Production of Biovanillin by One-Step Biotransformation Using Fungus *Pycnoporous cinnabarinus*

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The current study proposes a one-step biotransformation process for vanillin production from ferulic acid using the wild fungal strain *Pycnoporous cinnabarinus* belonging to the family Basidiomycete. Improvement of biotransformation conditions was performed in two steps; initially a one factor at a time method was used to investigate effects of medium composition variables (i.e., carbon, nitrogen) and environmental factors such as pH on vanillin production. Subsequently, concentrations of medium components were optimized using an orthogonal matrix method. After primary screening, glucose as carbon source and corn steep liquor and ammonium chloride as organic and inorganic nitrogen source, respectively, supported maximum biotransformation of ferulic acid to vanillin. Under statistically optimum conditions vanillin production from ferulic acid by *P. cinnabarinus* was 126 mg/L with a molar yield of 54%. The overall molar yield of vanillin production increased by 4 times.

KEYWORDS: Vanillin; *Pycnoporous cinnabarinus*; biotransformation; one factor at a time; orthogonal array

INTRODUCTION

Vanillin (3-methoxy-4-hydroxybenzaldehyde) is one of the most frequently used characteristic aromatic components extracted from the seedpods of *Vanilla planifola* and frequently used for the production of flavors for foods, confectionery, and beverages (approximately 60%), as a fragrance ingredient in perfumes and cosmetics (approximately 33%), and for pharmaceuticals (approximately 7%). It is produced on a scale of more than 10000 tons per year by the industry through chemical synthesis (*1*). The main portion is produced by chemical synthesis from guaiacol and lignin (*2*) and other alternative biotechnology approaches that are based on biotransformation with the use of fungi, bacteria, plant cells, or genetically engineered microorganisms, etc. The price of vanilla pods (between U.S. $3 and 120 per kg), which usually contains about 2% (w/w) vanillin, is greater (due to the limited availability of vanilla pods) as compared to the price of the chemically synthesized vanillin (about U.S. $12 per kg) (*1*). The price variation and increased consumer demand for natural flavors have attracted attention toward production of vanillin from other natural sources using biotransformation. Literature reports are available on the production of natural flavors using various biotechnological techniques (*3–7*). Usually biotransformation involves the use of enzymes or micro-organisms to perform chemical reactions in which the starting substances and products are of comparable chemical complexity. Biotransformation methods are distinct from biosynthesis, in which relatively complex products are assembled essentially de novo by whole cells, tissues, organs, or organisms from simple starting substances. In this process the substances utilized include carbon dioxide, ammonia, or glucose and those obtained from biodegradation. Biotransformation encompasses the field of microbial transformations of organic or inorganic compounds that result in a change in chemical structure (*8*). Ferulic acid (3-methoxy-4-hydroxycinnamic acid), a lignin-related aromatic acid, is of interest as a renewable resource for the production of useful chemicals due to its abundant availability. A number of attempts have been made to generate value-added products from ferulic acid by biotransformation (*9*). Sutherland et al. (*10*) reported that microorganism such as *Streptomyces setonii* are responsible for the metabolism of ferulic acid to vanillin, vanillic acid (4-hydroxy-3-methoxybenzoic acid), and protocatechuic acid (3,4-dihydroxybenzoic acid).

The production of vanilla beans is a time-consuming process and depends on the suitability of soil and climate conditions. The distinguishing vanilla essence develops in the fruit after a curing process that lasts additionally for 3−6 months (*11*). The annual demand for vanillin is 12000 tons, but only 1800 tons of vanillin is produced naturally, with the remaining demand fulfilled by chemical synthesis. Vanillin was first synthesized from eugenol in 1874−1875, and on commercial scale it was produced until the 1920s; later it was synthesized from lignin. Several routes exist for synthesizing vanillin from guaiacol. Currently, Rhodia (an intenational chemical company) utilizes the most efficient process for vanillin production, in which guaiacol is reacted with glyoxylic acid by electrophilic aromatic substitution to produce vanilmandelic. This was then converted to vanillin by oxidative decarboxylation (*12*).

Ferulic acid is found abundantly in plant cell walls covalently linked to either lignins or other polymers (*13*, *14*). It is a potential precursor for the production of vanillin as well as aromatic

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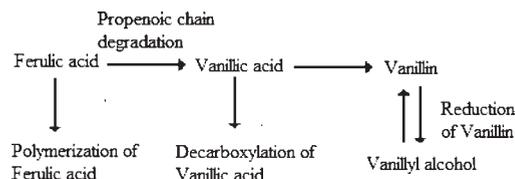
Figure 1. Biotransformation of ferulic acid into vanillin.

chemical compounds by microbial or enzymatic methods (*15*, *16*). During the biotransformation of ferulic acid into vanillin, it is released from raw materials by enzymatic treatment (*17*) and extraction (*18*) and then treated with various microorganisms (*19*−*21*).

As per European and U.S. legislations, vanillin produced through biotechnological (by biotransformation) protocol is considered as "natural", which generally originates from a natural source (*22*). Reports are available on the use of a two-step biotransformation process for the production of vanillin from ferulic acid using a combination of microorganisms such as *Aspergillus niger* and *Pycnoporus cinnabarinus* (*19*). Although coculture of *P. cinnabarinus* and other organisms has been used previously, a special emphasis has been given for the use of a single organism for one-step biotransformation of vanillin. *P. cinnabarinus* metabolizes ferulic acid into vanillin by propanoic acid side-chain degradation (**Figure 1**) to vanillic acid and then subsequent reduction into either vanillin or vanillyl alcohol or decarboxylated into methoxyhydroqinone (*23*).

The aim of present study was production of vanillin (the most universally used flavor in the food industry) using a single-step biotransformation process by the fungus *P. cinnabarinus*. The development of an efficient biotransformation process led to optimization of media using techniques such as one factor at a time followed by statistical methods (*24*). In the first step, we have used the one factor at a time method to examine effects of variables of medium composition (i.e., carbon, nitrogen) and environmental factors such as pH on vanillin production. Subsequently, the concentration of the medium components was optimized using an orthogonal array method.

MATERIALS AND METHODS

Materials. Media components such as glucose, maltose, fructose, lactose, sucrose, magnesium sulfate, calcium chloride, potassium dihydrogen orthophosphate, thiamin hydrochloride, ammonium chloride, diammonium tartarate, ammonium sulfate, ammonium chloride, triammonium citrate, ammonium phosphate, corn steep liquor, yeast extract, proteose peptone, casein peptone, soy peptone, beef extract, malt extract, and agar powder were purchased from M/S Hi-Media Laboratory India. HPLC grade acetonitrile and acetic acid were purchased from M/S E-Merck Pvt. Ltd., Mumbai, India. Ethyl acetate and sodium hydroxide were procured from M/S SD. Fine Chemicals Ltd., Mumbai, India.

Microorganisms and Maintenance of Cultures. *P. cinnabarinus* culture was procured from the National Centre for Industrial Microorganism, NCL, Pune, India, and maintained on sterile potato dextrose agar slants. Sterilization of maintenance medium was carried out at 121 °C for 20 min. The strain was subcultured and grown at 30 ± 2 °C for 72 h. The strain was subcultured after every month. The fully grown culture was maintained at 4 °C.

Inoculum Development. A solid medium (malt extract, 20 g/L; agar, 15 g/L; yeast extract, 2 g/L) was inoculated centrally with a mycelium fragment from culture on agar slants and incubated at 37 ± 2 °C until the mycelium completely covers the surface (USP 5262315). The mycelium fragments/circular disks of approximately 4 mm in diameter were prepared using a borer and subsequently used as an inoculum.

Media for Vanillin Production. The production medium consisting of 50 mL (maltose, 20 g/L; diammonium tartarate, 1.8415 g/L; KH_2PO_4, 0.2 g/L; $CaCl_2 \cdot 2H_2O$, 0.0132 g/L; $MgSO_4 \cdot 7H_2O$, 0.5 g/L; yeast extract,

0.5 g/L; thiamin hydrochloride, 2.5 mg/L) with pH adjusted to 7.0 was inoculated with 10 mycelium disks and incubated at 37 ± 2 °C on a rotary shaker at 120 rpm for 144 h. During incubation, after 72 h, 15 mg of sterile solution of ferulic acid was added to 50 mL of production medium. The sterile solution of ferulic acid was prepared by dissolving 15 mg of ferulic acid in 0.1 N NaOH and filter sterilized by passing through a 0.2 μm membrane. Harvesting of biomass was performed using simple filtration, and cell-free broth was analyzed for vanillin and ferulic acid content after extraction by HPLC.

Optimization of Media for Vanillin Production. The optimization of medium constituents for vanillin production by *P. cinnabarinus* was carried. Initially, the optimization studies were performed using the one factor at a time method, and then the concentration of each optimized constituent was determined by orthogonal matrix method.

One Factor at a time Method. *Effect of Initial pH.* To investigate the effect of initial pH on vanillin production, fermentation runs were carried out by adjusting the initial pH (before autoclaving) of the medium in the pH range of 4−7. The pH of the medium was adjusted using 1 N HCl and/or 1 N NaOH. Each medium was inoculated with 10 mycelium disks and placed at 37 ± 2 °C on a rotary shaker at 120 rpm for 144 h. During incubation, after 72 h, 15 mg of sterile solution of ferulic acid was added to 50 mL of production medium. Each fermentation run was performed in triplicates.

Effect of Carbon Sources. Maltose of the basal media was replaced with different carbon sources, namely, fructose, sucrose, dextrose, and lactose. Each of the carbon sources was used at a concentration of 2% (w/v), and its effect on the induction of ferulic acid bioconversion to vanillin was determined. The best carbon source was utilized for subsequent study.

Effect of Organic Nitrogen Sources. To investigate the effect of various organic nitrogen sources on the production of vanillin by *P. cinnabarinus*, yeast extract in the production media was substituted with different organic nitrogen sources such as corn steep liquor, soy peptone, beef extract, and proteose peptone at 0.05% (w/v).

Similarly, the effect of various inorganic nitrogen sources on the production of vanillin by *P. cinnabarinus* was evaluated. The diammonium tartarate of basal media was substituted with different organic nitrogen sources such as ammonium sulfate, triammonium citrate, ammonium phosphate, and ammonium chloride at 0.185% (w/v). Fifty milliliters of autoclaved medium was inoculated with 10 mycelium disks and placed at 37 ± 2 °C on a rotary shaker at 120 rpm for 144 h. During incubation, after 72 h, 15 mg of sterile solution of ferulic acid was added to 50 mL of production medium. Fermentation runs were performed in triplicates.

Optimization by L_{16}-Orthogonal Array Method. To investigate the relationship between variables of medium components and to optimize their concentration for maximal vanillin production, the orthogonal matrix L_{16} (4^4) method was used (*25*). Taguchi investigated a new method of conducting experiments, which were based on well-defined guidelines. This method uses a special set of arrays called orthogonal arrays. These standard arrays stipulate the way of conducting the minimal number of experiments, which could give the full information of all the factors that affect the performance parameter. The crux of the orthogonal arrays method lies in choosing the level combinations of the input design variables for each experiment (*25*), for example, pptimization of fermentation conditions for preparation of polygalacturonic acid transeliminase by *Erwinia carotovora* (*26*) and optimization of submerged culture conditions for mycelial growth and exobiopolymer production by *Paecilomyces tenuipes* (*27*).

We explain an optimization approach based on modified methods described by Taguchi (*28*, *29*), which overcomes many problems associated with conventional optimization strategies. The Taguchi method has found widespread use in industrial process design, principally in development trials, where it is used to generate enough process information to establish the optimal conditions for a particular process using the minimal number of experiments possible. The Taguchi method consists of three phases: (i) designing the experiments, (ii) running and analyzing, and (iii) confirming and validating the assumptions. In the Taguchi method, variables under optimization are arranged into an orthogonal array (L_{16} orthogonal array for the representative experiments), which is shown in **Table 1**. With respect to medium optimization, each column would correspond to individual medium components, and each row would represent individual levels. Each component was taken at five defined concentrations, covering

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Table 1. Orthogonal Project Design for Four Levels of Four Variables Used for Media Optimization for Vanillin Production

| sr. no.	A: glucose (g/L)	B: ammonium chloride (g/L)	C: corn steep liquor (g/L)	D: MgSO₄ (g/L)
1	1 (10)	1 (0.1)	1 (0.25)	1 (0.25)
2	1 (10)	2 (0.2)	2 (0.50)	2 (0.50)
3	1 (10)	3 (0.3)	3 (0.75)	3 (0.75)
4	1 (10)	4 (0.4)	4 (0.10)	4 (0.10)
5	2 (20)	1 (0.1)	2 (0.50)	3 (0.75)
6	2 (20)	2 (0.2)	1 (0.25)	4 (0.10)
7	2 (20)	3 (0.3)	4 (0.10)	1 (0.25)
8	2 (20)	4 (0.4)	3 (0.75)	2 (0.50)
9	3 (30)	1 (0.1)	3 (0.75)	4 (0.10)
10	3 (30)	2 (0.2)	4 (0.10)	3 (0.75)
11	3 (30)	3 (0.3)	1 (0.25)	2 (0.50)
12	3 (30)	4 (0.4)	2 (0.50)	1 (0.25)
13	4 (40)	1 (0.1)	4 (0.10)	2 (0.50)
14	4 (40)	2 (0.2)	3 (0.75)	1 (0.25)
15	4 (40)	3 (0.3)	2 (0.50)	4 (0.10)
16	4 (40)	4 (0.4)	1 (0.25)	3 (0.75)

the range over which its effect can be determined. The statistical independence of these arrays enables the effect of each factor to be separated from the others, the effects to be accurate and reproducible because the estimated effect does not include the effects of other factors, and the interactions between these factors to be determined. The vanillin produced with individual medium compositions (the yield of trials) was used to evaluate the effect of the components. These outcomes were obtained by scheming Taguchi's signal-to-noise ratios for each medium component. The objective of medium optimization was to maximize the yield of vanillin. For this Taguchi designed the following signal-to-noise ratio function

$$S/N = -10 \log\left[\frac{1}{n}\sum_{n=1}^{n}\left(\frac{1}{\mathrm{Yi}^2}\right)\right] \tag{1}$$

where *S/N* is the signal-to-noise ratio, *n* is the number of trials with given concentration, and Yi is the yield of correspondent trials. For each component the optimal conditions are those that give the largest *S/N* ratio. Using polynomial regression from the *S/N* ratio for each component to obtain curves for which the maximum represents yield optima can further refine the yield. All experiments were performed in triplicates. Fermentation using *P. cinnabarinus* was carried out under submerged condition at 37 ± 2 °C for 6 days. The nutritional requirement of *P. cinnabarinus* was determined. To the best of our knowledge no one has reported the optimization of medium composition for bioconversion of ferulic acid to vanillin by this method. The basic nutrition demanded by *P. cinnabarinus* is a carbon source, nitrogen source, and mineral salts. To carry out the L₁₆ (4⁴) orthogonal matrix, four factors were selected and varied at four levels as shown in **Table 1** along with the experimental conditions. Conditions such as temperature (37 ± 2 °C), agitation (120 rpm), incubation time (144 h), initial pH (6.5), and media volume ratio ($V_{\mathrm{media}}/V_{\mathrm{flask}}$) were fixed.

Analysis of Ferulic Acid and Vanillin. An aliquot of culture fluid was filtered and supernatant was extracted by ethyl acetate. The extracts were concentrated using a rotary vacuum evaporator, and the residue was redissolved in 50% (v/v) acetonitrile and water mixture. Analysis of ferulic acid and vanillin was performed by a Jasko HPLC system connected with a reverse phase column Waters Sperisorb 5 μm ODS₂ (250 × 4.6 mm i.d.) and protected by a guard column (50 × 4.6 mm i.d.). The ferulic acid and vanillin were eluted using a mobile phase (acetonitrile/water, 20:80) containing 1% (v/v) acetic acid with a flow rate of 1 mL/min. The detection was performed at wavelength 320 nm (UV detection). Molar yield (%) of vanillin was calculated by the following formula:

$$\text{molar yield (\%)} = \frac{\text{g of vanillin produced} \times \text{mol wt of ferulic acid}}{\text{g of ferulic acid added} \times \text{mol wt of vanillin}} \times 100 \tag{2}$$
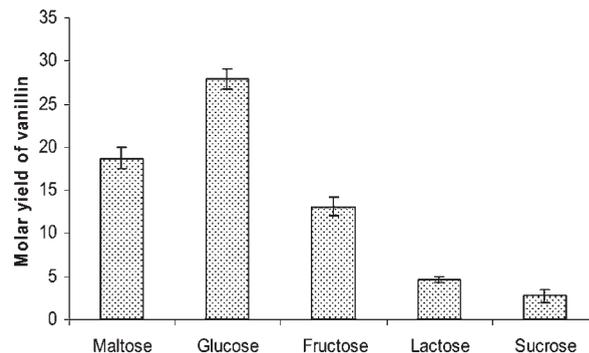
Figure 2. Optimization of carbon source.
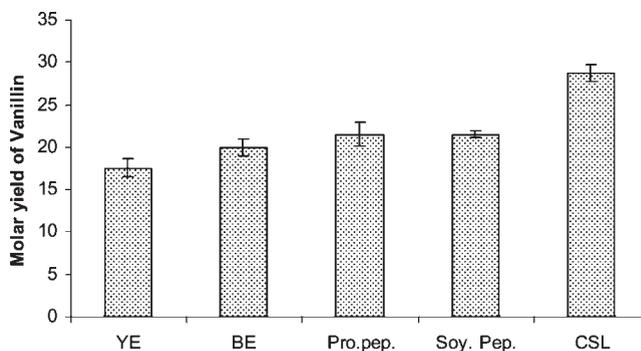
Figure 3. Organic nitrogen source optimization. CSL, sorn steep liquor; SM, soy peptone; YE, yeast extract; BE, beef extract; Pro Pep, proteose peptone.

Molar yield (%) gives the percentage yield of vanillin produced from ferulic acid.

RESULTS AND DISCUSSION

Optimization by One Factor at a Time Method. *Effect of Initial pH*. The pH of a medium always plays an important role during the production of biomolecules or secondary metabolites. At pH 6.5, maximum production of vanillin, 42.16% molar yield, was observed. It was found that vanillin production was less in all higher acidic pH values other than 6.5 (data not shown); hence, pH was found to be a significant factor for vanillin production. This could be because the enzyme decarboxylase, which is responsible for bioconversion of ferulic acid, shows optimum activity in the pH range of 4.5−7.5 (*30*).

Effects of Carbon Sources. During the fermentation process, the carbon source is essential for building of cellular material as well as an energy source (*31*). **Figure 2** shows the effect of different carbon sources on vanillin production. None of the carbon sources increased the production further, which could be due to the fact that in the bioconversion the supplemented carbon source is required only for growth of fungus. Glucose as a sole carbon source supported the maximum biomass as well as vanillin production (27.89% molar yield) in the current study. Maltose as a sole carbon source gave maximum production of vanillin (105 mg/L) using two-step fungal processes as reported earlier (*31, 32*).

Effect of Nitrogen Sources. The effects of nitrogen sources on vanillin production by *P. cinnabarinus* are shown in **Figure 3**. Corn steep liquor supported maximum vanillin production (30% molar yield). Hence, corn steep liquor was chosen as an organic nitrogen source for further studies. Ammonium chloride (**Figure 4**) was found to be the best among the different inorganic nitrogen sources screened for vanillin production (44.67% molar yield).
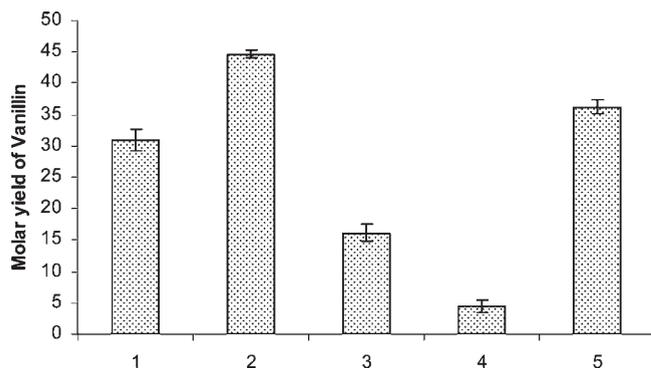
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Figure 4. Inorganic nitrogen source optimization. 1, Diammonium tartarate; 2, ammonium chloride; 3, ammonium sulfate; 4, triammonium citrate; 5, ammonium phosphate.

Table 2. Response Table for Means and *S/N* Ratio

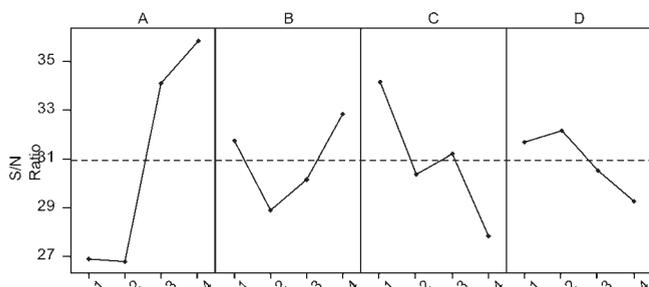| level	A mean	A *S/N*	B mean	B *S/N*	C mean	C *S/N*	D mean	D *S/N*
1	22.49	26.90	43.79	31.76	64.68	34.21	46.17	31.71
2	22.04	26.78	30.94	28.91	39.35	30.38	48.82	32.18
3	59.49	34.14	38.21	30.17	40.09	31.25	48.02	30.54
4	71.05	35.89	62.13	32.87	30.97	27.88	32.09	29.28
delta	49.01	9.11	31.18	3.96	33.71	6.33	16.73	2.90
rank	1		3		2		4	

Figure 5. Main effect plot for *S/N* ratios.

Lessage-Meessen et al. (*32*) reported yeast extract as organic nitrogen to support maximum vanillin production.

Optimization by L$_{16}$-Orthogonal Array Method. After selection of the best carbon and nitrogen (both organic and inorganic) sources using a one factor at a time method, the medium was subjected to final optimization using an L$_{16}$ orthogonal array. Using this method the parameters were optimized for their concentrations. The response of means and signal-to-noise ratio (larger is better) produced by the L$_{16}$ orthogonal array is given in **Table 2**. The values updated in the last two rows represent delta values and ranks for system. Delta values and ranks help to assess which factors have the greatest effect on the response characteristic of interest. Delta measures the size of the effect by taking the difference between the highest and lowest characteristic average for a factor. A higher delta value indicates greater effect of that component. Rank orders the factors from the greatest effect (depending on the delta values) to the least effect on the response characteristic. The main effect plots for *S/N* ratios are given in **Figure 5**, which shows how each factor affects the response characteristic.

The order in which the individual components selected in the present study affect the biotransformation process can be ranked
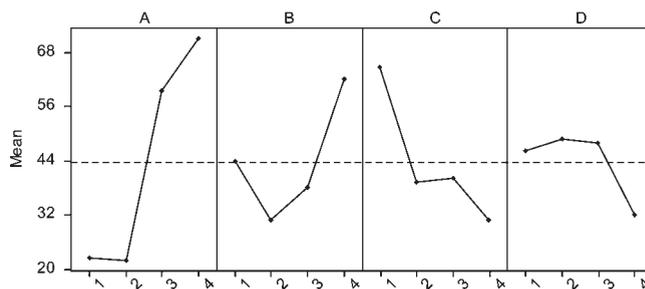

Figure 6. Main effect plot for means.

Table 3. Orthogonal Matrix Method Used for Optimizing Media for Vanillin Production by *Pycnoporus cinnabarinus*

	components				medium components (g/L)				
run	A	B	C	D	A	B	C	D	vanillin (mg/L)
1	1	1	1	1	10	1	0.25	0.25	25.60
2	1	2	2	2	10	2	0.50	0.50	22.13
3	1	3	3	3	10	3	0.75	0.75	25.89
4	1	4	4	4	10	4	1.00	1.00	16.36
5	2	1	2	3	20	1	0.50	0.75	21.88
6	2	2	1	4	20	2	0.25	1.00	26.84
7	2	3	4	1	20	3	1.00	0.25	17.19
8	2	4	3	2	20	4	0.75	0.50	21.54
9	3	1	3	4	30	1	0.75	1.00	55.99
10	3	2	4	3	30	2	1.00	0.75	17.89
11	3	3	1	2	30	3	0.25	0.50	79.89
12	3	4	2	1	30	4	0.50	0.25	84.22
13	4	1	4	2	40	1	1.00	0.50	71.22
14	4	2	3	1	40	2	0.75	0.25	56.93
15	4	3	2	4	40	3	0.50	1.00	29.16
16	4	4	1	3	40	4	0.25	0.75	126.40

as glucose > corn steep liquor > ammonium chloride > MgSO$_4$, suggesting that glucose had a major effect and MgSO$_4$ had least effect on vanillin production by *P. cinnabarinus*. The main effect plots for the means are given in **Figure 6**. These plots show how each factor affects the response characteristic. A main effect is present when different levels of a factor affect the characteristic differently. MINITAB software creates the main effects plot by plotting the characteristic average to each factor level. These averages are the same as those documented in the response in **Table 3**. A line connects the points for each factor. When the line is horizontal (parallel to the *x*-axis), then there is no main effect present. Each level of the factor affects the characteristic in the same way, and the characteristic average is the same across all factor levels. When the line is horizontal (parallel to the *x*-axis), then there is a minimal main effect present. Different levels of the factor affect the characteristic differently. The greater the difference in the vertical position of the plotted points (the greater the deviation from the parallel *x*-axis), the greater is the magnitude of the main effect. In the present study it can be seen that for each of the four variables at four levels, one level increases the mean compared to the other level. This difference is a main effect; that is, glucose at level 4, ammonium chloride at level 4, CSL at level 1, and magnesium sulfate at level 2 show a main effect. These levels also represent the optimal concentrations of the individual components in the medium. Response tables can also be used to predict the optimal levels of each component used in the study. To obtain the optimized levels or composition of each factor, the predictive analysis based on statistical calculations is shown in **Table 3**. Final medium for vanillin production by *P. cinnabarinus* is glucose, 40.0 g/L; ammonium chloride, 4.0 g/L; KH$_2$PO$_4$, 0.20 g/L; CaCl$_2$·2H$_2$O, 0.0132 g/L; MgSO$_4$·7H$_2$O, 0.50 g/L;

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corn steep liquor, 0.25 g/L; and thiamin hydrochloride, 2.50 mg/L. To confirm these results, experiments were carried out using these nutrient concentrations, and it was observed that the mean value obtained was 126 mg/L as compared to 115 mg/L predicted using MINITAB software for the same composition. This showed that the experimental value almost matches with predicted values. The final optimized medium produced 126 mg of vanillin at the end of 144 h as compared to 26 mg before optimization (data not shown). Gross et al. (*33*) patented a process for vanillin production that produced 64 mg/L vanillin. In this study, the conversion yield obtained in this study was 54% for ferulic acid. The one-step bioconversion of ferulic acid performed on medium optimized using the one factor at a time method followed by the orthogonal matrix method showed increased production of vanillin.

Conclusion. In the present study, a novel one-step biotransformation process developed using single filamentous fungi for the production of vanillin from ferulic acid was successful. The outcome of the one factor at a time method for ferulic acid biotransformation to vanillin using *P. cinnabarinus* revealed pH 6.5, glucose (as carbon source), corn steep liquor (as organic nitrogen source), and ammonium chloride (as inorganic nitrogen source) to support maximum vanillin production. Subsequent optimization with the orthogonal array method gave the optimum concentration medium components studied. The predicted values (115 mg/L) were effectively validated by experimental value (126 mg/L). Use of optimized medium for vanillin production resulted in increased molar yield up to 54% as compared to unoptimized medium. Utilization of ferulic acid for the production of highly valuable vanillin with increased molar yield was successfully performed.

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