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ORIGINAL ARTICLE

Statistically optimized production and characterization of vanillin from creosol using newly isolated *Klebsiella pneumoniae* P27

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Abstract The current research study deals with the screening of a potent vanillin-producing microorganism among 96 isolated strains. Biochemical characterization and molecular identification confirmed that the isolated strain belongs to the *Klebsiella pneumoniae* bacteria, so it was denoted as *Klebsiella pneumoniae* P27. The optimization of medium components for the enhanced production of vanillin was carried out using two-stage statistical experimental designs, in which the significant medium components for vanillin production were screened using a Plackett-Burman experimental design. And the optimal levels of those noteworthy factors were determined by using central composite design. The statistical optimization of medium components resulted in increases in vanillin production and vanillyl alcohol oxidase activity of 2.05-fold and 3.055-fold, respectively. The highest vanillin production (30.88 mg/L) and vanillyl alcohol oxidase activity (0.044 U/mL) was observed after 16 h of incubation in the presence of 0.26 mL/L creosol, 8.06 g/L yeast extract and 2.77 g/L NH_4NO_3 in the production medium. The optimally produced vanillin was extracted and confirmed using FTIR and LCMS spectral analysis. The results of the current study support a statistical process optimization approach as a potential technique for the enhanced production of vanillin from creosol by using newly isolated *Klebsiella pneumoniae* P27 bacterial strain.

Keywords Response surface methodology · Vanillyl alcohol oxidase · 16S rDNA sequencing · FTIR · HRMS

Introduction

Vanillin is one of the important flavoring agents and is a major constituent of the natural vanilla (Walton et al. 2003). The most traditional source of natural vanilla is bean or pod of the tropical vanilla orchid (*Vanilla planifolia*). Vanillin has tremendous applications in the food, pharmaceutical, beverage and perfume industries. More than 12,000 tons of vanillin are produced every year. Of that, <1% of it is extracted from vanilla pods; the remainder is synthesized via chemical processes (Priefert et al. 2003). Extraction of vanillin from vanilla pods is a very expensive and laborious process. Hence, the average cost of natural vanillin ranges from US $1200 to $4000/kg and that of the synthetic vanillin is <US $15/kg (Walton et al. 2003). Consumers are very conscious about their health and therefore prefer natural products over synthetic despite the higher market price (Schrader et al. 2004). To fulfill the enhanced industrial demand for natural vanillin, biotechnological researchers are busy exploring ways to biosynthesize vanillin. Biotechnology based approaches to vanillin synthesis use readily available and economically feasible natural feedstocks (Walten et al. 2000). Several potential feedstocks, such as curcumin, phenolic stilbenes, eugenol, isoeugenol, ferulic acid, have been reported. The controlled oxidation of lignin is another possible path to vanillin synthesis by utilizing lignin waste (Yoon et al. 2005; Zhao et al. 2005; Overhage et al. 2006; Shakeri et al. 2013). Furthermore, eugenol and isoeugenol are essential oil components that are used widely for vanillin synthesis. Isoeugenol has been reported to be biotransformed into vanillin by *Bacillus subtillis* B2 (Shimoni et al. 2000).

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Extraction from natural vanilla pods and biotransformation using natural feedstocks are quite expensive methods, so researchers turned to vanillin synthesis by a biocatalytic method employing microbial enzyme vanillyl alcohol oxidase (VAO) (EC 1.1.3.38). VAO is an oxidoreductase enzyme, and was first reported in the fungus *Penicillium simplicissimum* CBS 170.90 (Jong et al. 1992), then purified and characterized from *Byssochlamys fulva* V107 (Furukawa et al. 1999). The recombinant strain *Amycolatopsis* sp. HR 167, containing the VAO gene (vaoA), has been reported as a very efficient biotransformer to convert eugenol to coniferyl alcohol with the production of trace amount of vanillin (Overhage et al. 2006).

VAO is a versatile biocatalyst for vanillin biosynthesis, with a broad range of substrate specificity. It catalyzes vanillin synthesis using natural feedstocks, like creosol (2-methoxy-p-cresol), as a substrate. Creosol is a major component of creosote, which is obtained from heating wood or coal tar (van den Heuvel et al. 2001, 2004). Another potential feedstock for vanillin synthesis is vanillylamine derived from capsaicin (8-methyl-N-vanillyl-6-nonenamide)—a pungent principle of hot red pepper (van den Heuvel et al. 2001).

As the demand for vanillin is increasing day by day, most researchers have been in search of natural sources for vanillin production. Some earlier reports stated that certain fungal and bacterial species possess the ability to produce VAO—an enzyme responsible for vanillin biosynthesis. So it was hypothesized that these active microbial cultures could be screened from the environment for natural vanillin production, and such cultures could be directed to produce enhanced vanillin content during their growth by optimizing their metabolic conditions. Accordingly, the aim of the present study was to hunt for some environmental microorganisms with a potent ability to produce vanillin. The study was also intended to optimize the medium components for the increased production of vanillin using these environmental isolates. With the designed objectives and hypothesis, the screening, isolation and identification of the efficient bacterium were carried out here. The authors have successfully screened and identified an proficient VAO-producing bacterial strain from *Klebsiella pneumoniae*. This newly isolated bacterial isolate has been designated as *Klebsiella pneumoniae* P27. This is the first report of vanillin production by using an environmental isolate, *K. pneumoniae* P27. Microbial screening for the potency of VAO production was achieved by a rapid colony staining method (Kasabe et al. 2015). This method was modified by us and applied for the first time to the screening of VAO producers. The pre-optimization medium components and conditions were taken from earlier reports (Van Rooyen 2012). Statistical optimization studies were carried out using response surface methodology (RSM). The significant medium components affecting vanillin synthesis by this strain were selected by employing Plackett-Burman factorial experimental design, and were further optimized by using central composite design (CCD) (Omran 2014; Facchini et al. 2015; Jhample et al. 2015). After successful production optimization, the produced vanillin was extracted from fermentation broth for identification and characterization.

Material and methods

Chemicals and reagents

Standard vanillin, vanillyl alcohol, creosol and horse radish peroxidase were purchased from Sigma (Bangalore, India). 4-Aminoantipyrine, phenol, hydrogen peroxide (H_2O_2) and other media components used in this study were purchased from HiMedia and SD Fine Chemicals, Chennai, India. All the organic solvents (ethyl acetate, dichloromethane, chloroform, formic acid and methanol) were procured from Qualigens (Mumbai, India) and all other reagents were of analytical grade and obtained from local suppliers.

Isolation and culture conditions

The sampling was done at various locations involved in the wood processing and preservation industries as well as chemical and steel industrial sites of Kolhapur district, (M.S.), India, in order to isolate the vanillin-producing microorganisms. The samples were subjected to a five-stage enrichment technique, as described in our earlier reports (Kasabe et al. 2015). Briefly, the enrichment was achieved by maintaining various vanillyl alcohol concentrations ranging from 0.02% to 0.1% in three repeated cycles for each concentration stage. 1% of each sample was inoculated in the 100 mL mineral salt medium containing g/L: KH_2PO_4, 1.8; Na_2HPO_4, 1.0; NH_4Cl, 2.0; $(NH_4)_2SO_4$, 0.1; $MgCl_2{\cdot}6H_2O$, 0.07; 0.2 mL trace elements solution (Jong et al. 1990) and vanillyl alcohol as a sole carbon source with a particular concentration. After the enrichment process, 100 μL serially diluted broth was spread on the same medium containing 2.2% agar in sterile Petri plates. The inoculated plates were incubated at 30 °C for 24–48 h. After completion of incubation period, each isolated colony was further streaked out separately on the sterile Petri plates containing same solid medium and incubated at 30 °C for 24 h. Then, the grown colonies of individual isolated bacteria were further subjected to screening for VAO production as this enzyme plays a key role in vanillin biosynthesis.

Screening of VAO-producing bacteria

VAO-producing potent bacteria were screened using a H_2O_2-based rapid colony staining method with certain modifications (Kasabe et al. 2015). In order to select a potent VAO-producing microorganism, the isolated strains

were grown on enrichment medium plates. Previously sterilized strips of Whatmann filter paper No. 1 were soaked in filter-sterilized solution containing 0.5% vanillyl alcohol; 1.7% 4-aminoantipyrin; 6% phenol and 3000 U/L horseradish peroxidase (HRP). The soaked filter papers were then applied aseptically to the grown colonies, and the plates were incubated at 30 °C. The qualitative analysis of VAO was performed by measuring the time required for red color development due to the formation of quinoimine dye in the plate at colony site. The isolated strain showing maximum red color production within the least incubation time was taken to have capacity of highest VAO production and was selected for further studies.

Biochemical characterization, identification and phylogenetic analysis of the selected bacterial strain

The morphological, biochemical and physiological traits of the isolated strain were provisionally identified according to *Bergey's manual of systematic bacteriology*. Identification of the strain with matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry was performed by using instrument AUTOFLEX speed (Bruker Daltonik, Bremen, Germany) and MALDI biotyper software 3.0 (Bruker Daltonik) was used to identify the isolates and visualize the mass spectra. Molecular identification of the selected strain was performed using 16S rDNA sequencing. The DNA was extracted and the fragment of 16S rDNA sequence was amplified by polymerase chain reaction (PCR) using the primers 8F (5′-GGATCCAGACTTTGATYMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT- 3′). The PCR amplicon was purified by the ExoSAP IT (USB, Cleveland, OH) treatment. During this treatment, the ExoSAP IT removes left over primers and deoxynucleoside triphosphates by using combination of enzymes exonuclease I and shrimp alkaline phosphatase. The forward and reverse DNA sequencing reaction of PCR amplicon was carried out using 704F and 907R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. A consensus sequence of 1488 bp 16S rDNA was generated from forward and reverse sequence data using aligner software. The generated 16S rDNA sequence of the isolated bacterial strain was used to identify the bacterial strain. Molecular identification was done by multiple sequence alignment with 16S rDNA sequences of other known bacterial species deposited in the GenBank database. The alignment was carried out using Blastn—an online tool from the National Centre for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The phylogenetic tree was constructed using neighbor joining (NJ) method software MEGA version 6.0 with No. of bootstrap replication value of 500 with the maximum composite likelihood model (Saitou and Nei 1987).

Simultaneous vanillin production measurement with the growth curve

The isolated strain *K. pneumoniae* P27 was studied for vanillin biosynthesis in correlation with its growth curve in vanillin production medium. The medium was composed of 0.5% yeast extract, 0.2% NH_4NO_3, 0.02% K_2HPO_4, 0.03% $MgSO_4{\cdot}7H_2O$ and 0.05% (*v*/v) creosol. The bacterial culture was grown for 8 h at 37 °C in nutrient broth and 2% of it was inoculated in 100 mL vanillin production medium in multiple 250 mL Erlenmeyer flasks. The flasks were incubated at 120 rpm and 30 °C. At 1-h intervals, growth was measured at 660 nm; a flask containing production medium without inoculation of culture was used as a control. Vanillin production was determined spectrophotometrically at 340 nm in comparison with the calibration curve of standard vanillin (Jong et al. 1992). The optimal time of incubation was determined here, and large-scale vanillin production was then achieved.

Statistical optimization of vanillin biosynthesis by experimental design

Screening of significant medium components using Plackett-Burman experimental design

The Plackett-Burman experimental design was used to determine the major factors influencing vanillin production. The experimental design comprised a total of 13 experimental trials; among these, one run was carried out at the center point values and the remaining runs were conducted by combinations of high (+) and low (−) levels of all variables. In the Plackett-Burman experimental design, two levels were used to determine whether the maximum production was obtained at lower or higher concentration of the variables by comparing them with the experimental results obtained from center point values (Jhample et al. 2015). The medium components before optimization and conditions for vanillin production were taken from earlier reports (Van Rooyen 2012). These medium components were checked for vanillin production, and then were considered for further optimization studies. Six factors were studied, including yeast extract (A), NH_4NO_3 (B), K_2HPO_4 ©), $MgSO_4$ (D), creosol (E), and inoculum size (F) for statistical optimizations. In this experimental design, each of the factors was examined at two levels, low (−1) and high (+1). The low and high levels of these factors were taken as follows: yeast extract (4 g/L and 8 g/L), NH_4NO_3 (1 g/L and 2 g/L), K_2HPO_4 (100 mg/L and 300 mg/L), $MgSO_4$ (200 mg/L and 600 mg/L), creosol (0.3 mL/L and 0.7 mL/L), inoculum size (10 mL/L and 30 mL/L). The design was composed of 12 experimental trials (runs) with an additional one run at the center points as shown in Table 1. The Plackett-Burman

Table 1 Plackett-Burman experimental design of six variables for evaluating factors influencing vanillin production

Run no.	Yeast extract (g/L)	NH_4NO_3 (g/L)	K_2HPO_4 (mg/L)	$MgSO_4$ (mg/L)	Creosol (mL/L)	Inoculum size (mL/L)	Vanillin production (mg/L)	
							Experimental	Predicted
1	4	2	300	200	0.7	30	10.96	12.05
2	6	1.5	200	400	0.5	20	18.96	14.88
3	4	2	300	600	0.3	10	15.55	15.71
4	4	1	100	200	0.3	10	12.11	11.52
5	8	1	100	200	0.7	10	17.33	16.27
6	8	2	100	200	0.3	30	23.25	24.11
7	8	2	100	600	0.7	30	22.29	20.46
8	4	2	100	600	0.7	10	11.55	12.05
9	4	1	300	200	0.7	30	5.62	7.87
10	8	1	300	600	0.3	30	16.14	19.93
11	8	2	300	200	0.3	10	24.92	24.11
12	8	1	300	600	0.7	10	17.25	16.27
13	4	1	100	600	0.3	30	14.96	11.52

experimental design was based on the first order polynomial model as shown in Eq. 1 (Supplementary Table S1).

Where Y is the vanillin production, β_o is model intercept, β_i is the linear coefficient and χ_i is the level of the independent variable. The statistical significance of first order model was identified using analysis of variance (ANOVA), according to regression analysis of variables, factors with greater than 95% significance ($P < 0.05$) were considered to have a significant effect on vanillin production. Further optimization of critical factors for vanillin production was carried out using CCD.

CCD for optimization of medium components

Three significant medium components selected by the Plackett-Burman experimental design were further optimized here. RSM using full factorial CCD was employed to examine the effect of these significant components on vanillin production. The variables used were creosol, yeast extract and NH_4NO_3, each at five coded level ($-\alpha$, -1, 0, $+1$, $+\alpha$) as shown in Table S2. In the present study, the CCD was consisting of 20 experimental runs that included 6 runs at the center point with $\alpha = 1.682$. The full experimental set up with respect to the maximum and minimum values of each variable were listed in Table 2. The vanillin production was analyzed by using second order polynomial Eq. 2 (Table S1).

Where Y is the predicted response, β_o is the model constant, β_i is the linear, β_{ii} is the quadratic and β_{ij} is the interaction coefficients. The coefficient of determination, R^2 determines the quality of fit of polynomial equation, all the experiments were performed in triplicate, and average vanillin produced was used as response. The results of the experimental design were analyzed and interpreted using 'Design Expert' STAT Ease software 9.0 version (STAT Ease Minneapolis, MN).

Table 2 Experimental design and results of central composite design (CCD) for vanillin production

Run no.	Creosol (mL/L)	Yeast extract (g/L)	NH_4NO_3 (g/L)	Vanillin production (mg/L)	
				Experimental	Predicted
1	0.1	4	1	21.18	19.57
2	0.3	4	1	16.14	15.77
3	0.1	12	1	11.18	11.28
4	0.3	12	1	11.08	9.56
5	0.1	4	3	7.14	7.60
6	0.3	4	3	18.22	17.94
7	0.1	12	3	9.70	9.89
8	0.3	12	3	20	21.43
9	0.03	8	2	13.85	13.76
10	0.37	8	2	19.92	20.27
11	0.2	1.27	2	7.14	7.60
12	0.2	14.72	2	3.77	3.57
13	0.2	8	0.31	20.11	22.05
14	0.2	8	3.68	24.40	27.43
15	0.2	8	2	24.40	22.72
16	0.2	8	2	25	27.43
17	0.2	8	2	28.70	27.43
18	0.2	8	2	28.70	27.43
19	0.2	8	2	30.81	27.43
20	0.2	8	2	27	27.43

Table 3 Biochemical test results for the isolated strain

Biochemical tests	Result
Gram stain	Gram negative
Motility	Non motile
Indol production	–
Methyl red	+
Voges-Proskauer	+
Citrate	+
Urease	+
H_2S production	–
Catalase	+
Starch hydrolysis	–
Gelatin hydrolysis	–
Glucose	+
Fructose	+
Xylose	+
Sucrose	+
Maltose	+
Lactose	+
Arabinose	+
Gas production	+
Probable strain	*Klebsiella* sp.

Validation of optimization for vanillin biosynthesis

After the successful optimization of vanillin biosynthesis, the comparative vanillin production profile was studied. The unoptimized medium and the statistically optimized medium were inoculated with 2% of *K. pneumoniae* P27 seed culture and incubated at 120 rpm and 30 °C for 24 h. Vanillin production in both media was assessed simultaneously at 1-h intervals for up to 24 h using a spectrophotometric method at 340 nm wavelength.

In order to validate the results obtained from statistically optimized medium and to achieve mass production of vanillin, an experiment was conducted in a 2-L fermenter containing 1 L optimized fermentation medium. The medium was inoculated with 2% of *K. pneumoniae* P27 seed culture and incubated at 120 rpm and 30 °C for 16 h. After completion of the incubation period, the fermentation broth was centrifuged at 8000 rpm for 15 min at 4 °C and vanillin production was assessed quantitatively.

In addition to vanillin production, we also studied the VAO production in the unoptimized medium and in the statistically optimized medium. VAO enzyme activity was assessed before and after optimization of medium components by the method reported by Jong et al. (1992). Briefly, the reaction mixture was composed of 1.5 mL carbonate buffer (pH 10) and 1 mL 0.1% vanillyl alcohol solution and 0.5 mL enzyme solution. The reaction mixture was incubated at 30 °C for 1 min, and then the vanillin production was measured spectrophotometrically at 340 nm. One unit of enzyme activity was defined as the amount of enzyme that forms 1 μmol min^{-1} vanillin under standard assay conditions.

Extraction of vanillin from fermentation broth

The fermentation broth was centrifuged at 8000 rpm for 15 min. The filtrate was extracted three times with equal volumes of ethyl acetate and dichloromethane. The organic phase containing vanillin was evaporated at 55 °C, concentrated, and reconstituted in 2 mL methanol. In order to obtain the fingerprint of extracted sample, the sample was applied to a TLC plate. Ten microliters of standard vanillin at concentration of 1 mg/mL in methanol was spotted on the TLC plate as a reference. Similarly, a 10 μL aliquot of sample solution was spotted on the TLC plate. The plate was dried for 10 min and then subjected to chromatographic separation. The solvent system composed of chloroform: ethyl acetate: formic acid (10:8:1) was used as a mobile phase. After chromatographic separation, the chromatogram was developed in an iodine chamber. An orange colored spot for vanillin appeared in the standard and the sample lane. The spot appearing in the sample lane was scratched from TLC plate. The scratched silica was suspended in 0.5 mL methanol for 10 min to dissolve the extracted vanillin in the methanol. The solution was centrifuged at 5000 rpm for 5 min, the pellet was discarded and supernatants pooled for further characterization studies.

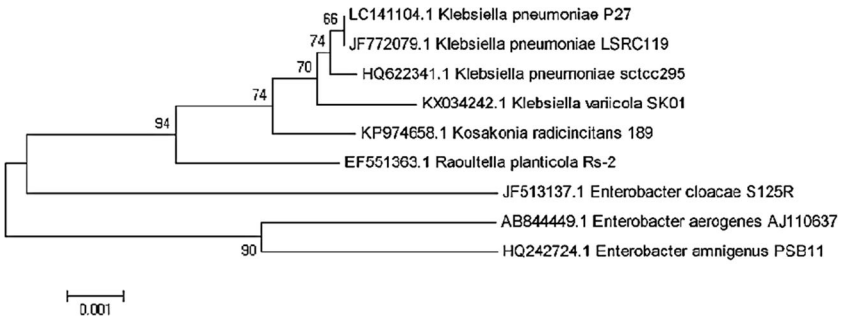


Fig. 1 Phylogenetic tree based on the partial 16S rRNA sequence of the isolated *Klebsiella pneumoniae* P27. The tree shows the phylogenetic affiliation of *K. pneumoniae* P27 with other related bacterial species. The tree was constructed using the NJ method, and bootstrap values based on 500 replications are shown at the nodes of the tree

Characterization of extracted vanillin

The extracted vanillin was characterized and validated by using Fourier transform infrared (FTIR) spectroscopy (Gunasekaran and Ponnusamy 2005; Voitl and von Rohr 2010) and liquid chromatography mass spectrometry (LCMS) analysis. These validations of extracted vanillin were performed in comparison with the standard vanillin solutions prepared in methanol with final concentration of 5 μg/mL.

The FTIR spectrum was recorded using a JASCO FT/IR-4600 in the range of 4000–500 cm^{-1}, recorded at 4 cm^{-1} resolution. Standard vanillin displays a broad absorption band in the region of 3332.39 cm^{-1}, 2806–2871 cm^{-1}, 2832.92 cm^{-1}, 1625–1430 cm^{-1}, 2947.66 cm^{-1}, and weak intensity bands at 536.11 cm^{-1} and 637.36 cm^{-1}. In order to validate the results, absorption band patterns of the standard and extracted vanillin were compared.

Further identification of the extracted vanillin was performed by mass spectrometric techniques by using a Bruker Impact HD (Impact II-UHR-ESI-Q-ToF) mass spectrometer. The acquired mass spectra were analyzed using Bruker Compass Data Analysis 4.2 software.

Statistical analysis

Results of isolations, screenings, vanillin production, enzyme production and characterization of vanillin were acquired as an average of three or more determinations. ANOVA was carried out on all data at $P < 0.05$ using Graph Pad software (GraphPadInStat version 3.00, GraphPad Software, San Diego, CA).

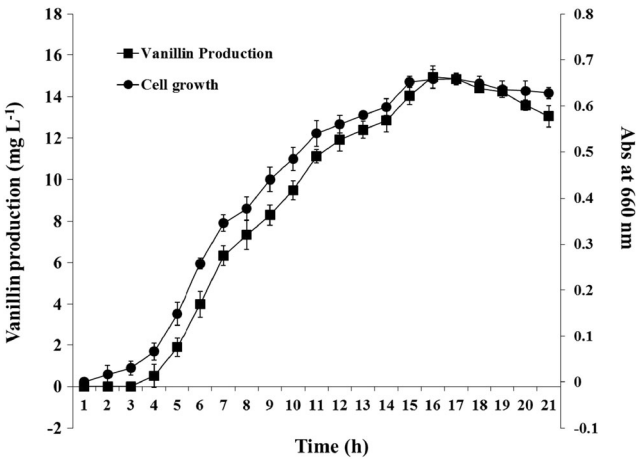


Fig. 2 Simultaneous measurement of growth of *K. pneumoniae* P27 and vanillin production in the fermentation medium. Growth of *K. pneumoniae* P27 (●) and vanillin production (■) were measured at 660 nm and 340 nm, respectively. All values are expressed as mean ± SD ($n = 3$)

Fig. 3 **a–c** Contour plots and response surface plots for optimization of vanillin production by *K. pneumoniae* P27, showing the interaction effects of yeast extract, creosol and NH_4NO_3 on vanillin production. **a** Effect of yeast extract and creosol; **b** effect of NH_4NO_3 and creosol; **c** effect of NH_4NO_3 and yeast extract

Results and discussion

Isolation, screening and identification of vanillin-producing *K. pneumoniae* P27

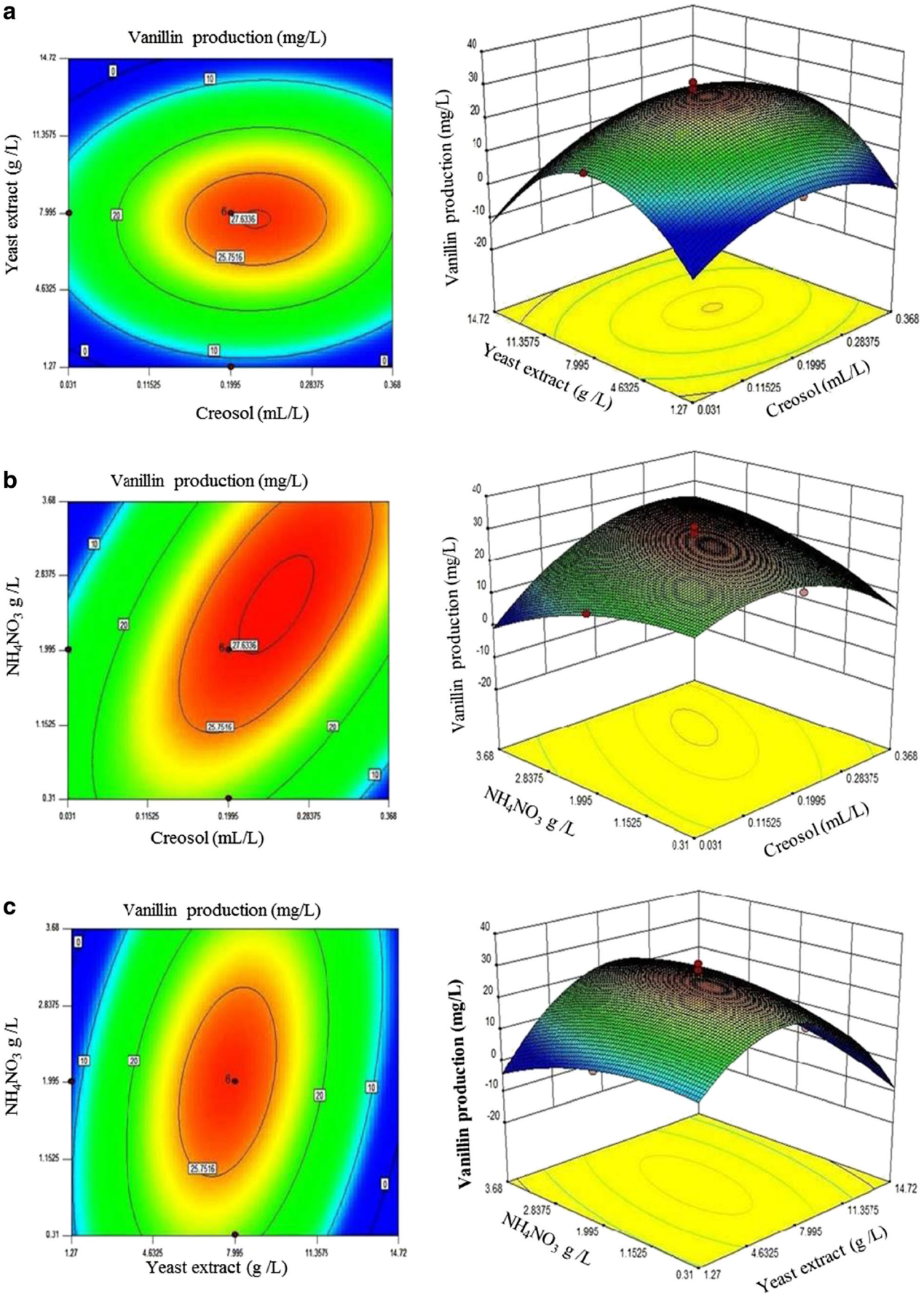
In the present study, 96 microbial strains were isolated from 29 samples collected from 7 different locations in the Kolhapur area. Of these, 25 isolates were selected on the basis of colony staining methods in correlation with their VAO enzyme production ability. These 25 isolates were further pooled for biochemical characterization and microbial identification. Of the 25 selective VAO-producing strains, 10 showed formation of maximum red color within a short period of time (10 min). From VAO quantitative activity assessment data of these selective strains, isolate P27 showed the most significant enzyme activity, and also the best results in the colony staining procedure. Therefore this isolate was selected for further studies.

Phenotypic and biochemical characterization revealed that the isolated strain was a rod-shaped Gram-negative bacterium. The results of some biochemical tests (Table 3) suggested that this isolated strain belongs to *Klebsiella* sp. Hence it was preliminary designated as *Klebsiella* sp. P27. The identification based on MALDI-TOF analysis gave a mean MALDI-TOF MS log (score) value of 2.303 ± 0.031, which confirmed the culture up to species level as *K. pneumoniae*.

Molecular identification by 16S rDNA gene sequence revealed that the isolated strain was a *K. pneumoniae*, so it was named *K. pneumoniae* P27. The partial sequence of 16S rDNA gene from the newly isolated *K. pneumoniae* P27 strain was deposited for global access with the DNA Data Bank Japan (DDBJ) under accession no. LC141104.1. A phylogenetic tree was constructed using some of the neighbors having maximum homologous gene sequences, which confirm its closer relationship with its neighborhood organisms (Fig. 1).

Simultaneous vanillin production measurement with the growth curve

The vanillin production profile of isolated *K. pneumoniae* P27 was studied with its growth curve in vanillin production medium (Fig. 2). With inoculation of 2% inoculum, actual vanillin production was initiated after 4 h of incubation. Production increased further with the subsequent increment in cell mass, reaching a maximum after 15 h. The change in protein content in the growing culture medium was recorded at time intervals of 1 h (Table S4). According to the growth


a
Vanillin production (mg/L)
Yeast extract (g /L)
Creosol (mL/L)
Vanillin production (mg/L)
Yeast extract (g /L)
Creosol (mL/L)
b
Vanillin production (mg/L)
NH4NO3 g /L
Creosol (mL/L)
Vanillin production (mg/L)
NH4NO3 g /L
Creosol (mL/L)
c
Vanillin production (mg/L)
NH4NO3 g /L
Yeast extract (g /L)
Vanillin production (mg/L)
NH4NO3 g /L
Yeast extract (g /L)

curve, after incubation of *K. pneumoniae* P27 for 15 h with this inoculation set up, the bacterial growth reaches the beginning of its stationary phase. All these experiments were performed in triplicate and results are expressed in mean ± SD format ($n = 3$). The results suggest that production of vanillin can be achieved only in the exponential phase of growth, and vanillin synthesis was observed to decrease with further passage of incubation time. As vanillin is synthesized by *K. pneumoniae* P27 in its exponential phase, it may be concluded that it is a primary metabolite of the culture. Thus, the incubation time required for optimum vanillin production by newly isolated *K. pneumoniae* P27 was standardized here in order to obtained mass production of vanillin.

Experimental design for statistical optimization of vanillin production

In the Plackett-Burman design, the independent variables (medium components) were studied with their respective high and low levels. The results of Plackett-Burman design indicated that yeast extract, NH_4NO_3 and creosol were the significant medium components showing P values <0.05 significance level obtained by regression analysis, and the remaining components were found to be insignificant. The obtained model F value was 29.02, indicating that the model was significant. In order to produce vanillin, first order polynomial Eq. 3 is derived from regression analysis (Table S1).

The significant components, yeast extract (A), NH_4NO_3 (B) and creosol (E) short-listed in the Plackett-Burman design were subjected to further optimization of their required concentration and their interaction effects leading to maximum vanillin production.

In the present work, full factorial CCD was employed to identify optimal conditions for enhanced production of vanillin. The experimental design and the response for each individual experiment are summarized in Table 1. The results showed that the production of vanillin corresponding to the combined effect of all three components in the specified ranges, and is best predicted by the Eq. 4 (Table S1).

The effect on vanillin yield of interactions of variables was studied against any two independent variables, while keeping the remaining third independent variable at a constant level. Contour plots or response surface plots can be used to predict the optimum values of each of the significant variables for vanillin production. The interaction effects of yeast extract, creosol and NH_4NO_3 amongst each other are well explained in Fig. 3 with the help of the respective three-dimensional (3D) response plots and contour plots. The shape of the response surface curve shows that vanillin production was considerably affected by the change in concentration of the two variables. In the case of the interactive effects of yeast extract and creosol (Fig. 3a), lower vanillin yield was obtained at the higher and lower concentrations of both variables. The interaction of creosol and NH_4NO_3 (Fig. 3b) indicates moderate interactions between the tested variables. The effect of interaction between NH_4NO_3 and yeast extract (Fig. 3c) shows that NH_4NO_3 and yeast extract concentrations markedly affect vanillin production. The typical response surface plots shown in Fig. 3 affirm that concentrations of 0.26 mL/L creosol, 8.06 g/L yeast extract and 2.77 g/L NH_4NO_3 are significant for maximum vanillin production.

VAO activity and vanillin production

Klebsiella pneumoniae P27 is the first bacterial source reported for the biosynthesis of vanillin using creosol as substrate. The isolated *K. pneumoniae* P27 is significant because of the potential for high vanillin production within a short period of time. The initiation of vanillin production occurred after 4 h, reaching a maximum after 16 h of incubation. The vanillin production profile of *K. pneumoniae* P27 before and after medium optimization is shown in Fig. 4. According to the results, maximum vanillin yield before medium optimization was 14.96 mg/L (molar yield 6.19%) after 15 h of incubation. In contrast, production in RSM-based optimized medium was 30.88 mg/L (molar yield 12.89%) after 16 h of incubation. Thus, a 2.05-fold increase in vanillin production was achieved using the RSM-based optimized medium. This far greater molar yield of vanillin production by *K. pneumoniae* P27 after 16 h of incubation is much more than the amount of vanillin produced by the bioconversion of isoeugenol by *Bacillus subtilis* B2 after 24 h of incubation (molar yield 12.4%) (Shimoni et al. 2000). In contrast, the yield is lower than vanillin production by *Bacillus fusiformis* (molar yield 17.43%) and *Bacillus pumilus* (molar yield 40.5%) strains using isoeugenol as substrate (Zhao et al. 2005; Hua et al. 2007) after 72 h and 150 h incubation, respectively. The significance of the present study lies in the time of vanillin production by *K. pneumoniae* P27.

The statistically optimized medium contained 8.06 g/L yeast extract and 2.77 g/L NH_4NO_3, while the unoptimized medium has 5 g/L yeast extract and 2 g/L NH_4NO_3. This enhanced concentration of yeast extract and NH_4NO_3 in the optimized medium may cause a delayed cell death phase and slight increment in the exponential phase, which may lead to changes in the optimal time of vanillin production after optimization.

Similarly, VAO activity during vanillin production before medium optimization was 0.014 U/mL, which increased to 0.044 U/mL (3.055-fold enhancement) after optimization of the medium. This confirms that enhancement of vanillin production can be obtained due to enhancement of VAO enzyme production.

For the validation of the results, laboratory scale studies (flask level) were compared with a pilot scale plant using a 1 L medium in a fermenter of 2 L volume. The results show that the statistical

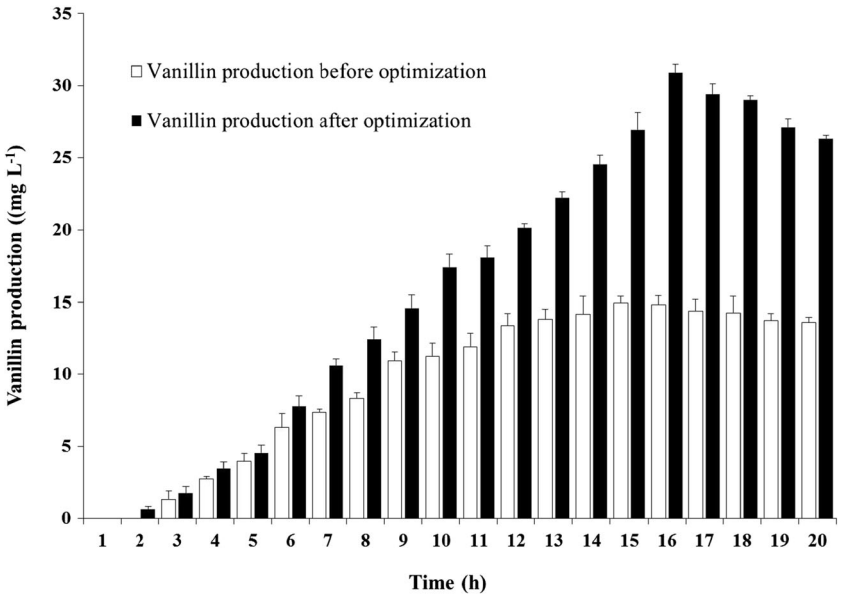


Fig. 4 Enhancement in vanillin production (mg/L) before (□) and after (■) optimization of medium components using response surface methodology (RSM). All values are expressed as mean ± SD (n = 3)

optimization method worked well, and that vanillin production was optimized successfully in the current study. Indeed, production in the pilot level plant (32.11 ± 0.56 mg/L) was even higher than in the laboratory scale plant (30.88 ± 0.26 mg/L).

Characterization of vanillin production

The extracted vanillin was characterized by FTIR spectroscopy in comparison with standard vanillin. The FTIR spectrum indicates the high degree of resemblance in the main absorption peaks of standard and extracted vanillin, confirming that the extracted sample is vanillin (Fig. S1). The results are in accordance with data reported earlier by Gunasekaran and Ponnusamy (2005).

The high-resolution mass spectrometry (HRMS) spectrum for extracted vanillin is shown in Fig. 5. The spectrum, showing the molecular ion at m/z 153.05 [M_+H], corresponds to the molecular formula $C_8H_9O_3$. As vanillin has same molecular formula, the results confirmed the presence of vanillin in extracted sample.

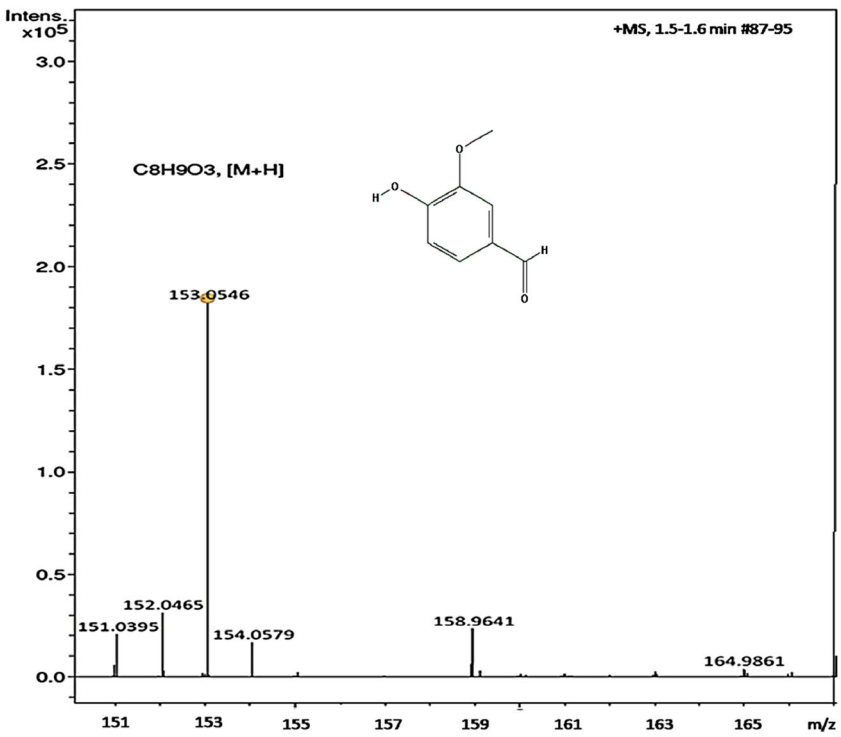


Fig. 5 Identification of vanillin. A high-resolution mass spectrometry (HRMS) spectrum obtained by a Bruker Impact HD (Impact II-UHR-ESI-Q-ToF) mass spectrometer shows that the molecular ion at m/z 153.05 [M_+H] corresponds to vanillin (molecular formula $C_8H_9O_3$)

Conclusion

The present study addressed the problems of fulfilling the increased demand for natural vanillin by society. The outcome of the current research has taken us very close to obtaining a good solution to this problem. Here, vanillin was synthesized using a bacterial culture with the investment of simple efforts. The bioconversion of a natural feedstock, creosol, to vanillin was carried out here using a simple medium and by a newly isolated *K. pneumonia* P27 strain. This biotransformation can be achieved within a relatively very short period as compared to earlier reported bacterial sources such as *B. subtilis* B2, *B. fusiformis* and *B. pumilus*. This is the principal advantage of the current study. The isolated novel bacterial culture was screened from among 96 bacterial strains isolated from various environmental samples. As vanillin biosynthesis is the product of the enzyme VAO, microbial screening for potent VAO production was targeted here. This screening was carried out by a very rapid colony staining method that has been modified by us and applied for the first time to the screening of VAO producers (Kasabe et al. 2015). It can be concluded that the H_2O_2-based rapid colony staining method is a very effective strategy for exploration of predominant vanillin-producing bacteria. The strain *K. pneumoniae* P27 has a naturally enhanced vanillin production ability in a simple medium under suitable environmental conditions. This ability is present naturally in the strain just as it would be in a genetically engineered bacterial culture. The successful screening and isolation of such a potent bacterium is the most striking achievement of this study, and suggests that the strain *K. pneumoniae* P27 could serve as a potential candidate for the industrial production of vanillin. The RSM-based statistical optimization approach increased vanillin production two-fold as compared to unoptimized production conditions. This is another fruitful achievement of the current research work. FTIR and HRMS spectral analysis confirmed the integrity of the synthesized vanillin. So it can be concluded that natural vanillin production is now well optimized by the current research study. The strategies designed by the authors could be of great help to industries seeking natural vanillin production.

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Compliance with ethical standards

Conflict of interest All the authors declare that they have no conflict of interest.

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