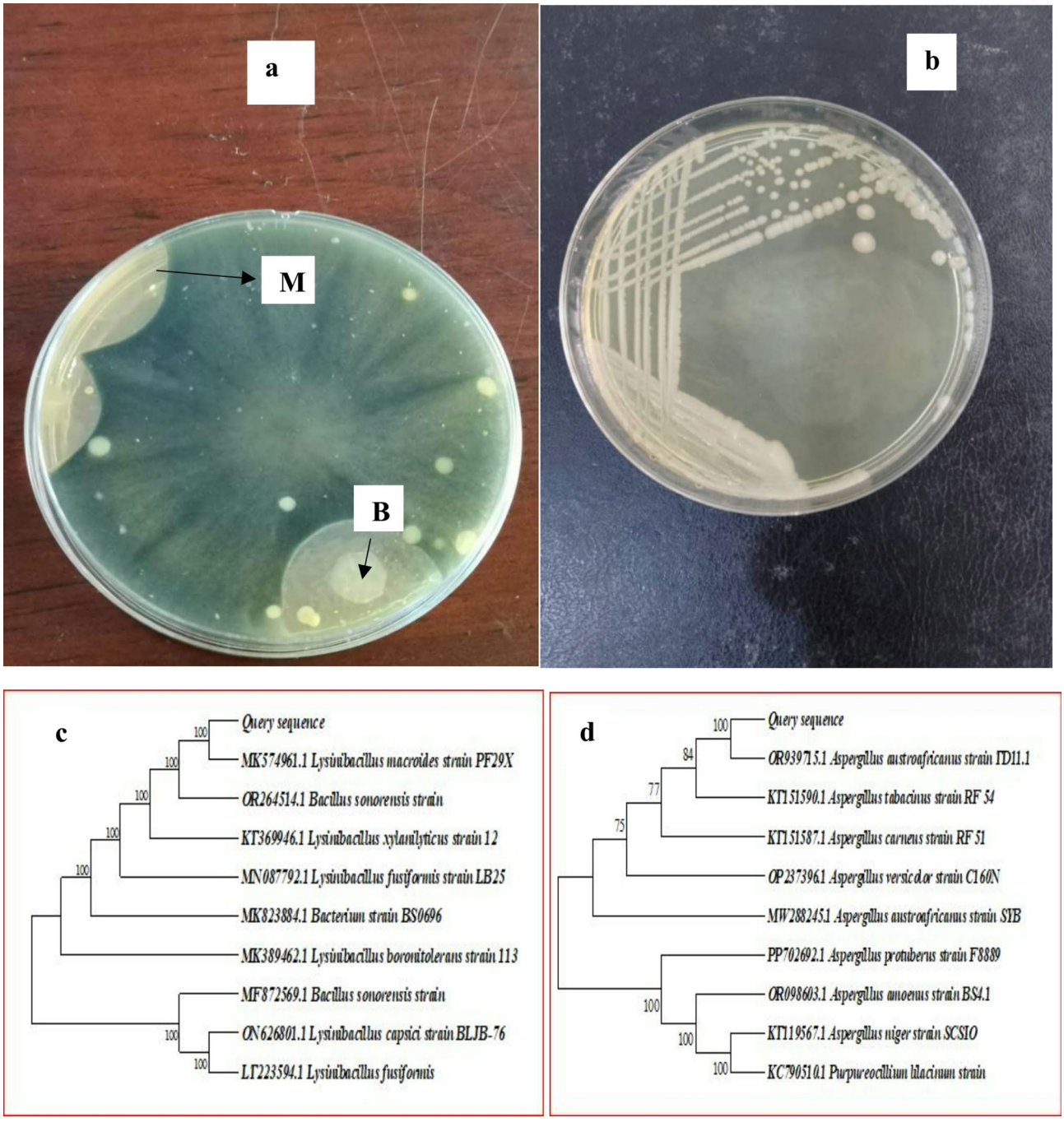


Fig. 1 (**a**) Primary isolation plate showing bioactive compound-producing bacterium (**B**) and the primary susceptible mould (**M**); (**b**) Bioactive compound producing isolate on tryptic soy agar at 24 h (**c**) Phylogenetic tree showing the query sequence of *Lysinibacillus macroides* in relationship with close relatives (**d**) Phylogenetic tree showing the query sequence of the primary susceptible mould *Aspergillus austroafricanus* in relationship with close relatives

nature of the producing organism and product of interest [41].

Plackett–Burman screening for significant variables

The results of the PBD revealed that 6 factors significantly contributed to improved production of the BAC. The highest ZND of 43.0 mm was observed in run 15, representing a 1.24-fold improvement from OFAT and a 2.53-fold improvement from baseline (Table S1). The model was significant ($F = 45.58$, $p < 0.0001$), with an adjusted R^2 of 94.69% and a predicted R^2 of 85.24%, both of which were in reasonable agreement (Table S4). The model had a nonsignificant curvature of $F = 3.77$; $p = 0.0842 > 0.05$, suggesting that a path of steepest ascent

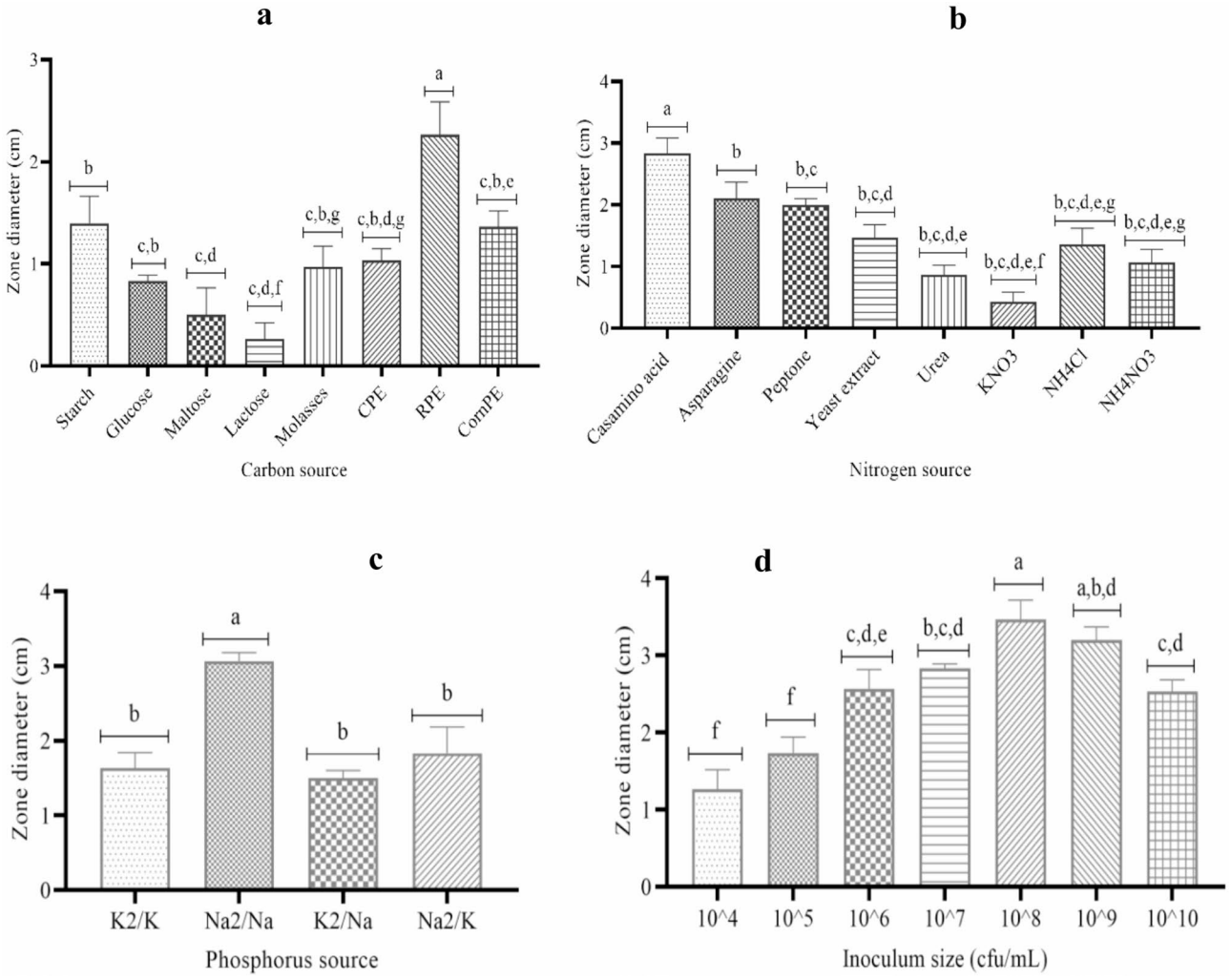


Fig. 2 OFAT screening of carbon (**a**), nitrogen (**b**), phosphorus (**c**) sources and inoculum sizes (**d**) bioactive compound production by *Lysinibacillus macroides* strain UCCM 00159; figure bars are means of triplicate determinations and error bars are standard deviations from the means; bars with similar alphabets indicate non-significant means and dissimilar alphabets indicate significantly different means at 5% significance level

(PSA) had to be conducted to build up to the RSM [42]. The first-order model for BAC production via the PBD is presented in Eq. 9:

$$y = 2.66 + 0.18RPE + 0.44CAA - 0.48Mg^{2+} - 0.23Ca^{2+} + 0.48INV + 0.38PO_4{}^{2-} \quad (9)$$

where y is the zone diameter (ZND), RPE is the rice processing agent, CAA is the casamino acid, and INV is the inoculum volume.

Path of the steepest ascent

When all other factors are held constant, a one-unit change in a particular factor results in a certain change in the entire response, which is represented by the coefficient estimates in the PBD. These coefficients are adjusted around the average (intercept) on the basis of factor settings. The results of the PSA experiment are presented in Table 1. Experiment 5 resulted in the largest inhibition zone diameter of 46.7 ± 0.20 mm, where variable levels were set at 44.72% (v v^{-1}) rice processing effluent, 10.44 g L^{-1} casamino acid, 1.50 g L^{-1} $MgSO_4.7H_2O$, 2.54 g L^{-1} $CaCl_2.2H_2O$, 12.00% (v v^{-1}) inoculum volume, and 9.26 g L^{-1} Na_2HPO_4/NaH_2PO_4 (2:1). PSA improved the bioactivity from 17 mm in the crowded plate to 46.7 mm (4.61-fold increase), which was 1.82-fold greater than that of PBD.

CCRD-RSM modelling

The results of the extraction of the bioactive compounds via different solvents revealed that the highest mean amount of extract (8.4 g L^{-1}) was obtained via ethyl acetate, followed by 7.1 g L^{-1} via petroleum ether (Table 2). There are several reports on the extraction of

Table 1 Path of steepest ascent experimentation leading to optimum Inhibition zone diameter of bioactive compound

Significant factors from first-order model	x_1	x_2	x_3	x_4	x_5	x_6	Response
[a] Base point (coded)	0	0	0	0	0	0	
[b] Base point (actual)	30	3	3.5	3.5	2	4.5	
[c] Original step unit	10	2	0.5	0.5	2.5	1.5	
[d] Coefficient, β	0.175	0.442	-0.475	-0.225	0.475	0.375	
[e] Steepest ascent step distance, Δ (coded)	0.368	0.931	-1	-0.474	1	0.79	
[f] Steepest ascent step distance, Δ (actual)	3.68	1.86	0.5	0.24	2.5	1.19	ZND (mm)
Exp 1 (origin)	30	3	3.5	3.5	2	4.5	37.5 ± 0.0
Exp 2 (origin + Δ)	33.68	4.86	3	3.26	4.5	5.69	39.2 ± 0.1
Exp 3 (origin + 2Δ)	37.36	6.72	2.5	3.02	7	6.88	42.8 ± 0.2
Exp 4 (origin + 3Δ)	41.04	8.58	2	2.78	9.5	8.07	45.9 ± 0.1
Exp 5 (origin + 4Δ)	**44.72**	**10.44**	**1.5**	**2.54**	**12**	**9.26**	**46.7 ± 0.2**
Exp 6 (origin + 5Δ)	48.4	12.3	1	2.3	14.5	10.45	42.1 ± 0.1
Exp 7 (origin + 6Δ)	52.08	14.16	0.5	2.06	17	11.64	36.7 ± 0.2

x_1 = rice processing effluent; x_2 = casamino acid; x_3 = $MgSO_4.7H_2O$; x_4 = $CaCl_2.2H_2O$; x_5 = inoculum volume; x_6 = Na_2HPO_4/NaH_2PO4; [a] = Coded levels of variables used in the Plackett-Burman design (PBD) experiment that gave the highest inhibition zone diameter (ZND) which have now assumed the levels of center points (0,0,0,0,0,0) in PSA; [b] = Actual levels of variables used in the PBD experiment that gave the highest inhibition zone diameter (ZND) now used as levels at center points; [c] = The guessed variable step distances used in PBD experiments; [d] = coefficients of significant variables used to build the first-order PBD model; [e] = Coded step units calculated from slope of first-order model; [f] = Actual step distance calculated using the relationship between coded and actual levels (Myers et al. 2009); Exp. = Path of steepest ascent experiments

bioactive compounds from fermentation broth by ethyl acetate, mostly because of its more compatible polarity and extraction efficiency [19, 43]. Chloroform extracted the least amount of bioactive compound (3.6 g L^{-1}) from fermentation broth. One-way ANOVA revealed that the BAC concentration extracted with ethyl acetate was significantly greater ($p < 0.05$) than those extracted with all other solvents and were used for CCRD-RSM modelling and optimization.

The results of BAC production and the corresponding ZND as directed by CCRD-RSM are presented in Table 3, and the ANOVA results are presented in Tables S5a and b for the ZND (Y_1) and BAC concentration (Y_2) models, respectively. Analysis of variance for model Y_1 yielded an adjusted R^2 of 0.8971 and a predicted R^2 of 0.7146. The model itself was significant at $F = 14.73$, $p < 0.0001$, with a nonsignificant lack-of-fit statistic of $F = 0.6723$, $p = 0.7629 > 0.05$, suggesting model adequacy. The BAC concentration (Y_2) model revealed an adjusted R^2 of 0.8261 and a predicted R^2 of 0.6331. This model was also significant at $F = 9.97$, $p < 0.0001$, with a nonsignificant lack-of-fit statistic of $F = 0.4843$, $p = 0.8943 > 0.05$ relative to the pure error. All linear terms contributed significantly to the two models. However, some two-way interactions did not make significant contributions to either of the two models. Figure 3 shows the two-way interactions that contributed significantly to the Y_2 model. Since more than 50% of the model terms were significant, model reduction was not necessary, and the suggested model could be used to navigate the design space, as suggested by its adequate precision.

Table 2 Solvent extraction of crude bioactive compound and their corresponding Inhibition zone diameters against primary susceptible mould, *Aspergillus Austroafricanus* strain UCCM 00160

S/N	Solvent	Amount of extract (g/L)	Inhibition zone (mm) ± standard deviation
1	Chloroform	3.6 ± 0.0	31.6 ± 0.2
2	Ethanol	4.9 ± 0.3	36.2 ± 0.5
3	Methanol	5.6 ± 0.0	32.3 ± 0.5
4	Ethyl acetate	8.4 ± 0.2	44.1 ± 0.8
5	Petroleum ether	7.1 ± 0.4	27.6 ± 0.1
6	Acetone	6.7 ± 0.3	25.5 ± 0.3

A sensitivity analysis of the bioprocess with respect to the Y_2 model revealed that the model terms could be arranged in order of significance as follows: X_3^2, X_4^2, $X_2 \times_3$, X_5^2, $X_1 \times_4$, X_2^2, X_5, $X_4 \times_5$, $X_4 \times_6$, X_3, $X_2 \times_6$, X_4, X_1, X_6, and X_2. The above order reveals that the quadratic term of Mg^{2+} (X_3^2) made the largest contribution (first-order sensitivity) to the bioprocess and that the linear term of casamino acid (X_2) had the least significant effect on the bioprocess. The model itself indicates that increasing the concentrations of casamino acids, Mg^{2+}, Ca^{2+}, and phosphates while decreasing the rice processing effluent and inoculum volume increases the yield of bioactive compounds. The second-order sensitivity given by the significant two-way interaction terms (Fig. 3) revealed that the casamino acid/Mg^{2+} interaction ($X_2 \times_3$) made the most significant contribution to the Y_2 model ($F = 60.11$, $p < 0.0001$). These findings suggest that increased synthesis of bioactive compounds depends heavily on effective manipulation of the concentrations of these nutrients [44].

The second-order functions of the two models are presented as Eqs. 10 and 11 for the ZND and BAC concentration models, respectively.

Table 3 Design matrix of actual input variable levels, experimental and predicted responses of a surface methodology for optimized bioactive compound production

Run	x_1	x_2	x_3	x_4	x_5	x_6	eZND	pRSM	eBAC conc	pRSM
1	44.72	10.44	1.5	2.54	12.0	9.26	45.8	43.3	9.3	9.4
2	41.04	12.3	1.0	2.3	9.5	10.45	50.3	48.56	9.45	9.43
3	41.04	8.58	2.0	2.78	9.5	8.07	41.6	40.19	8.95	8.95
4	48.4	8.58	2.0	2.3	9.5	8.07	29	27.15	9.23	9.19
5	48.4	8.58	1.0	2.78	14.5	10.45	45.1	44.11	9.53	9.5
6	48.4	12.3	1.0	2.78	9.5	10.45	36.1	35.3	9.35	9.35
7	44.72	10.44	1.5	2.54	12.0	11.12	46.9	47.92	9.56	9.53
8	44.72	10.44	1.5	2.54	12.0	9.26	40.7	43.3	9.55	9.4
9	44.72	10.44	1.5	2.16	12.0	9.26	39.1	40.61	9.66	9.71
10	41.04	8.58	1.0	2.78	9.5	10.45	30.6	32.7	9.51	9.52
11	48.4	12.3	1.0	2.3	9.5	8.07	38	38.92	9.34	9.26
12	48.4	8.58	2.0	2.78	14.5	8.07	42.8	44.3	9.19	9.21
13	44.72	10.44	2.28	2.54	12.0	9.26	32.9	33.21	9.06	9.02
14	41.04	8.58	2.0	2.3	9.5	10.45	24.2	23.85	9.55	9.45
15	48.4	12.3	1.0	2.78	14.5	8.07	32.8	32.91	9.06	9.16
16	48.4	12.3	2.0	2.3	14.5	8.07	29.5	27.15	9.28	9.27
17	38.96	10.44	1.5	2.54	12.0	9.26	34.2	35.58	9.42	9.35
18	44.72	10.44	1.5	2.54	12.0	9.26	44	43.3	9.27	9.4
19	41.04	8.58	1.0	2.3	9.5	8.07	38.5	39.17	9.44	9.53
20	48.4	12.3	2.0	2.78	14.5	10.45	42.2	41.29	9.72	9.63
21	44.72	13.35	1.5	2.54	12.0	9.26	47.2	46.48	9.8	9.69
22	44.72	10.44	1.5	2.54	12.0	9.26	43.1	43.3	9.44	9.4
23	41.04	12.3	2.0	2.3	14.5	10.45	44.8	44.2	9.34	9.38
24	41.04	12.3	2.0	2.78	14.5	8.07	40	40.64	9.61	9.55
25	41.04	8.58	2.0	2.3	14.5	8.07	32	32.55	9.11	9.11
26	50.48	10.44	1.5	2.54	12.0	9.26	30.7	30.92	9.15	9.22
27	44.72	10.44	0.72	2.54	12.0	9.26	39.9	41.18	8.81	8.85
28	41.04	12.3	2.0	2.78	9.5	10.45	38.8	39.33	9.62	9.72
29	48.4	8.58	1.0	2.3	9.5	10.45	36.7	35.81	9.39	9.45
30	41.04	12.3	2.0	2.3	9.5	8.07	34.2	34.95	9.75	9.78
31	44.72	10.44	1.5	2.54	12.0	9.26	41	43.3	9.54	9.4
32	48.4	8.58	2.0	2.78	9.5	10.45	38.9	37.94	9.53	9.49
33	48.4	8.58	1.0	2.78	9.5	8.07	40.2	40.56	9.34	9.3
34	48.4	12.3	1.0	2.3	14.5	10.45	46.9	48.06	8.64	8.64
35	41.04	8.58	2.0	2.78	14.5	10.45	45	43.84	9.28	9.36
36	48.4	8.58	2.0	2.3	14.5	10.45	38.4	40.3	8.74	8.77
37	44.72	7.53	1.5	2.54	12.0	9.26	41.7	44.02	9.45	9.57
38	44.72	10.44	1.5	2.92	12.0	9.26	44	44.08	9.9	9.85
39	48.4	12.3	2.0	2.78	9.5	8.07	38.9	40.64	9.66	9.73
40	44.72	10.44	1.5	2.54	8.09	9.26	41.8	42.81	9.31	9.27
41	44.72	10.44	1.5	2.54	12.0	7.4	44.8	45.38	9.36	9.4
42	44.72	10.44	1.5	2.54	12.0	9.26	46.1	43.3	9.36	9.4
43	41.04	12.3	1.0	2.78	14.5	10.45	43.8	45.4	9.18	9.22
44	44.72	10.44	1.5	2.54	15.91	9.26	49.7	50.29	8.95	8.99
45	44.72	10.44	1.5	2.54	12.0	9.26	47.8	43.3	9.57	9.4
46	44.72	10.44	1.5	2.54	12.0	9.26	43.6	43.3	9.22	9.4
47	41.04	8.58	1.0	2.78	14.5	8.07	50.6	49.56	9.26	9.22
48	41.04	12.3	1.0	2.78	9.5	8.07	47.9	45.76	9.03	9
49	48.4	12.3	2.0	2.3	9.5	10.45	36.9	37.69	9.45	9.49
50	41.04	8.58	1.0	2.3	14.5	10.45	53.3	51.32	9.51	9.44
51	48.4	8.58	1.0	2.3	14.5	8.07	41.6	40.82	9.2	9.1
52	41.04	12.3	1.0	2.3	14.5	8.07	47.8	48.52	9.16	9.2

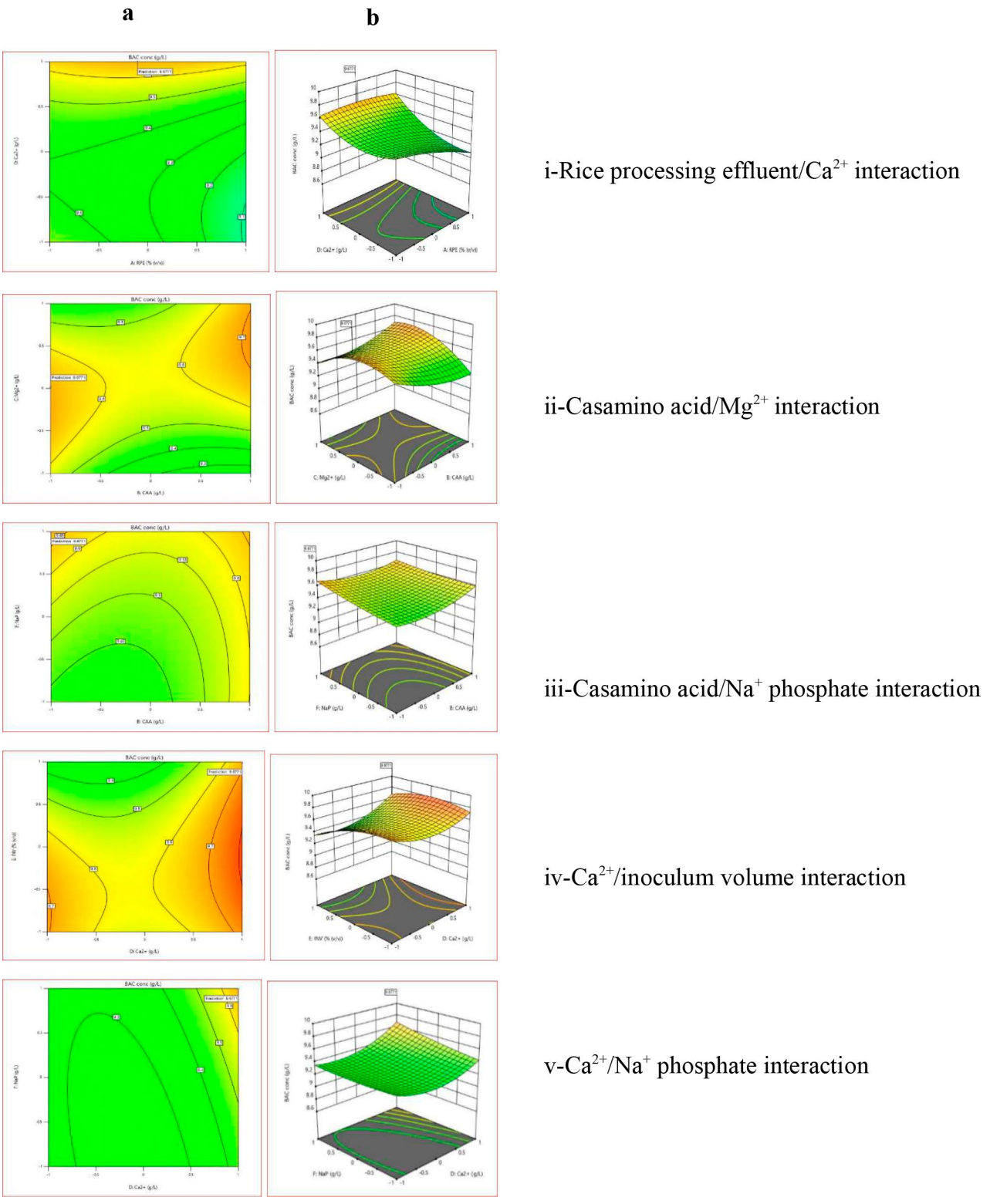


Fig. 3 Contour (**a**) and their corresponding surface (**b**) plots of significant two-way interaction terms of BAC concentration (Y_2) model of response surface methodology (RSM)

$$
\begin{aligned}
y_1 &= 7.38 - 0.15x_1 + 0.08x_2 - 0.26x_3 \\
&+0.11x_4 + 0.24x_5 + 0.08x_6 - 0.14x_1x_2 \\
&+0.13x_1x_3 + 0.02x_1x_4 - 0.08x_1x_5 + 0.09x_1x_6 \\
&+0.02x_2x_3 - 0.15x_2x_4 - 0.20x_2x_5 + 0.11x_2x_6 \\
&+0.27x_3x_4 - 0.04x_3x_5 + 0.05x_3x_6 - 0.05x_4x_5 \\
&-0.17x_4x_6 + 0.18x_5x_6 - 0.41{x_1}^2 + 0.08{x_2}^2 \\
&-0.25{x_3}^2 - 0.04{x_4}^2 + 0.13{x_5}^2 + 0.14{x_6}^2
\end{aligned} \tag{10}
$$

$$
\begin{aligned}
y_2 &= 9.40 - 0.04x_1 + 0.04x_2 \\
&+0.06x_3 + 0.04x_4 - 0.09x_5 \\
&+0.04x_6 - 0.01x_1x_2 + 0.01x_1x_3 \\
&+0.09x_1x_4 - 0.034x_1x_5 - 0.034x_1x_6 \\
&+0.15x_2x_3 + 0.013x_2x_4 - 0.017x_2x_5 \\
&-0.046x_2x_6 + 0.03x_3x_4 - 0.01x_3x_5 \\
&-0.01x_3x_6 - 0.08x_4x_5 + 0.064x_4x_6 \\
&-0.033x_5x_6 - 0.05{x_1}^2 + 0.09{x_2}^2 - 0.19{x_3}^2 \\
&+0.155{x_4}^2 - 0.111{x_5}^2 + 0.024{x_6}^2
\end{aligned} \tag{11}
$$

Model optimization

The multiobjective approach of optimization by the response optimizer adopted the approach of composite desirability, *D*. The optimal solution of variable levels was set to (x_1, x_2, x_3, x_4, x_5, x_6) = (-0.051, -1, -0.147, 0.979, 1, 1) = (44.81% [v v^{-1}], 8.58 g L^{-1}, 1.57 g L^{-1}, 2.775 g L^{-1}, 14.5% [v v^{-1}], 10.45 g L^{-1}), corresponding to the rice processing effluent, casamino acid, $MgSO_4.7H_2O$, $CaCl_2.2H_2O$, inoculum volume and Na_2HPO_4/NaH_2PO_4 concentrations, respectively. Under these conditions, a BAC concentration of 9.674 ± 1.26 gL^{-1} was obtained, with a composite desirability, *D*, of 0.883.

Validation of optimum conditions in real time

A real-time triplicate validation study of the predicted optimum levels of medium variables for improved production of the bioactive compound (BAC) by strain UCCM 00159 yielded a BAC concentration of 9.58 ± 1.08 g L^{-1}. Since the difference between the predicted BAC and real-time concentrations was not up to 5%, the optimized levels of the significant variables were adopted for all subsequent fermentations of the bioactive compound by the study bacterium [15].

The RSM is by far the most exploited yield improvement technique at both the laboratory and industrial levels and has been applied to solve complex bioprocess problems in engineering and science. Specifically, successful exploitation of RSM to improve the yield of microbial bioactive compounds has been reported by previous researchers [1, 45]. The optimized solution for BAC production by the axenic culture of *Lysinibacillus macroides* strain UCCM 00159 revealed an inoculum volume greater than 10% (v v^{-1}), which is rarely reported [20]. Lertcanawanichakul and Sahabuddeen [46] reported that at inoculum volume higher than 1% (v v^{-1}), bioactive compound synthesis was reduced, and the final concentration was low. However, strain UCCM 00159 required a high inoculum volume for increased production of the metabolite. The nutritional, metabolic/physiological, environmental and chemical cues necessary to induce the expression of silent biosynthesis-related gene clusters for bioactive compound production in bacteria are achieved earlier, with high initial cell density [6]. Very few studies have reported inoculum volumes greater than that reported in this study. A typical study by Yanti et al. [39] reported a 25% (v v^{-1}) inoculum for optimum production of bacterial cellulose by *Acetobacter xylinum* strain LKN6 from Sako liquid waste.

Biologically-induced overproduction of bioactive compound

The concept of silent biosynthesis-related gene clusters (BGCs) involved in bioactive compound synthesis necessitated experimentation with the biological induction of the biosynthesis of bioactive compounds by primary susceptible microorganisms [6]. The axenic culture of strain UCCM 00159 (10:0, % v v^{-1}) produced 9.6 g L^{-1} of crude BAC. However, a small challenge through coculture with its PSM (9:1% v v^{-1}) spiked an increase in the BAC concentration to 11.97 g L^{-1}. The BAC concentration increased along the coculture gradient until it reached 4:6 (% v v^{-1}), when the BAC concentration reached a high value of 22.2 g L^{-1}, representing a 13.69-fold increase in shake flasks (Fig. 4a). This increase may arise from chemical cues, including secondary metabolites and extracellular enzymes, from susceptible organisms [47]. Interestingly, the sterile filtrate of the axenic culture of *Aspergillus austroafricanus* strain UCCM 00160 demonstrated bioactivity against the fish pathogen *Vibrio alginolyticus*, and *Bacillus subtilis* and *Staphylococcus aureus* but not against strain UCCM 00159 at the concentration tested (data not shown).

The results of the 5-L bioreactor bio-induced production revealed that the BAC concentration by axenic culture increased from 9.65 ± 0.18 g L^{-1} in the shake flask to 37.21 ± 0.21 g L^{-1} (3.86-fold) under controlled bioreactor conditions. However, the concentration of the bioactive compounds produced by coculture increased from 22.2 ± 0.09 g L^{-1} in shake flasks to 187.09 ± 0.83 g L^{-1} (8.11-fold increase) in the bioreactor (Fig. 4b). A major limitation of this study is the possibility that in a bacterium–bacterium coculture or other bacterial–fungal cocultures, the BAC concentration from strain UCCM 00159 may be higher than that obtained in the present study [6]. Sun et al. [47] reported the induction of the production of 7 bioactive metabolites by *Aspergillus sydowii* through coculture with *Bacillus subtilis*, and

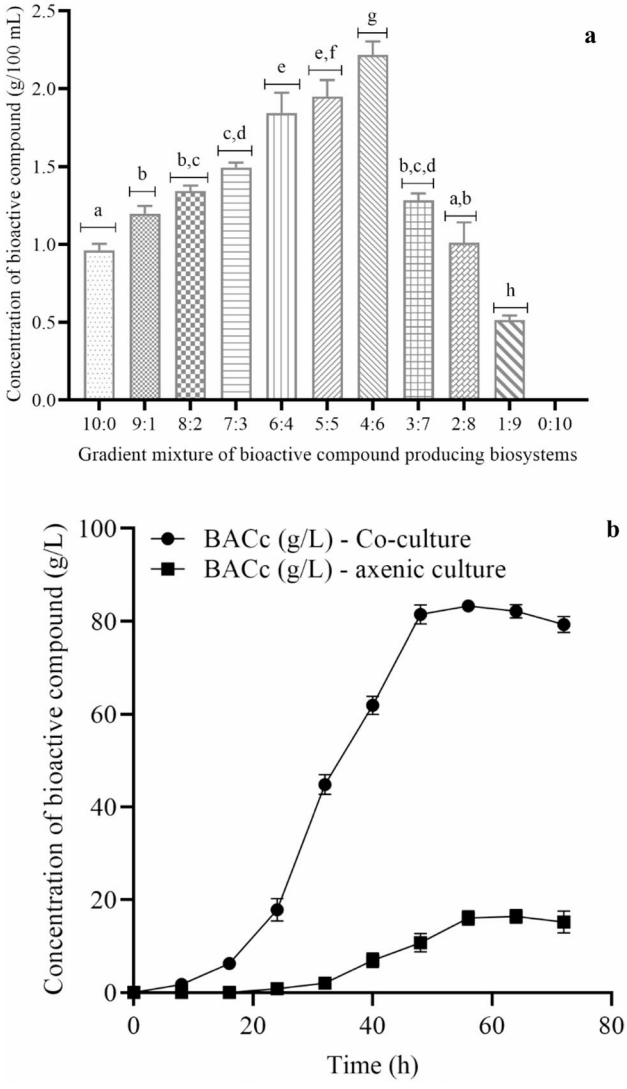


Fig. 4 Time course of BAC production in 5-L bench-scale bioreactor by an axenic culture of *Lysinibacillus macroides* and co-culture with the primary susceptible mould *Aspergillus austroafricanus* strain UCCM 00160; data points are means of triplicate determinations and error bars are standard deviations from means at 5% significant level

many more are discussed in the brilliant review of Peng et al. [48].

Identity of the bioactive compound

The ethyl acetate extract of the BAC was first purified by silica gel column chromatography, and semipreparative HPLC identified 9 bioactive fractions from the coculture BAC extract (Table 4). This finding indicates that many silent biosynthesis-related gene clusters leading to the synthesis of BAC were activated. Interestingly, the ethyl acetate extracts of axenic cultures of strains UCCM 00159 and UCCM 00160 revealed 3 and 4 BACs, respectively, with strain UCCM 00160 BACs being mostly active against Gram-positive bacteria.

The bioactivity of the fractions was concentrated in fraction C, which was semipreparative HPLC-identified as a heterocycloanthracene-like compound by comparing its retention time to that of standard bioactive chemicals. Heterocyclic compounds constitute one of the largest groups of compounds employed by chemists in organic synthesis and are prevalent among a variety of pharmacologically-active synthetic and natural compounds. Anthracene (anthracin) is a polycyclic aromatic hydrocarbon with three fused benzene rings, and its toxicity (bioactivity) to microorganisms, animals and humans is well established [49]. The detoxification process of anthracin frequently involves the biosynthesis of antibiotic monooxygenases, suggesting that the majority of microorganisms that degrade anthracin do so by synthesizing bioactive compounds from it [50].

Tricine SDS-PAGE analysis revealed a 10.5 kDa peptide that was well within the molecular weight range of class II nonmodified peptide bacteriocins [51, 52]. The compound was named 'macroidin' and defined as a heterocycloanthracin-like class II bacteriocin from *Lysinibacillus macroides* strain UCCM 00159. Chopra et al. [53] reported the production of a 6.5 kDa heterocycloanthracin bacteriocin, called sonorensin, from *Bacillus sonorensis*. This is the first report of the production of a heterocycloanthracin-like bacteriocin by a *Lysinibacillus* strain. Ahmad et al. [54] reported the production of a novel 51 kDa class III bacteriocin by *Lysinibacillus* sp. JX402121 with 97% sequence similarity with *Lysinibacillus macroides*.

In silico characterization of bioactive peptide

In silico analysis confirmed that the molecular weight of the peptide was 10.5 kDa, with an isoelectric point (pI) of 8.52, suggesting that optimum activity of the compound occurred under slightly alkaline conditions. This was further confirmed by the net balance of positively charged amino acids (Arg + Lys). The peptide sequence was dominated by glycine (18.6%), cysteine (16.5%) and phenylalanine residues (13.4%), with a molecular formula of $C_{442}H_{645}N_{137}O_{131}S_{17}$. The instability index of 'macroidin' was 45.38 > 40, indicating that the peptide was unstable in vitro. The aliphatic index, defined as the relative volume occupied by aliphatic side chains (alanine, valine, isoleucine, and leucine), was 25.15, suggesting that 'macroidin' was stable to high-temperature treatments, a property that is characteristic of class I and II bacteriocins. The grand average of hydropathicity (GRAVY) of 'macroidin' was −0.097, indicating that the compound was soluble. The GRAVY index describes the solubility of a protein, with an increasing positive score indicating greater hydrophobicity. A low or negative GRAVY value, as observed for 'macroidin', denotes better interaction between the peptide and water, indicating

Table 4 Summary of semi-preparative HPLC purification and spectral retention times of Ethyl acetate crude extract fractions of *Lysinibacillus macroides* strain UCCM 00159 bioactive compound

Fractions	Chromatogram Peaks	ZND (mm)	Pooled Fractions	Spectral Retention time (min)	ZND (mm)	Spectral Retention time (min)
1	100.84	-	A	3.65	2.7	
2	64.28	-	B	5.63	1.9	
3	727.64	14.7	**C**	**9.02**	**38.4**	**9.01**
4	63.92	-	D	11.5	4.2	
5	0.93	-	E	19.6	4.3	
6	1.48	-				
7	984.23	-				
8	163.58	-				
9	152.85	19.5				
10	177.45	-				
11	1142.66	26.7				
12	5.67	-				
13	75.34	-				
14	1542.52	43.2				
15	8.83	-				
16	846.38	22.4				
17	1809.67	32.5				
18	453.57	28.1				
19	0.43	-				
20	663.28	31.4				
21	11.48	-				
22	15.57	-				
23	291.65	11.7				

ZND– Inhibition zone diameter; HPLC– High performance liquid chromatography

the hydrophilicity and solubility behaviour of the peptide [55].

Stability of 'macroidin' with respect to hydrolytic enzymes, pH and temperature

An assessment of the stability of bacteriocins to hydrolytic enzymes, pH and temperature (Fig. 5) is needed to determine the biochemical nature of the compound. The stability of 'macroidin' to α-amylase indicated the absence of a carbohydrate moiety in the molecule [56]. Macroidin is also stable to catalase and RNase (Fig. 5a), suggesting that its bioactivity is due to neither hydrogen peroxide nor ribonuclease activities [57]. The bacteriocin was sensitive to lipase, as indicated by the significant ($p < 0.05$) reduction in the mean ZND, suggesting that the lipid moiety was partly essential for biological activity. This may be derived from the hydrophobic heterocyclic anthracin component of the compound, which is itself biologically active [50]. The biological activity of bacteriocins is intricately linked to the presence of hydrophobic regions in the bacteriocin molecule, which mediate hydrophobic interactions between bacterial cells and bacteriocin molecules [58]. The almost complete loss of bioactivity in the presence of proteinase K, pepsin, trypsin and protease suggested that a peptidic compound was primarily responsible for the antimicrobial activity of 'macroidin'. This characteristic is desirable for bacteriocin since its potential for inhibiting beneficial microbiota in the gut is significantly reduced, thus enhancing its safety. These results are corroborated by Chopra et al. [53] and Wayah and Philip [57], who reported the stability of sonorensin and fermencin, respectively, to these enzymes.

The results of the effect of pH on bacteriocin activity (Fig. 5b) suggest that the bacteriocin described in this report may be applied in many areas. However, optimal bioactivity was observed at alkaline pH, confirming the isoelectric point from in silico analysis. These results agree with those of sonorensin [53] and fermencin [57] but differ markedly from those of nisin, which is reported to be stable in a narrow acidic pH range and would lose 90% of its activity at pH 7 [59].

The mean residual inhibition zone diameter of 'macroidin' exposed to different temperatures (Fig. 5c) for 30 min remained largely unchanged, suggesting great stability of the bioactive compound, as previously predicted by in silico analysis. One very important property of bacteriocin is its stability to high-temperature treatments. This has been attributed to the low molecular weight and aliphatic index of the peptide [55]. Nisin is reported to be stable at 100 °C and will lose < 10% of its activity after autoclaving at 121 °C for 20 min. This was not the case in this study, as 'macroidin' lost all of its activity at 121 °C but retained 91.25% of its activity at 90 °C and 81.75%

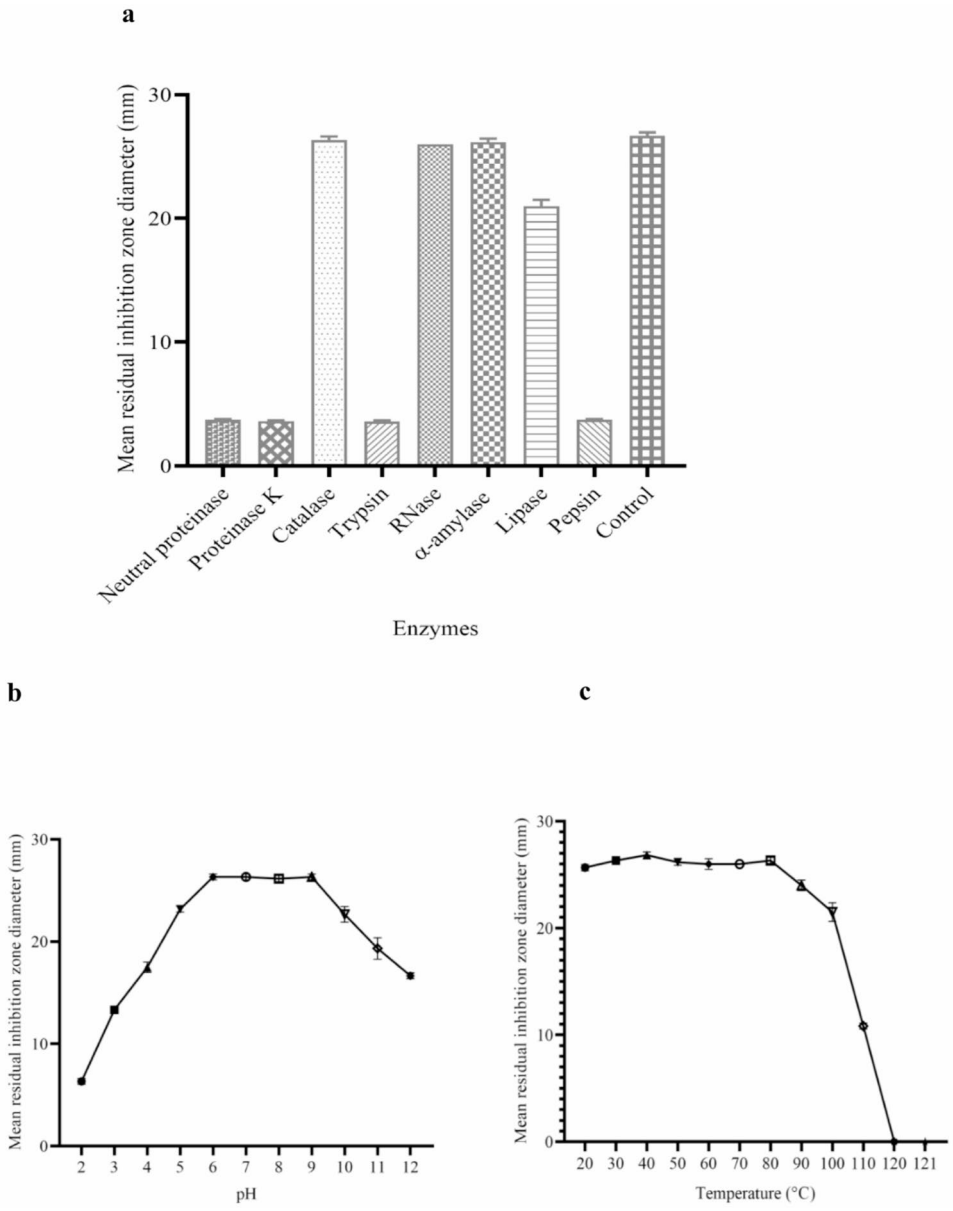


Fig. 5 Stability of 'macroidin' to (**a**) hydrolytic enzymes, (**b**) pH and (**c**) temperature; data points are means of triplicate determinations; error bars are standard deviations from means at 5% significance level

at 100 °C. Chopra et al. [53] reported that the activity of sonorensin decreased completely at autoclaving temperatures. However, Wayah and Philip [57] reported that fermencin retained almost all its antimicrobial activity at 100 °C.

Activity spectrum and MICs of Macroidin

The antimicrobial spectrum of 'macroidin' from *Lysinibacillus* (formerly *Bacillus*) *macroides* covered both Gram-positive and Gram-negative bacteria, yeasts and moulds but to varying degrees (Table 5). The activity was greater against Gram-positive bacteria and filamentous fungi (moulds) than against unicellular fungi (yeasts) and Gram-negative bacteria, as evidenced by the MICs. The limited activity against Gram-negative bacteria may arise from the presence of an outer membrane, which poses an extra barrier in the complex cell wall against the uptake of the bacteriocin. By high-affinity binding to lipid II, bacteriocins form pores in the cytoplasmic membrane of Gram-positive cells and inhibit peptidoglycan formation and transportation of peptidoglycan units to the site of cell wall assembly [60]. Nisin is considered a typical bacteriocin owing to its narrow activity spectrum against Gram-positive bacteria and closely related strains [61, 62]. However, the search for broader spectrum bacteriocins has been ongoing for decades, especially among

Table 5 Minimum inhibitory concentrations of purified bioactive compound against susceptible microorganisms

S/N	Test organism	Mean MIC ± SD (µg/mL)
1	*PSM (*Aspergillus austroafricanus*)	0.98 ± 0.04
2	*Aspergillus flavus* ATCC 21882	1.95 ± 0.14
3	*Fusarium oxysporum* ATCC 16322	1.95 ± 0.06
4	*Saccharomyces cerevisiae* UCCM 00054	62.5 ± 0.78
5	*Listeria monocytogenes* ATCC 23074	0.98 ± 0. 01
6	*Staphylococcus aureus* ATCC 33591	31.3 ± 1.02
7	*Erwinia carotovorum* ATCC 39048	7.8 ± 0.43
8	*Pseudomonas stutzeri* NCTC 12262	500.0 ± 1.11

*PSM = Primary susceptible microorganism; MIC = Minimum inhibitory concentration; ATCC = American Type Culture Collection; UCCM = University of Calabar Collection of Microorganisms; NCTC = National Collection of Type Cultures; Values are means of five replications; SD = standard deviation

Table 6 Comparative antioxidant activity of bioactive compound from *Lysinibacillus macroides* strain UCCM 00159 with ascorbic acid standard

S/N	Parameters	Ascorbic acid (Mean ± SEM)	Bioactive compound (mean ± SEM)
1	IC_{50} of DPPH assay (µg/mL)	64.34 ± 0.46	77.41 ± 0.54
2	IC_{50} of $ABTS^{*+}$ assay (µg/mL)	42.32 ± 0.74	46.46 ± 0.11
3	IC_{50} of FRAP assay (µg/mL)	53.66 ± 0.25	50.13 ± 0.18
3	Maximum scavenging power (%) against DPPH	92.05 ± 0.22	89.67 ± 0.04
4	Maximum scavenging power (%) against $ABTS^{*+}$	96.56 ± 0.41	88.47 ± 0.38
5	Maximum FRAP value (mM equivalents of $FeSO_4$)	77.01 ± 0.27	74.18 ± 0.73

the GRAS genera *Bacillus* and lactic acid bacteria. The broadness of the activity spectrum of an antimicrobial peptide is a major criterion for the selection of a bacteriocin as a biopreservative in the food industry [63].

Several studies have reported different MICs for bacteriocins against different microorganisms. The sonorensin of Chopra et al. [53] demonstrated an MIC of 1 µg mL^{-1} against the Gram-positive food pathogen *Listeria monocytogenes*, which compares favourably with 0.98 µg mL^{-1} 'macroidin'. However, their MIC of 45 µg mL^{-1} against *Staphylococcus aureus* did not compare favourably with the value of 31.5 µg mL^{-1} reported in this study. Henderson et al. [64] reported an MIC of 8 µg mL^{-1} nisin against *L. monocytogenes*, which is several orders greater than the MIC observed in this study. Overall, the antimicrobial activity spectrum of bacteriocins from strains of *Bacillus* is comparable to that reported in this study [65, 66].

The activity of 'macroidin' against plant pathogenic bacteria and fungi is very noteworthy. *Aspergillus flavus* and *Fusarium oxysporum* are responsible for mycotoxin contamination and spoilage, respectively, of stored foods. Both developments lead directly or indirectly to food shortages as well as increased health burdens arising from toxin-mediated infections and diseases. *Pectobacterium carotovorum* is a globally-recognized plant pathogen that causes soft rot of carrots and cabbage, blackleg of potatoes and spoilage of fresh vegetables before/after harvest [67, 68]. The low MICs of 'macroidin' against these pathogens indicate the potential for biological control towards sustainable agricultural practices. The major agricultural significance was the poor inhibition of *Pseudomonas stutzeri* (MIC = 500 µg mL^{-1}) by 'macroidin', which appears promising from the perspectives of enhanced seed germination and crop growth promotion [69].

Antioxidant potential of 'macroidin'

The antioxidant potential of *Lysinibacillus macroides* bacteriocin against three radicals revealed that the DPPH radical scavenging power of 'macroidin' was 89.67%, with an IC_{50} of 77.41 µg mL^{-1} (Table 6). This activity was significantly different from that mediated by the bacteriocin from *Lactobacillus lactis* strain CH3, where DPPH scavenging activity occurred at an IC_{50} of 12.5 µg mL^{-1} [70]. However, the bacteriocin from *Streptomyces variabilis* strain RD-5 could scavenge only the DPPH radical at a rate of 82.86% at 5000 µg mL^{-1} [71]. Ascorbic acid was not significantly superior in scavenging the DPPH radical at 92.05% and total free radical at 77.01 mM equivalents of $FeSO_4$. However, it was significantly better at scavenging the $ATBS^{*+}$ radical than the studied BAC. Bacteriocins mediate antioxidant activity by inhibiting lipid peroxidation metabolites present in the cell membrane [72]. Free radicals are formed from uncontrollable metabolic processes and are capable of damaging cells and tissues, causing major disorders such as mutagenesis, carcinogenesis, Alzheimer's disease, circulatory disturbances and aging problems [73]. Therefore, there is a need to source biologically-active compounds with inherent abilities to remove these radicals from the system. This prevents them from mediating oxidative stress in cells or tissues, thereby circumventing the complications that may arise therefrom.

Biopreservation potential of macroidin

The study bacteriocin demonstrated good biopreservation against *Staphylococcus aureus* and *Listeria monocytogenes* in mixed fruit juice at their MICs [74]. The 'macroidin' at specific MICs preserved the bacteriological quality of the mixed fruit juice, allowing it to remain for 4 months at 4 °C without significant changes in bacterial counts (Table 7). Many reports on biopreservation consider the reduction in microbial count over time as preservation, which may not be the case. Cell lysis in a food material is not a welcome development, as it releases intracellular materials into the food matrix, which may lead to significant organoleptic changes in the food. The biopreservation potential of

Table 7 Bio-preservation potential of purified heterocycloanthracin-like bacteriocin from *Lysinibacillus macroides* strain UCCM 00159

Duration (d)	Concentration (μgmL^{-1})	Nisin (standard bio-preservative)		Study bioactive compound	
		S. aureus $cfumL^{-1}$	L. monocytogenes $cfumL^{-1}$	S. aureus $cfumL^{-1}$	L. monocytogenes $cfumL^{-1}$
	0	8.6×10^{8}	9.2×10^{8}	8.1×10^{8}	8.2×10^{8}
	0.98	5.1×10^{3}	9.8×10^{4}	3.1×10^{7}	8.6×10^{5}
	1.95	4.4×10^{3}	7.2×10^{4}	9.4×10^{6}	6.3×10^{6}
	3.9	7.2×10^{4}	7.2×10^{5}	3.2×10^{5}	7.8×10^{4}
30	7.8	3.9×10^{6}	9.0×10^{5}	7.3×10^{4}	7.5×10^{2}
	15.6	2.9×10^{7}	7.6×10^{4}	6.5×10^{4}	6.0×10^{0}
	31.3	3.1×10^{7}	3.0×10^{2}	6.3×10^{5}	0
	62.5	9.1×10^{5}	5×10^{0}	7.8×10^{3}	0
	0	5.8×10^{6}	8.7×10^{6}	5.2×10^{6}	6.7×10^{6}
	0.98	7.6×10^{6}	4.2×10^{4}	3.9×10^{4}	6.7×10^{5}
	1.95	4.6×10^{5}	5.9×10^{5}	5.1×10^{4}	5.1×10^{4}
	3.9	3.3×10^{5}	7.3×10^{6}	4.2×10^{3}	7.8×10^{2}
60	7.8	4.8×10^{6}	8.5×10^{5}	4.1×10^{3}	4.0×10^{0}
	15.6	3.3×10^{7}	6.2×10^{2}	5.7×10^{2}	0
	31.3	4.2×10^{7}	4.0×10^{0}	5.9×10^{5}	0
	62.5	6.2×10^{5}	0	3.1×10^{2}	0
	0	8.4×10^{4}	8.2×10^{4}	8.7×10^{4}	7.7×10^{4}
	0.98	8.2×10^{8}	9.4×10^{4}	6.5×10^{3}	5.1×10^{5}
	1.95	3.4×10^{7}	4.4×10^{3}	3.8×10^{2}	4.8×10^{2}
	3.9	6.4×10^{6}	6.6×10^{3}	8.7×10^{3}	7.0×10^{0}
90	7.8	7.1×10^{6}	5.8×10^{5}	6.8×10^{3}	0
	15.6	2.8×10^{6}	4.6×10^{0}	5.4×10^{2}	0
	31.3	4.7×10^{7}	0	4.1×10^{5}	0
	62.5	5.3×10^{5}	0	4.0×10^{0}	0
	0	4.6×10^{1}	7.4×10^{1}	4.2×10^{1}	4.4×10^{1}
	0.98	6.3×10^{5}	5.0×10^{5}	5.3×10^{1}	4.0×10^{5}
	1.95	8.6×10^{6}	3.2×10^{4}	5.5×10^{2}	3.6×10^{3}
	3.9	5.3×10^{7}	4.0×10^{4}	4.8×10^{2}	5.0×10^{0}
120	7.8	4.1×10^{7}	3.4×10^{5}	4.8×10^{1}	0
	15.6	2.0×10^{8}	3.2×10^{1}	4.7×10^{0}	0
	31.3	3.9×10^{7}	0	3.5×10^{5}	0
	62.5	4.3×10^{5}	0	0	0

MIC– minimum inhibitory concentration defined as the lowest concentration that inhibited visible growth of the bacterium

'macroidin' was comparable to that of nisin, which was also comparable to that of the sonorensin bacteriocin of Chopra et al. [32]. The use of bacteriocin as a biopreservative in the food industry has been pursued since the 1960s, especially with nisin, which is best used at low temperatures [63]. Gassericin, a bacteriocin synthesized by *Lactobacillus gasseri* strain LA39, was reported to be stable at 4 °C for 3 months [75]. This huddle technology is recommended for *Lysinibacillus macroides* 'macroidin' when used as a candidate bacteriocin for the food industry because of its potential in the control of foodborne pathogens and spoilage organisms.

In conclusion, a 10.5 kDa class II heterocycloanthracin-like bacteriocin named 'macroidin' was isolated from the culture filtrate of *Lysinibacillus macroides* for the first time. The bacteriocin demonstrated antimicrobial activity against its primary susceptible mould, *Aspergillus austroafricanus*, and against foodborne and phytopathogenic strains of bacteria and moulds. Its free radical scavenging power was comparable to that of ascorbic acid, and its biopreservation activity was commendable. Although the axenic culture of the bacterium could produce the bioactive compound under RSM-optimized medium conditions, biological induction by the primary susceptible mould increased the bioactive compound yield 8-fold and is recommended for its large-scale fermentative production.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12896-025-00979-1.

Supplementary Material 1

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Author contributions
M.E., A.A. and P.E. conceived and designed the study; P.E., C.E., D.E. and U.E. conducted the investigations, collected and analyzed data; M.E. and A.A. wrote the first draft of the manuscript; S.A. reviewed the draft. All authors commented on previous versions of the manuscript and approved the final manuscript.

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Data availability
Data is provided within the manuscript or supplementary information file.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
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Competing interests
The authors declare no competing interests.

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