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Endophytic bacteria of *Catharanthus roseus* as an alternative source of vindoline and application of response surface methodology to enhance its production

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Abstract: The requirement for novel, cost-effective pharmaceutical compounds is growing. We screened endophytic bacteria of *Catharanthus roseus* for the production of vinca alkaloids. Vindoline-producing endophytic bacteria was identified as *Microbacterium* sp. Vindoline was purified and characterized using column chromatography, TLC, HPLC and electrospray ionization mass spectrometry. The subsequent aim of this study was to design a cost-efficient media that can maximize vindoline production from *Microbacterium* sp. Initial optimization of the nutritional requirement and of the process parameters were carried out by monothetic analysis. Key factors obtained were optimized by the central composite design of response surface methodology. This model was also validated by repeating the experiment under the optimized conditions, which resulted in maximum production of 82 μg/L (predicted response was 76.21 μg/L). Reliability of the experiments was confirmed by ANOVA analysis, regression coefficient, prediction vs actual chart and a 3D response plot, which determined the fit of the model. This study should serve as a reference point for the use of statistical techniques in bacterial systems for production of bioactive, anticancer compounds by plant endophytes.

Keywords: endophyte; alkaloid; vindoline; monothetic; response surface technology

INTRODUCTION

Endophytes are microorganisms such as bacteria and fungi that spend either all or part of their lifespan inside the healthy tissues of a living plant without causing disease [1,2]. We are familiar with the fact that endophytic microorganisms can be exploited in agriculture, medicine and industry as they are promising, less explored and useful sources of novel, natural, bioactive products such as antibacterial, antifungal, antiviral, antitumor, antioxidant, antiinflammatory, immunosuppressive drugs and many related compounds [3,4].

Catharanthus roseus belongs to the family Apocynaceae and it is an important medicinal plant being a vital source of alkaloids. It can be called an important herbal drug because different parts of the plant are used for treating various diseases such as cancer, diabetes, hypertension and menstrual disorders [5]. Widely used alkaloids such as vindoline, vinblastine, vincristine, ajmalicine and serpentine have been isolated from different parts of *C. roseus* [6]. The terpenoid indole alkaloids vincristine and vinblastine, derived from the coupling of vindoline and catharanthine monomers, are used in the treatment of Hodgkin's disease and acute leukemia [7]. Various anticancer drugs, such as those derived from natural products, target microtubules in growing cancerous cells and induce cell death by blocking mitosis at metaphase/anaphase transition. Vincristine has anti-angiogenesis activity, manifesting as disrupted intracellular transport and decreased tumor blood flow via interference with microtubule formation and mitotic spindle dynamics [8,9]. Vinblastine suppresses treadmilling and affects the dynamic instability of microtubules by inducing a conformational change in tubulin, forming harmful vinblastine-tubulin spiral oligomers and paracrystals [10].

Since the discovery of the terpenoid indole alkaloids as antitumor drugs in the 1970s, aerial parts of *C. roseus* have been the only source of vindoline [11], which has negatively impacted ecosystems. To treat various type of cancer, 3 kg of vinca alkaloids are required yearly and 500 kg of dried leaves should be processed in order to

obtain 1 g of these compounds [12]. The second problem is its high price, which ranges from $1 million to $3 million per kg due to the limitations associated with extraction and production protocols [13]. One way to positively meet the demands of the industry is to look for a potential precursor of the anticancer drugs, such as vindoline, in organisms excluding plants. Microorganisms have several advantages over plants and animals. Microbes can be easily cultured and maintained, and they can be produced in large-scale fermentation processes, which make them a possible alternative source of important bioactive metabolites [14]. The endophytic fungi, *Talaromyces radicus*, *Eutypella* spp. and *Fusarium* isolated from *C. roseus* have been reported to produce vincristine and vinblastine [6,15,16].

Statistical optimization techniques such as response surface methodology (RSM) are generally used to study responses after simultaneous variation of several factors [17]. The most popular response surface statistical experimental designs are the Box-Behnken design (BBD), central composite design (CCD) and Plackett-Burman design (PBD) [18,19]. The aim of this study was to explore the bacterial endophytes of *C. roseus* for the production of alkaloids and to optimize the nutritional requirement of isolated endophytes in order to enhance the production of the desired compound. The optimum nutritional requirements for enhanced vindoline and biomass production, employing economical carbon and nitrogen sources, were determined. Consequently, this article should serve as an innovative reference point in endophytic biology, as it provides information concerning the culture condition of *Microbacterium* sp., providing useful information for the pharmaceutical industry, which is yet to be discussed in the literature. This should aid the drug discovery program since this article is the first report on the production and optimization of media components for vindoline production by endophytic bacteria.

MATERIALS AND METHODS

Chemicals and instrumentation

The nutrient, tryptic soya and Luria Bertani (LB) broths were obtained from Hi-media, Mumbai, India. The solvents used in the study (ethanol, chloroform, ethyl acetate, methanol and ammonia) were obtained from Merck, Germany. The standard vindoline was obtained from Sigma-Aldrich Co. USA. DNA sequencing was performed using Veriti® 99 well Thermal Cycler (Model No. 9902). Thin-layer chromatography was performed using silica gel plates Merck 60 F254. A Waters (Milford, MA, USA) HPLC was used for chromatographic separation and peak purification. Electrospray ionization mass spectroscopy (Thermo Fisher Scientific, USA) was performed in order to determine the molecular weight and fragmentation patterns of ions. Optimization studies were performed statically using design expert 10.0 (Stat-Ease Inc., Minneapolis, MN, USA) software. Chemical structure was drawn with Chem Draw software (PerkinElmer, USA).

Isolation and identification of endophytic bacteria

The isolation procedures of endophytic bacteria from different parts of *Catharanthus roseus* and the characterization of the isolated endophytic bacteria were discussed and published in our earlier work [4]. Identification of the vindoline-producing endophytic bacteria was performed by culture, morphological and molecular approaches. The bacterium was grown in nutrient agar media to study the culture and morphological characters. Total genomic DNA was extracted, and UV-Vis spectrophotometer was used to determine the concentration by measuring the absorbance at 260 nm. The quality of the DNA was evaluated by 1.2% agarose gel electrophoresis. Isolated DNA was amplified using the Veriti® 99-well thermal cycler (Model No. 9902) with 16S rRNA specific primers (8F and 1492R). The PCR amplicon was enzymatically purified and further subjected to Sanger sequencing. Bi-directional DNA sequencing reaction of the PCR amplicon was performed using the BDT v3.1 cycle sequencing kit on the ABI 3730xl genetic analyzer with 704F and 907R primers. The consensus sequence of 1436 bp 16S rDNA was generated using Aligner software from forward and reverse sequenced data. The 16S rDNA consensus sequence was used to carry out the BLAST alignment search tool of the NCBI GenBank database. ClustalW was used for multiple alignments of the first fifteen sequences with maximum identity score. The distance matrix was generated using the RDP database and a phylogenetic tree was constructed using MEGA5. Sequencing was performed by Xcelris Labs ltd., Ahmedabad, India, and the sequence data were deposited in the GenBank database using the NCBI BLASTN program.

Screening and extraction of vindoline

Preliminary studies on the production of vinca alkaloids were carried out in 3 liquid culture media. Among the media tested, the production of vinca alkaloids was established in significant quantities in one medium. Hence, further studies were carried out for the production and isolation of the alkaloids using the described medium. *Microbacterium* sp. isolated from *C. roseus* was cultured in 500 mL nutrient broth (NB) medium, pH 7.0. In order to screen the isolates for the production of various bioactive compounds, shake flasks were inoculated with 50 µL of inoculum and suspension cultures were established at 37°C at 160 rpm in an orbital shaker incubator (REMI CIS 24 BL) for 45 h. After incubation, the culture broth of the bacterium species was centrifuged at 7000 xg for 15 min at 4°C to obtain the cell-free supernatant. The supernatant was collected and used for extraction of the alkaloids. For the extraction process of alkaloids, a recognized methodology involving acid and base extraction has been proposed [20,21]. However, the extraction method was slightly modified from the earlier published work. The pH of the supernatant was adjusted to 2.0 with 0.1 M HCl and extracted with 100 mL of petroleum ether. Two phases were placed in a separating funnel and allowed to stabilize. The petroleum ether layer was removed to eliminate lipophilic molecules. The lower aqueous layer was transferred to a conical flask and treated with 25% ammonium hydroxide to adjust the pH to about 8.5, and an equal volume of ethyl acetate was used for extraction. After extraction, two phases were separated using a separating funnel, and the ethyl acetate layer was dried in a rotatory evaporator and collected in 2 mL of methanol to obtain the alkaloid-rich fraction [22]. The alkaloid-rich extract was subjected to phytochemical analysis using Mayer's and Wagner's reagents for the detection of alkaloids [23].

Purification of bacterial vindoline

Bacterial vindoline was purified using silica gel column chromatography. The slurry was made using silica gel (60-120 mesh size, length x diameter 50 cm x 2 cm) and chloroform. It was slowly charged into column side walls to avoid cavitation and the bottom valve was opened while charging. The flow rate was fixed per column height and diameter. The chloroform was eluted up to the level of packing. The methanolic extract, i.e. the alkaloid-rich fraction, was charged at the top of packing. Non-adsorbing cotton was placed immediately to avoid bed disruption. The column was eluted with a gradient of chloroform: methanol.

Thin-layer chromatography (TLC) analysis

The alkaloid-rich extract, column chromatography-purified vindoline and standard vindoline were subjected to TLC analysis. TLC was performed according to the method described in [24] on silica gel plates (Merck 60 F254) with minor changes. We modified the ratio of ethyl acetate:methanol:25% ammonia as the solvent system. The TLC chamber was saturated with solvent systems of different polarity and ratios. Forty µL of crude ethyl acetate extract, 20 µl of purified vindoline and 10 µl of standard vindoline were spotted on the TLC plate just 1 cm above the edge of the plate. The TLC plates were dipped in a saturated chamber in such a way that the applied spot remained above the solvent level and the plates were allowed to develop. When the solvent front reached the desired level, the TLC plates were taken out and allowed to air dry. The chromatogram was developed by observing the plates at 254 nm. The retention factor (Rf) value of each band was obtained as the ratio of distance travelled by the solute to that of the solvent front.

High performance liquid chromatography (HPLC) analysis

A Waters (Milford, MA, USA) HPLC equipped with a photodiode array detector, autosampler and pump were used for chromatographic separation and peak purity. Waters Millennium Software was used to record and process the chromatographic data. The optimal chromatographic conditions were obtained after testing different compositions of 3 different mobile phases in a linear gradient elution of solvents A and B from 20:80 to 80:20 in 20 min, using a Waters RP-18 column (4.6 x 150 mm, 5 µm particle size). According to method I, sodium phosphate buffer (5-100 m*M*) with a pH range of 2-8 was used with an organic solvent (methanol and acetonitrile). In method II, potassium phosphate buffer with a pH range of 2-8 was used with an organic solvent (methanol and acetonitrile). According to method III,

the mobile phase was water and methanol. A constant flow rate of 1 mL/min was maintained during analysis and detection was performed at 210 nm. The column temperature was maintained at 25°C. The sample injection volume was 20 µL. Vindoline was identified by comparing the UV spectra and retention time of the sample with the reference vindoline.

Electrospray ionization mass spectrometry analysis

The molecular masses and fragmentation patterns of purified and standard vindoline were determined by mass-spectrometric analysis. From accurate mass values, elemental compositions were calculated using Xcalibur 2.0.7. software. Initially, the samples were dissolved in HPLC-grade methanol and filtered using a 13-mm nylon syringe filter of 0.22-µm pore size. The vindoline-rich extract, purified vindoline and the standard vindoline were analyzed using the Linear Ion Trap Mass Spectrometer (Thermo Fisher Scientific, USA) by direct infusion in ESI positive ion mode, with 5 kV capillary voltage. The mass range in the ESI-MS experiments used for the detection of vindoline was 100-500 m/z. Multiple mass spectrometry (MS/MS) studies were performed to obtain the fragmentation pattern of the sample and standard vindoline under the same conditions [25].

Selection of the medium and optimization of parameters

NB, tryptic soya broth and LB broth media were selected based on previous works related to endophytes [26,27]. The growth curves of bacteria were analyzed in the three different media by measuring the absorbance at 600 nm at different time intervals. The optimum growth condition was determined by employing five different temperatures ranges (28°C-45°C), six different pH values (5-8), and incubation in an incubator shaker for 45 h at five different rpm values (80-240). The quantity of the constituents of the selected medium, i.e. NB (peptone, yeast extract, beef extract and sodium chloride), was optimized to increase the production of vindoline using one factor. The selected bacterial cultures were inoculated into the respective media and incubated at 33°C in an orbital shaker incubator (REMI CIS 24 BL) at 160 rpm for 45 h, and their respective biomass and vindoline production were quantified and the one producing the best results was chosen for further optimization. After analyzing the results, we decided to use additional carbon and nitrogen sources to further enhance the bacterial biomass and vindoline production.

Optimization of the medium using various carbon and nitrogen sources using a monofactorial search

Different carbon sources such as dextrose, fructose, galactose, sucrose, lactose, maltose, starch, glycerol and citric acid were used at a concentration of 1% in the basal media. After selecting the best carbon source, its concentration was optimized, and the best concentration was used in the next process. Various nitrogen sources such as glycine, urea, sodium nitrate, potassium nitrate, ammonium nitrate, asparagine, soybean meal and tryptone were used at concentrations containing the same equivalent weight of nitrogen at 0.5% in the medium containing the already optimized carbon source at a concentration of 1%. The cultures were then screened for their respective biomass and vindoline production. The optimized carbon and nitrogen source that produced the best results was selected for further optimization.

Response surface methodology

RSM is an empirical technique that aids in determining the relationships between a group of precise experimental factors and the responses thus obtained according to diverse criteria. To achieve a more realistic model, previous information and an understanding of the process variables under analysis are essential. Based on the result obtained after one-factor-at-a-time-approach, the time, temperature, pH, tryptone and dextrose concentrations were found to be key variables for vindoline production. The central composite rotatable design (CCRD) of RSM was employed in the present study involving four different factors. The experimental design was applied after selecting a range of each variable (maximum and the minimum). Each of the parameters was coded at three levels: -1, 0 and +1. For the optimization RSM study, four independent variables, pH (6.0-7.0), temperature (28-37°C), concentration of dextrose (1-1.5%) and tryptone (1-2%) were taken as per the experimental design. The

experimental design consisted of a total of 25 trials with varying condition. The optimization experiments were done in 100-mL Erlenmeyer flasks on a rotary shaker incubator at the already optimized condition and were quantified using a spectrophotometer.

Statistical analysis

The statistical and graphical analysis of the obtained result was done using Design Expert 10.0 (Stat-Ease Inc., Minneapolis, MN, USA) software. The Microsoft Office data analysis tool pack was used to obtain graphs in which the error bars denote standard errors. Once the experiments were completed, the coefficient of the polynomial was calculated, and Student's t-test was used to determine the significance of each coefficient [18]. Model terms were selected or rejected on the basis of the Student's t-value and its significance. To evaluate the optimum yield of vindoline, three-dimensional plots were obtained based on the effect of the levels of four parameters and their interactions. The hump in the 3D plots was used to study the interactions between the parameters and the optimum concentrations of each parameter with respect to the desired response.

RESULTS

Isolation of endophytic bacteria from *C. roseus*

Nine different endophytic bacteria were isolated and purified from the stem, leaves and roots of *C. roseus*, as published earlier [4].

Screening, extraction and partial purification of vindoline

A creamy precipitate in Mayer's reagent and a brownish precipitate in Wagner's reagent were observed in one bacterial extract, which is a positive result for the production of alkaloids from endophytic bacteria. Specific acid-base extraction is required for the recovery of alkaloids. The amount of vindoline production was studied during changes in the pH from 2-10. As the pH was increased, the production vindoline was also increased up to pH 8, with a further increase in pH leading to a decreased production of vindoline. Column chromatography was used for the purification of vindoline. Twelve samples were collected at different time intervals from each of seven different mobile phases ranging from 2:8 to 8:2 ratios of solvent (chloroform:methanol). Vindoline was successfully separated as a pure compound in the third fraction with a 7:3 chloroform:methanol ratio.

Identification of endophytic bacteria

The bacterial strain CSR-3 exhibiting a positive result for the production of vindoline was identified using culture, morphological and molecular approaches. A single band of 1500 bp was observed in agarose gel electrophoresis, as shown in Fig. 1A. The bacterium was assigned to a genus based on a 99-100% similarity index. The culture CSR-3 showed similarity with *Microbacterium* sp. HBUM178514 based on nucleotide homology and phylogenetic analysis, as shown in Fig. 1B. The evolutionary distances were computed using

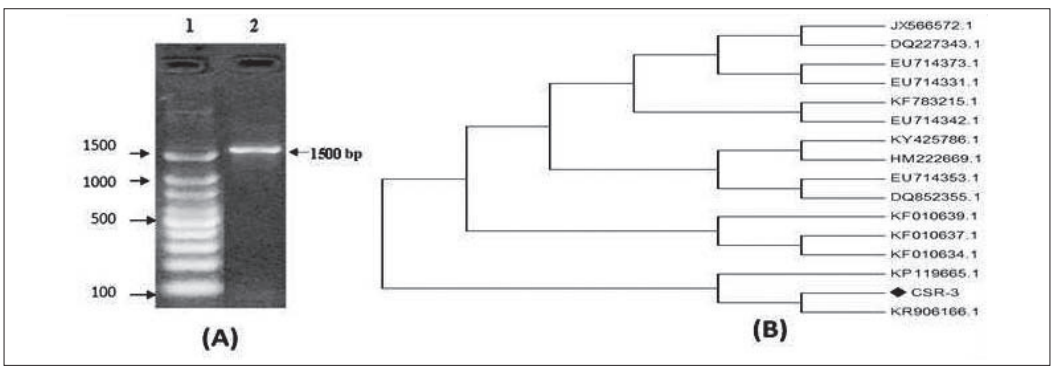

Fig. 1. Identification of bacterial endophyte. **A** – 1.2% Agarose gel electrophoresis showing the single band of 1500 bp of the 16S rDNA amplicon. Lane 1: 100bp DNA ladder; lane 2: 16S rDNA amplicon. **B** – The evolutionary relationship of CSR-3 showing 99-100% similarity index with *Microbacterium* sp. HBUM178514 based on nucleotide homology and phylogenetic analysis.

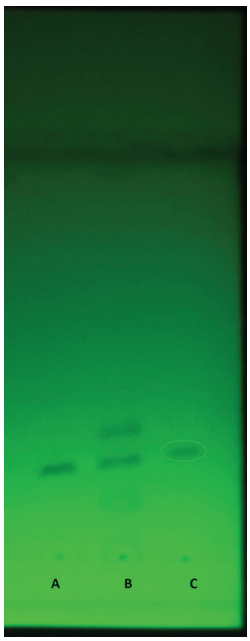

Fig. 2. TLC analysis. Lane A – standard vindoline with an Rf value of 0.35; lane B – ethyl acetate extract having with an Rf value of 0.36; lane C – purified vindoline with an Rf value of 0.37 with the optimized mobile phase containing ethyl acetate:methanol:ammonia (6:0.3:0.1) as a solvent system.

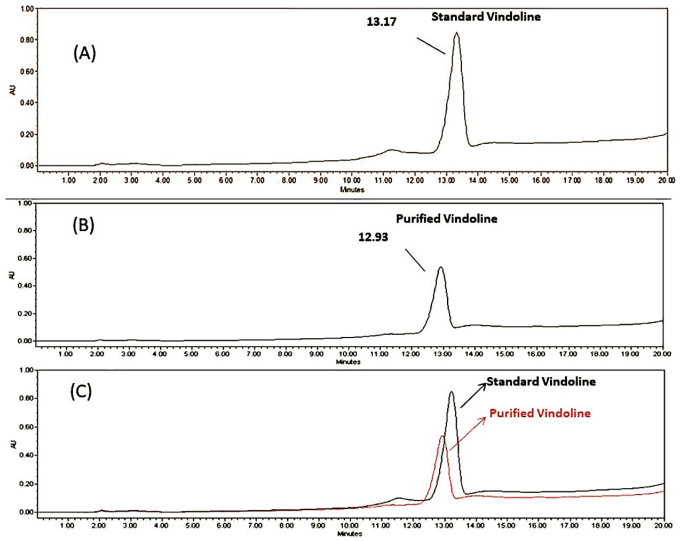

Fig. 3. HPLC analysis. **A** – Standard vindoline with a single peak at a retention time of 13.17 min. **B** – Bacterial purified vindoline with a single peak at a retention time of 12.93 min. **C** – Overall peak of both standard and purified vindoline.

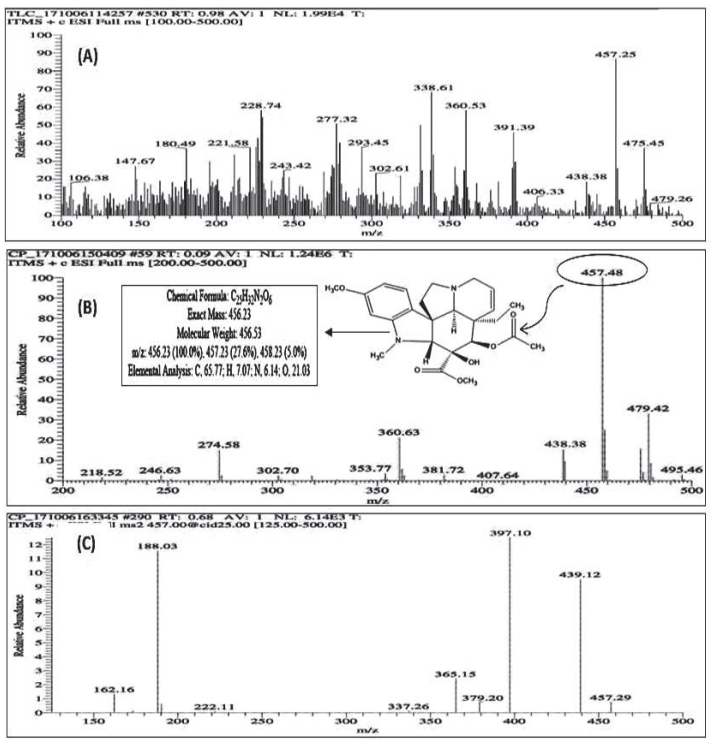

Fig. 4. Electrospray Ionization Mass Spectrometry analysis. **A** – Mass spectra of bacterial alkaloid-rich extract; the respective major ions $[M^+H]^+$ at m/z 457 indicate the presence of vindoline in the bacterial alkaloid rich extract. **B** – Mass spectra of purified vindoline; the single major ions $[M^+H]^+$ at m/z 457. **C** – MS/MS fragmentation pattern of purified vindoline; ions at m/z 438, 397, 188 and 162, respectively, related to the molecular structure of vindoline.

the Jukes-Cantor method [28], and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA5 [29]. The sequenced nucleotide was submitted to NCBI GenBank database and they provided the GenBank accession number as MF924391.

Thin-layer chromatography (TLC) analysis

A new method for the separation of vindoline in a silica gel TLC plate was developed after optimizing the mobile phase with a solvent system of ethyl acetate:methanol:ammonia (6:0.3:0.1). Distinct bands were observed when exposed to UV light (254 nm). The result of TLC analysis is presented in Fig. 2.

HPLC analysis

A novel method for HPLC analysis has been developed using a PDA detector to evaluate the peak purity and retention time of the compound. The optimum mobile phase composition was achieved using method III, with water and methanol in linear gradient mode and an increasing methanol concentration from 20%-80% during 20 min. Fig. 3 depicts the HPLC profile

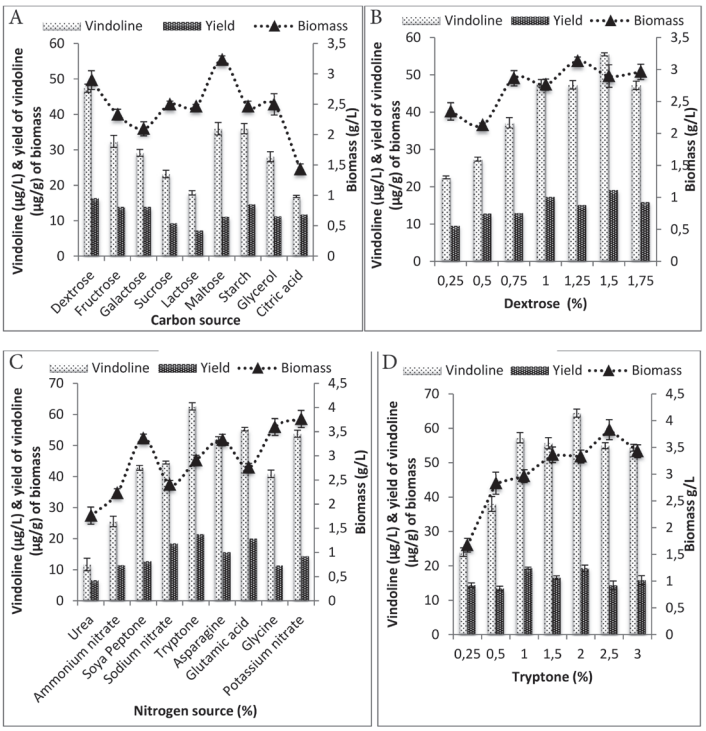

Fig. 5. Optimization of media using various carbon and nitrogen source employing a monofactorial search. **A** – Optimization of a suitable carbon source in which dextrose gave the best result, producing about 47 µg/L vindoline at a biomass of 2.8 g/L. **B** – Dextrose concentration optimization in which dextrose was used at different concentrations from 0.25-1.75%, and the maximum yield of vindoline was at 1.5% dextrose concentration, about 55 µg/L. **C** – Optimization of different nitrogen sources in which tryptone gave the best result, producing about 60 µg/L vindoline at a biomass of 3.2 g/L. **D** – Tryptone concentration optimization at which it was found that 2% tryptone yielded an optimum concentration of vindoline at 65 µg/L and about 3.5 g/L bacterial biomass.

of standard vindoline (Fig. 3A), having a single peak at a retention time of 13.17 min; purified vindoline (Fig. 3B) has a single peak at a retention time of 12.93 min; the overall peak of both standard and purified vindoline is presented in Fig. 3C.

Electrospray ionization mass spectrometry (ESI/MS) analysis

The positive-mode ESI/MS yield of the respective major ions $[M^+H]^+$ at m/z 457 was similar to the molecular weight of vindoline in the bacterial alkaloid-rich extract (Fig. 4A), and to purified vindoline (Fig. 4B) under the conditions described in the experimental section. The MS/MS fragmentation pattern of the purified vindoline also matched exactly with the molecular structure of vindoline, with ions at m/z 438, 397, 188 and 162, respectively (Fig. 4C).

Selection of medium and optimization of its composition and parameters

The biomass and vindoline quantity produced by *Microbacterium* sp. was analyzed in NB, tryptic soya and LB broth media. It was cultured in the 3 media for 45 h, with all other parameters being constant. We found that *Microbacterium* sp. spends a maximum of 9 h in lag phase when cultured in the NB medium as compared to 15 and 12 h in the tryptic soya broth medium and LB broth media, respectively. The optimum growth of bacteria was observed in NB medium by measuring the OD at 600 nm at regular intervals. After optimization, we observed that 33°C provided the best result but the amount of biomass was maximum at 37°C. Both the amount and yield of vindoline were maximum (29.23 µg/L) at pH 6.5, 33°C and 160 rpm. After optimizing the components of the selected medium, the yield and quantity of vindoline were maximum in the medium containing 0.3% yeast extract, 0.1% beef extract, 0.4% peptone and 0.6% NaCl, with 32.09 µg/L vindoline and optimum biomass of 2.96 g/L.

Optimization of media using various carbon and nitrogen source employing a monofactorial search

Different carbon sources were screened for enhanced production of vindoline in the cultures, and for their effect on the bacterial biomass. Experiments were performed in triplicates and results are shown as mean±standard error (SE) in Fig. 5(A) and (B); a statistically significant higher biomass was produced by maltose followed by dextrose, while the yield of vindoline was highest in media containing dextrose followed by starch and maltose. Dextrose was chosen as the best carbon source producing around 47 µg/L of vindoline at a biomass of 2.8 g/L. Dextrose was used at a different concentration from 0.25-1.75% and maximum yield of vindoline was found to be 55 µg/L at 1.5% dextrose concentration.

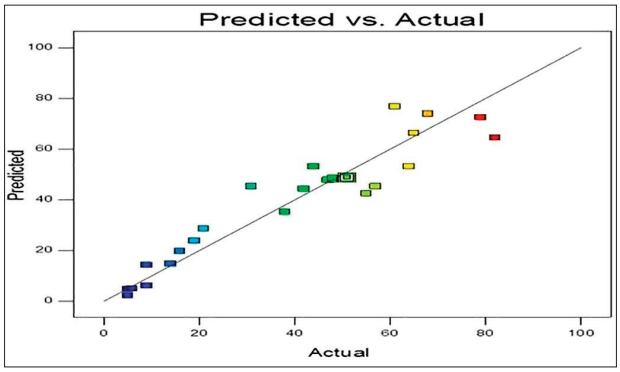

Fig. 6. Response surface predicted vs actual plot for optimizing the yield of vindoline. Plots of predicted vs actual concentrations of vindoline (µg/L) produced from *Microbacterium* sp. The predicted value is in reasonable agreement with the actual value of vindoline, which indicates that the model is in good agreement with the predicted and actual responses.

Different nitrogen sources were screened for enhanced production of vindoline in the culture and their effect on the growth of biomass, with the results presented as the mean±SE in Fig. 5C and D. The statistically significant highest biomass and vindoline were produced by tryptone, followed by glutamic acid, while urea produced the lowest amount of vindoline and biomass. Tryptone was chosen as the optimum nitrogen source, and after further optimization, it was found that 2% tryptone yields an optimum concentration of vindoline at 65 µg/L and around 3.5 g/L bacterial biomass, as presented in Fig. 6B.

Response surface and statistical analysis for optimizing the yield of vindoline

The use of statistical experimental design methods such as RSM for optimization can reduce process variability, overall cost and enhance product yields [19]. RSM is not yet used for the optimization of media components

Table 1. Analysis of variance (ANOVA) for the response surface quadratic model. The current model with an F-value of 6.08 suggests the model was statistically significant. The values of Prob>F fewer than 0.05 specifies that the model terms are significant. The Lack of Fit F-value of 0.44 implies the lack of fit is not significant relative to the pure error.

ANOVA for Response Surface Quadratic model						
Source		**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p-value Prob > F**	
Model		**12705.95**	**14**	**907.57**	**6.08**	**0.0034**	**significant**
A-Temperature		*172.77*	*1*	*172.77*	*1.16*	*0.3074*	
B-pH		*5638.31*	*1*	*5638.31*	*37.74*	*0.0001*	
C-Tryptone		*200.56*	*1*	*200.56*	*1.34*	*0.2735*	
D-Dextrose		*101.48*	*1*	*101.48*	*0.68*	*0.4290*	
AB		*50.00*	*1*	*50.00*	*0.33*	*0.5757*	
AC		*152.86*	*1*	*152.86*	*1.02*	*0.3356*	
AD		*111.49*	*1*	*111.49*	*0.75*	*0.4079*	
BC		*5.03*	*1*	*5.03*	*0.034*	*0.8581*	
BD		*0.11*	*1*	*0.11*	*7.074E-004*	*0.9793*	
CD		*569.47*	*1*	*569.47*	*3.81*	*0.0794*	
A^2		*2717.36*	*1*	*2717.36*	*18.19*	*0.0016*	
B^2		*662.23*	*1*	*662.23*	*4.43*	*0.0615*	
C^2		*420.26*	*1*	*420.26*	*2.81*	*0.1244*	
D^2		*487.68*	*1*	*487.68*	*3.26*	*0.1009*	
Residual		1493.81	10	149.38			
	Lack of Fit	*955.81*	*8*	*119.48*	*0.44*	*0.8324*	**not significant**
	Pure Error	*538.00*	*2*	*269.00*			
Cor Total		**14199.76**	**24**				

A=temperature B=pH, C=Tryptone, D=Dextrose, df=degree of freedom

for vindoline production by endophytes. Analysis of variance (ANOVA) helped us to study the statistical significance of the quadratic model and as per the quadratic model, the mean value of vindoline was found to be 38.36 µg/L, having a standard deviation of 12.22. The statistical significance of the model was checked by the F-test and ANOVA of the response (Table 1). The current model, with an F-value of 6.08, suggests that the model and the regression were statistically significant. There is only a 0.34% possibility that an F-value this large could arise due to noise. The values of "Prob>F" less than 0.05 specifies that the model terms are significant. The "Lack of Fit F-value" of 0.44 implies that the lack of fit is not significant relative to the pure error. A non-significant lack of fit is desirable; hence we consider the model to be fit. The acceptability of the model was also checked by the determination of the regression coefficient. In this work, the value of the R square 0.8948 specified that only 0.10% of the entire variable was not described by the model. The value of adjusted R^2 and predicted R^2 is also high to support a high significance of the model. The difference between the value of Adj R-square (0.7475) and pred R-Square (0.5515) is less than 0.2. This also revealed that the predicted R^2 of 0.7475 is in reasonable agreement with the adjusted R^2 of 0.5515 and indicates a good accuracy and credibility of the experiments [18]. The actual and predicted yields of vindoline by the model equation obtained in the experiments are given in Fig. 6.

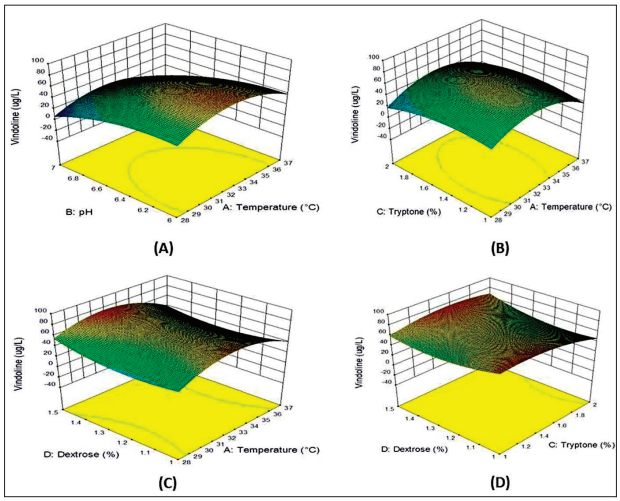

Fig. 7. Response surface 3D plot and interaction among nutrients. **A** – The effect of pH and temperature on the concentration of vindoline. **B** – The effect of tryptone and temperature on the concentration of vindoline. **C** – The effect of dextrose and temperature on the concentration of vindoline. **D** – The effect of dextrose and tryptone on the concentration of vindoline. From the response surface plot, it can be observed that both high pH and temperature adversely affected the vindoline yield. A pH value of about 6.5 and 33°C temperature is required to produce vindoline at an optimum level. Increasing the concentration of dextrose and at the same time decreasing the concentration of tryptone, decreases the yield of vindoline. Dextrose at a concentration of 1.5% and tryptone at a concentration of 2% are required for optimal production of vindoline at 82 µg/L.

Response surface plot and interaction among nutrient

To evaluate the relationship between the test variables and response, the fitted polynomial equation was separately expressed as 3D-response surfaces curves. The response surface plot provides an easy and convenient way to represent the effect of the significant variables, their interaction in the response variable and their optimum levels. The variations in the yields of vindoline from the response surface as a function of varying factors, the temperature, pH, dextrose and tryptone concentrations (obtained through analysis of variance) are shown in Fig. 7A-D. From the response surface plot, it can be seen that both high pH and temperature adversely affected vindoline yield, suggesting that a pH around 6.5 and temperature of 33°C are required to produce vindoline at an optimum level. The yield of vindoline is optimum at the intermediate concentration of tryptone and temperature, as per the experimental design. It is understood that that the yield of vindoline is lower at low temperature and low dextrose concentration, and that after increasing the concentration of dextrose from 1% to 1.5% and the temperature from 28 to 33°C, the vindoline yield increases from 40 to 65 µg/L. It was also observed that when increasing the concentration of dextrose while at the same time decreasing the concentration of tryptone, the yield of vindoline decreases. Hence, we can say that dextrose at a concentration of 1.5% and tryptone at a concentration of 2% are the required quantities for the production of 82 µg/L vindoline by *Microbacterium* sp.

DISCUSSION

Every bacterium has its own requirements, and its growth curve depends on both the physical and chemical nature of its surroundings. For working with endo-

phytic bacteria, complex media having high amounts of undefined nutrients are usually chosen over a defined medium having significantly fixed amounts of nutrients [30]. Eevers et al. [31] used tryptic soy agar medium for growing endophytic bacteria and optimization of the isolation process. Gupta et al. [32] also used nutrient agar media and successfully isolated *Bacillus subtilis* and *Stenotrophomonas maltophilia* from the root and leaves of *Prosopis cineraria*. Kumar et al. [33] used eight different media to optimize vinca alkaloid production from *Fusarium solani* isolated from *C. roseus*. In another study, vinca medium-1 was optimized for the production of vinblastine and vincristine, respectively, from an endophytic fungus *Fusarium oxysporum* isolated from *C. roseus* [15]. Although the selection of an appropriate growth medium is crucial for the isolation and maintenance of endophytes, a comparative study of different nutrient media types needs to be performed. The complex media that were selected for the present study contained tryptone, soytone, beef extract, yeast extract and NaCl. The media used for the growth of endophytes provided a rich environment, albeit one that was very dissimilar to the atmosphere inside the plant. There are reports on the isolation of fungal endophytes from *C. roseus* but there are only a few reports on the isolation of endophytic bacteria and optimization of the components of nutrient agar medium. Yeast extract has been reported as biotic elicitor; it has been recognized as a signaling element in enriching indole alkaloids in various protoplast-derived tissues and plantlets of *C. roseus* [34]. But yeast extract is mainly known to provide carbon and nitrogen to the bacteria, and such elicitation activities for the production of useful secondary metabolites has not been studied yet in endophytes.

There is no such work on the production of vindoline from bacterial endophytes. Vindoline content was enhanced by 229-403% when *Curvularia* sp. and *Choanephora infundibulifera* were inoculated into healthy non-infected, endophyte-free plants of *C. roseus*. In endophyte-inoculated plants of *C. roseus* the expression of the terpenoid indole alkaloid (TIA)-pathway genes was upregulated [35]. The endophytic fungus *T. radicus* is reported to produce vinblastine and vincristine, and its production has been optimized in nine different liquid media [6]. In earlier studies, statistical optimization techniques have been successfully used in the optimization of media for vinca alkaloid production from endophytes, such as the optimized production of vinblastine (70 µg/L) and vincristine (670 µg/L) by *T. radicus* isolated from *C. roseus* [6]. An endophytic fungus *Fusarium oxysporum* isolated from *C. roseus* was also reported to produce vinblastine (76 µg/L) and vincristine (67 µg/L) after optimization [15].

Phytochemical analysis is generally carried out in the extracts of plants but very few reports are available on endophytes. In a study of endophytes, phytochemical analysis of ethyl acetate extracts of *C. gloeosporioides* isolated from *Plumeria acuminata* showed the presence of alkaloids [23]. Alkaloids have also been detected in endophytes isolated from *Salvadora oleoides*, *Tabebuia argentea*, *Pinus roxburghii* and *Urospermum picroides* by phytochemical analysis [36]. Verma et al. [37] studied vinblastine and catharanthine yields as a function of pH and similar results were obtained, which is to be expected because vindoline, vinblastine and catharanthine have a similar nature as they belong to the group of terpenoid indole alkaloids. In earlier studies, HPLC analysis of the secondary metabolite profiles of *C. roseus* gave a similar result in which the retention time of vindoline was found to be 13.5 min. The mobile phase used was a mixture of phosphate buffer and acetonitrile with a linear gradient solvent system from 80:20 (v/v) to 20:80 (v/v) in 20 min [38]. In another study, the retention time of vindoline was found to be 13.22 min after optimization of the mobile phase of acetonitrile and 0.1 M phosphate buffer containing 0.5% glacial acetic acid, 21:79 (v/v) [39]. Kumar et al. [33] also used TLC and ESI/MS to confirm the production of vincristine and vinblastine by an endophytic fungus *Fusarium solani* isolated from *C. roseus*.

The cost of growth media accounts for 25-35% of the total cost of metabolite production, hence the concentrations of factors such as carbon and nitrogen sources is of prime importance, as is the case here when information on the growth requirements of an important strain are lacking. After selecting a suitable medium for the optimum production of vindoline by *Microbacterium* sp., the components of the selected medium, yeast extract, beef extract, peptone and NaCl, were optimized. Although the amount of vindoline was increased, it was not to a satisfactory level, and so more the optimized nutrient agar medium was supplemented

with different additional carbon and nitrogen sources using a "one-factor-at-a-time-approach". Although the "one-factor-at-a-time approach" is frequently used for optimization studies, it is unsuitable for multifactor optimization and it also neglects the complex interaction among various parameters used in the study. The inadequacy of the "one-factor-at-a-time" method can be overcome by using a potent and suitable statistical experimental design tool such as RSM, which can reduce the process unevenness and explain the interactions between the different variables [40].

We believe that this study will serve as a reference point for the use of the statistical techniques in bacterial systems and vital research in this area. Also, it should offer researchers flexibility for selecting improved conditions, such as optimum carbon and nitrogen sources, pH, rpm and temperature. The production of vindoline by *Microbacterium* sp. was detected by TLC, HPLC and ESI/MS. The current work provides evidence that the information about the molecular mass of a compound can be obtained by direct-injection ESI-MS and MS/MS fragmentation patterns, which provide specific structural information for its identification. Secondly, the use of the response surface methodology for media optimization successfully increased vindoline production from 29 μg to 82 μg per L of bacterial culture and also assisted in the identification of the most significant and optimal nutrient levels. In order to authenticate the suitability of the model equations, experiments were repeated twice at the predicted optimum conditions. The mean values of the predicted responses and the experimental data displayed a worthy correlation between the predicted and experimental yield of vindoline, specifying a good fit of the model. The reliability of the experiments is supported by the ANOVA table, regression coefficient, and response surface 3D plot, which concluded the model could be considered to fit.

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