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Process Optimization and Characterization of Enhanced Vanillin Yield Using *Bacillus aryabhattai* NCIM 5503

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Abstract

Background and Objective: Vanillin is a strong flavor used widely in food industries, but the quantity of this compound from plant sources is minimal. In the present study, vanillin was produced as bio-vanillin using biotechnological techniques and effects of the process parameters (carbon-source, nitrogen-source and pH) on ferulic acid bioconversion to vanillin for enhancing vanillin concentration were studied using *Bacillus aryabhattai* NCIM 5503.

Material and Methods: Briefly, culture media included 5 g l^{-1} each carbon (glucose, sucrose, fructose, sorbitol, lactose, xylitol and mannitol) and nitrogen (ammonium sulphate, peptone, beef extract, yeast extract and urea) sources in distilled water supplemented with 5% (w v^{-1}) of ferulic acid and 1% (v v^{-1}) of *Bacillus aryabhattai* NCIM 5503 as inoculum at a pH range of 4.5-12. Fermentation broth was extracted using centrifuge and further analyzed for the presence of vanillin using spectrophotometry and high-performance liquid chromatography.

Results and Conclusion: This study revealed that a maximum vanillin concentration of 0.87 g l^{-1} was achieved under optimum conditions (culture media with fructose and beef extract at pH 9) of 30 ºC and 150 rpm. Furthermore, vanillin in the extracted fermented broth was characterized using high-performance liquid chromatography and spectrophotometric analysis with thiobarbituric acid assay at 55 ºC for 10 min followed by 20 min of incubation at room temperature.

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1. Introduction

Vanillin is a commonly used flavoring agent extracted from vanilla (*Vanilla planifolia*) pods. This flavoring agent is used in food, pharmaceutical and cosmetic industries [1]. However, the natural vanillin production is not more than 1% and supports only 0.2% of the market needs due to expensive processing costs [2]. Thus, chemical and biotechnological synthesis are alternatives for vanillin production. Chemically derived vanillin is economical but produces undesirable racemic mixtures [3,4]. In contrast, microbially derived vanillin does not produce racemic mixtures [4,5]. Vanillin is produced as an intermediate product by microbial degradation of various substrates such as ferulic acid, isoeugenol, lignin and eugenol [6,7]. Ferulic acid is the most potent precursor for the biotechnological production of vanillin from tyrosine and phenylalanine metabolisms [5,7]. Several bacterial strains have shown abilities of catabolizing ferulic acid to produce vanillin. Strains such as *Amycolatopsis* ATCC 39116 [8], *Amycolatopsis* HR167 [9], *Bacillus* (*B.*) *subtilis* [10], *Lactobacillus* spp. [11] and *Streptomyces setonii* ATCC 39116 [12] can produce vanillin. *Bacillus* spp. are reported to produce vanillin from ferulic acid rich substrates [10,13-15]. This strain includes enzymes responsible for vanillin production. The *B. aryabhattai* is reported to produce ligninolytic enzymes (laccases, lignin peroxidases and manganese dependent peroxidase) [13]. These enzymes are needed for the microbial degradation of lignin-rich substrates to produce vanillin. Relatively low yields have been reported for the biosynthesis of vanillin using fructose as carbon source [16]. Process parameters such as carbon source, nitrogen source

and pH affect the vanillin yield. The present study was varied out to optimize the process parameters (pH, carbon and nitrogen sources) using *B. aryabhattai* NCIM 5503 for enhancing vanillin concentration. Characterization of the extracted broth was carried out using high-performance liquid chromatography (HPLC) and spectrophotometry to investigate the presence of vanillin.

2. Materials and Methods

2.1. Chemicals

Vanillin (99%) (Sigma-Aldrich, USA), ferulic acid, 2-thiobarbituric acid (99%) (HiMedia Laboratories, Mumbai, India) were used in this study. The HPLC reagents, carbon sources, nitrogen sources, HCl (35-38%) of analytical grades were purchased from HiMedia Laboratories, Mumbai, India.

2.2. Inoculum preparation

The *B. aryabhattai* NCIM 5503 was used as the primary culture (NCIM, Maharashtra, India). The culture was recovered in nutrient broth (5 g l^{-1} peptone; 5 g l^{-1} NaCl; 2 g l^{-1} yeast extract; 1 g l^{-1} beef extract) at 30 ℃ for 48 h. After twice subculturing, 1% (v v^{-1}) of the inoculants was transferred into Erlenmeyer flask (250 ml) with 50 ml of nutrient broth and then cultivated overnight [17].

2.3. Bioconversion experiment

Briefly, 1% (v v^{-1}) of *B. aryabhattai* NCIM 5503 was added into Erlenmeyer flasks (250 ml), containing 100 ml of bioconversion media with 5% (w v^{-1}) of ferulic acid. Bioconversion media consisted of 0.5 g of carbon, 0.5 g of complex nitrogen and 1 ml of trace metal solution g l^{-1} (0.37 g l^{-1} $CaCl_2.2H_2O$; 0.62 g l^{-1} $CuSO_4.5H_2O$; 0.60 g l^{-1} $FeSO_4.7H_2O$; 0.59 g l^{-1} $MnSO_4.4H_2O$; 0.42 g l^{-1} $ZnSO_4.7H_2O$; 0.79 g l^{-1} $CoCl_2.6H_2O$; 0.70 g l^{-1} $NaMoO_4$). Glucose, sucrose, fructose, sorbitol, lactose, xylitol and mannitol were used as carbon sources. Ammonium sulphate, peptone, beef extract, yeast extract and urea were used as nitrogen sources. Bioconversion media were incubated (150 rpm, 30 ℃, 96 h) using rotary incubator (Remi, New Delhi, India) and analyzed every 24 h to assess the vanillin production. All experiments were carried out in triplicate. In general, pH of the bioconversion media was set at 4.5, 5.5, 7, 9 and 12. Furthermore, the best carbon-source, nitrogen-source and pH were chosen to optimize the vanillin production. Additionally, 0.5 g of glucose and 0.5 g of beef extract at pH 7 were used as controls. Vanillin concentration was assessed using thiobarbituric acid (TBA) method and spectrophotometric assay and characterized using HPLC with UV detector [14].

2.4. Thiobarbituric acid assay and spectrophotometric analysis

The TBA assay [18] was used to assess the vanillin concentration in fermented broth.

Briefly, 1-ml aliquots of the fermented media were centrifuged (Sigma 3-30 KS, Germany) at 5009 g for 20 min at 24 h intervals up to 96 h of incubation. Supernatants were used to investigate formation of vanillin in the reaction mixture using TBA method. In test tubes, 5 ml of 24% HCl, 2 ml of 1% TBA solution and 0.5 ml of sample were mixed and the final volume was adjusted to 10 ml. Test tubes were incubated at 55 ºC for 10 min using water bath and then stored at room temperature for 20 min. Absorbance was measured at 434 nm using blank solution as reference and UV/Vis spectrophotometer (EI, Himachal Pradesh, India). Vanillin concentration was assessed using linear regression equation obtained from vanillin standard curve at 434 nm [14].

2.5. High performance liquid chromatography analysis

Standard solutions of vanillin for HPLC analysis were prepared in ranges of 0.2, 0.4, 0.6, 0.8, and 1.0 mg ml^{-1} filtered using 0.2-µm membrane filters. The filtered extracts were injected into the HPLC (Waters India, New Delhi, India) fitted with silica reversed-phase C-18 column (25 cm * 4.6 mm * 5 µm) (Waters India, New Delhi, India). The mobile phase included Solvent A (acetonitrile) and Solvent B (0.2% acetic acid) in a ratio of 60:40. The flow rate was 1 ml min^{-1}, injection volume was 20 µl and the maximum run pressure was 3000 psi. Moreover, UV detection was carried out at 280 nm. Computerized integration was carried out using Empower 2 Software (Waters India, New Delhi, India) to calculate specific compound peak areas at specific retention times. For the analysis of vanillin in broth, 500 µl of aliquots were centrifuged at 5009 g for 20 min. Supernatant was freeze-dried and dissolved in 500 µl of methanol and filtered using 0.2-µm filters before injecting into the HPLC system. Vanillin in the extracted broth was characterized by comparing sample retention time with standard solution [14].

2.6. Statistical analysis

Data were statistically analyzed using OriginPro 2019b Statistical Software (OriginLab, USA). For the analysis of data, one-way analysis of variance was used to show variations between the treatments in each test. All experiments were carried out in triplicate. Gaussian peak function was used to analyze the experimental data. Differences were significant when $p<0.05$.

3. Results and Discussion

Shake-flask experiments were carried out to assess effects of operational conditions (carbon-source, nitrogen-source and pH) on ferulic acid bioconversion using *B. aryabhattai* NCIM 5503. Of the three parameters, vanillin bioconversion process was optimized to achieve the maximum concentration.

3.1. Effects of various carbon sources on vanillin

Bioconversion of ferulic acid to vanillin by *B. aryabhattai* NCIM 5503 was comparatively studied using various media. Carbon is mostly the energy source of

microorganisms [19]. Preliminary experiments showed that fructose included higher vanillin concentrations of 0.71 g l^{-1}, compared to that the carbon sources did (Table 1). In a study by Oddou et al. [16], fructose as a sole carbon source yielded lesser vanillin concentrations, compared to that the glucose-phospholipid media did. Gunnarsson et al. [12] reported that glucose produced the maximum vanillin yield at pH 8.2 using *Streptomyces setonii* ATCC 39116, compared to that arabinose did. Tilay et al. [19] reported that carbon sources were unable to enhance the bioconversion process and only needed for the growth of microorganisms. Yoon et al. [20] investigated effects of various carbon sources (glucose, fructose, galactose and glycerol) on vanillin yield. They reported that galactose increased the vanillin yield (550 mg l^{-1}) while fructose decreased the vanillin yield [20].

Table 1. Effects of carbon sources on vanillin concentration

Carbon sources	Vanillin concentration (g l^{-1})
Glucose	0.24±0.01^{d}
Sucrose	0.28±0.01^{c}
Fructose	0.71±0.01^{a}
Sorbitol	0.20±0.01^{e}
Lactose	0.12±0.01^{f}
Xylitol	0.08±0.01^{g}
Mannitol	0.64±0.02^{b}

(SD±0.05; n=3) (Different superscript letters in column indicate significant difference between samples at $p < 0.05$).

3.2. Effects of various nitrogen sources on vanillin

In this study, nitrogen-source of the culture media was optimized using various complex nitrogen-sources. The shake-flask study showed that a maximum of 0.65 g l^{-1} vanillin was produced using beef extract followed by yeast extract and peptone (Table 2). Yan et al. [15] reported a maximal vanillin quantity of 0.04 g l^{-1} using yeast extract and peptone as nitrogen-source at 35 ºC with an agitation rate of 200 rpm. In another study, Hua et al. [21] stated that *Streptomyces* V1 yields 19.2 g l^{-1} vanillin using yeast extract supplemented with 1% (w v^{-1}) of ferulic acid at pH 8.5. Tilay et al. [19] reported that use of organic and inorganic nitrogen sources increased the vanillin yield.

Table 2. Effects of nitrogen sources on vanillin concentration

Nitrogen sources	Vanillin concentration (g l^{-1})
Ammonium sulphate	0.03±0.01^{e}
Peptone	0.27±0.01^{d}
Beef extract	0.64±0.01^{a}
Yeast extract	0.37±0.01^{c}
Urea	0.45±0.01^{b}

(SD±0.05; n=3) (The concentration of nitrogen-sources was 0.5%) (Different superscript letters in column indicate significant difference between samples at p<0.05).

3.3 Effects of pH on vanillin

Effects of pH in culture media were studied by varying the pH of culture media at 4.5-12 (Table 3). The pH of fermentation broth normally plays critical roles in production of extracellular metabolites [19]. Preliminary experiments have shown that vanillin production increases with increases in pH from acidic to alkaline. The *Bacillus* spp. can endure a pH range of 6-12 but need a pH range of 8-10 for their growth [15]. Paz et al. [22] described effects of pH on bioconversion of ferulic acid to vanillin. They reported that ferulic acid did not degrade under pH 5 [22]. Thus, *B. aryabhattai* was unable to convert ferulic acid into vanillin under acidic conditions [13]. Reports showed that vanillin production was less under acidic conditions. This might be due to decarboxylase enzymes responsible for ferulic acid conversion, showing activity at pH 4.5-7.5 [19]. The shake-flask study showed that the vanillin yield increased with increasing pH 7 to pH 9 and then decreased. Mazhar et al. [23] reported a higher vanillin yield by *Enterobacter hormaechei* at pH 7. Furthermore, a maximum quantity of 0.18 g l^{-1} vanillin was achieved at alkaline pH (pH 9), showing positive effects on vanillin production. Results of this study were similar to those of studies by Chen et al. [10]. Zhao et al. [24] reported that *B. fusiformis* CGMCC1347 produced 8.10 g l^{-1} vanillin at pH 7, when urea was used as nitrogen source. Based on the maximum vanillin concentration, carbon-source, nitrogen-source and pH were selected for the optimal vanillin production. This study demonstrated that vanillin concentration of the optimized condition (0.87 g l^{-1}) was maximum, compared to control (0.36 g l^{-1}). In control samples, glucose and beef extract were respectively used as carbon and nitrogen sources at pH 7. In a previous study, a molar yield of 42.16% of vanillin was seen at pH 6.5 [19]. Table 4 compares the optimal conditions of vanillin yield.

Table 3. Effects of pH on vanillin concentration

pH	Vanillin concentration (g l^{-1})
4.5	0.03±0.01^{e}
5.5	0.04±0.01^{d}
7	0.12±0.01^{c}
9	0.18±0.01^{a}
12	0.16±0.01^{b}

(SD±0.05; n=3) (Different superscript letters in column indicate significant difference between samples at p<0.05).

3.4. Characterization of vanillin

Presence of vanillin was initially detected using spectrophotometric analysis and TBA [18]. The TBA responded to standard vanillin solutions by forming yellow-colored solutions. Light yellow-colored solutions were detected in the extracted broth, demonstrating the presence of vanillin. Absorbance of the standard vanillin solutions was measured at 434 nm. Linear regression equation was used with correlation coefficient of 0.99. Vanillin concentration in the extracted broth was calculated using the linear regression equation [14].

Figure 1 shows vanillin concentration (λ_{max}) in the extracted fermented broth with a peak at 434 nm. Under optimal conditions, *B. aryabhattai* NCIM 5503 produced 0.87 g l^{-1} vanillin.

Table 4. Comparison of the optimum cultural conditions for enhancing vanillin yield from ferulic acid as substrate

Organisms	Cultural Conditions	Vanillin Yield (g l^{-1})	Reference
Amycolatosis sp. ATCC 39116	pH: 8 Carbon source: Glucose Nitrogen source: Yeast extract	19.5	[25]
Aspergillus niger K8 *Phanerochaete chrysosporium* ATCC 24725	pH: 5 Carbon source: Cellobiose Nitrogen source: beef extract	0.44	[26]
Bacillus aryabhattai BA03	pH: 8.5 Carbon source: Glucose Nitrogen source: Yeast extract	0.14	[13]
Bacillus subtilis	pH: 9 Carbon source: Glucose Nitrogen source: Yeast extract, Peptone	0.04	[15]
Bacillus subtilis (MTCC 1427)	pH: Nil Carbon source: Ferulic acid Nitrogen source: Nil	1.04	[14]
Enterobacter hormaechei	pH: 7 Carbon source: Glucose Nitrogen source: Beef extract	5.2	[23]
Pseudomonas putida (DSM 12585) *Streptomyces setonii* (ATCC 391161)	pH: 7.2 Carbon source: Glucose Nitrogen source: Yeast extract	6.4	[27]
Pycnoporus cinnabarinus	pH: 7 Carbon source: glucose	0.12	[19]
Streptomyces sp. strain V-1	pH: 8.5 Carbon source: Glucose Nitrogen source: Yeast extract	19.2	[21]
Bacillus aryabhattai NCIM 5503	pH: 9 Carbon source: Fructose Nitrogen source: Beef extract	0.87	This study

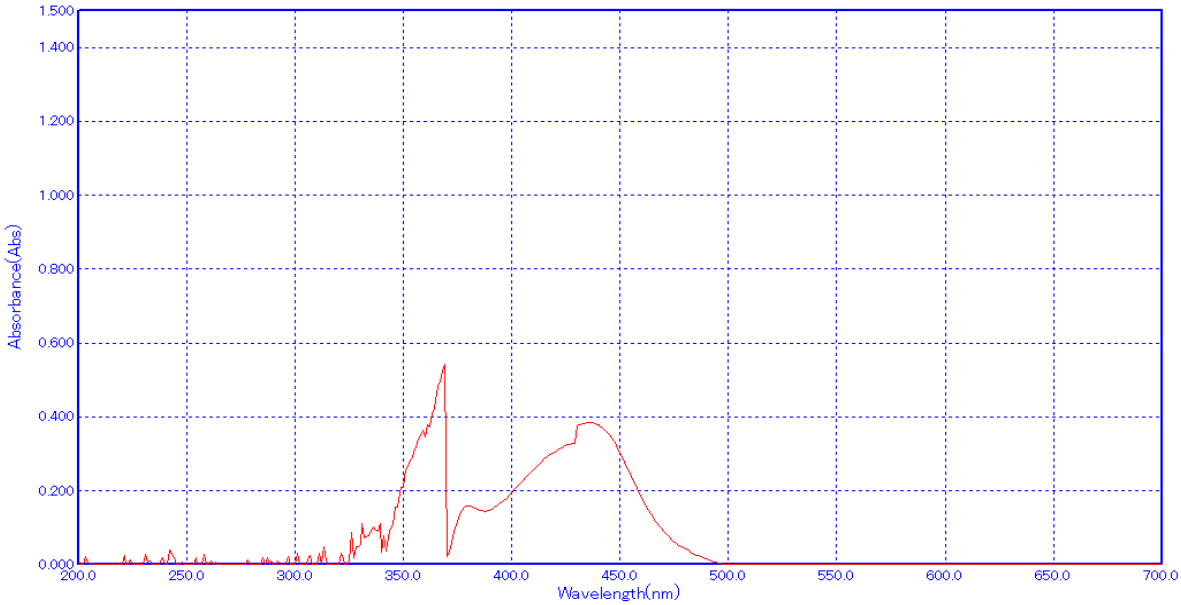

Figure 1. Spectrophotometric analysis of the extracted broth

The HPLC analysis was carried out for the identification/quantification of products formed after submerged fermentation. Quantification was carried out using standard vanillin graph (Fig. 2). The HPLC analysis of vanillin showed a retention time of 1.44 min (Fig. 3), comparing to the standard graph. Rana et al. [14] reported that vanillin was detected at a retention time of 2.76 min, similar to this study. Combined peaks near the retention time were seen because of the compounds that were structurally homologous to vanillin. This was due to the formation of vanillic acid or other substances produced by vanillin oxidation, which might delay vanillin characterization. However, the merged peak near the retention time of vanillin needs further screening.

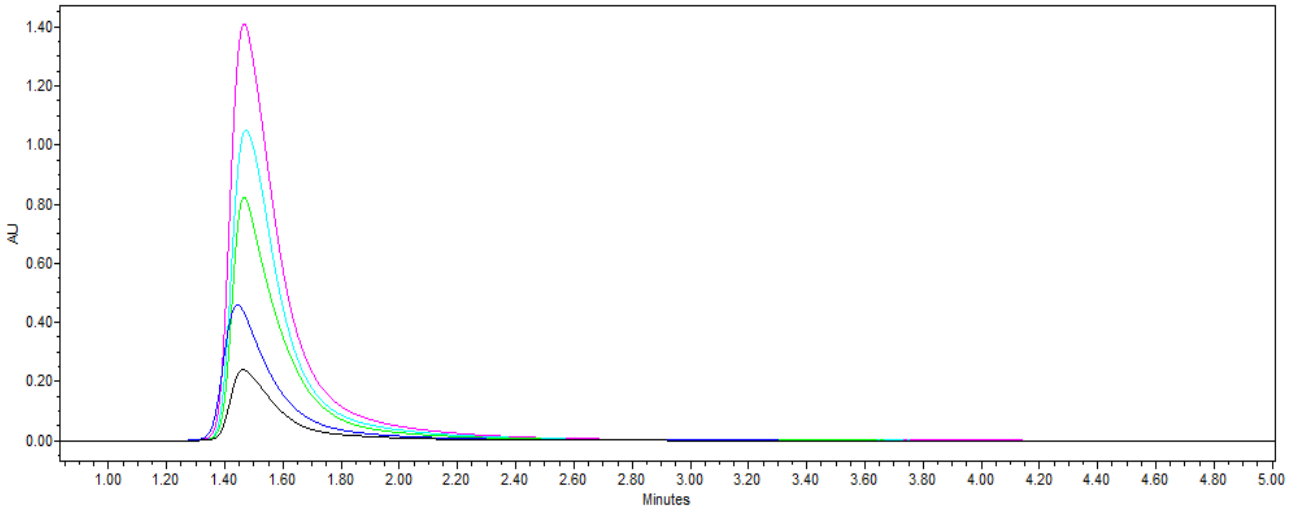

Figure 2. High-performance liquid chromatography chromatogram of the standard vanillin

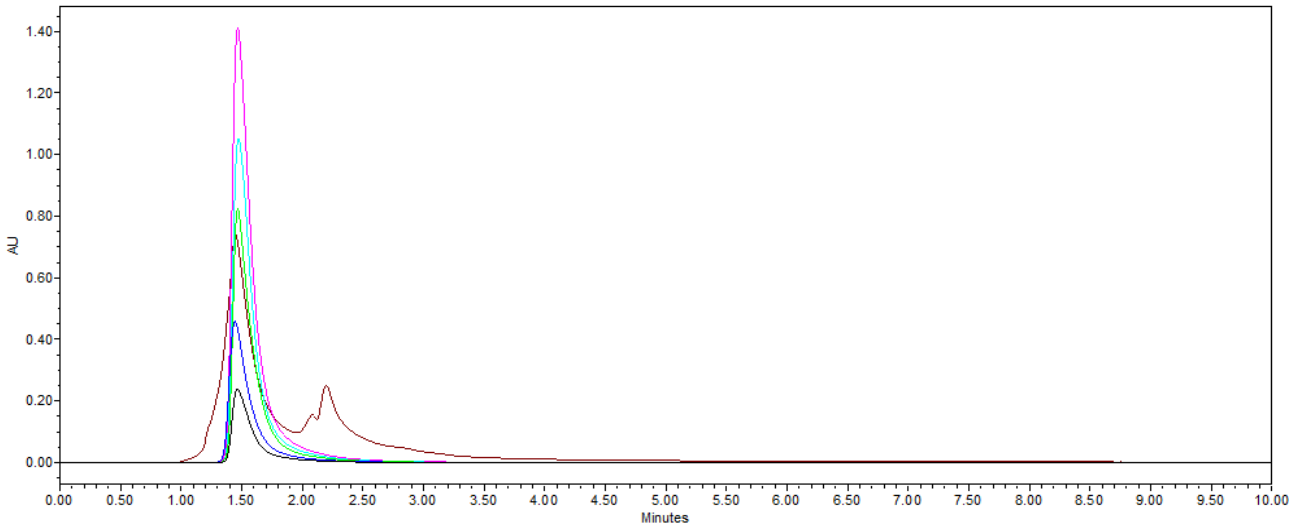

Figure 3. High-performance liquid chromatography chromatogram of the extracted broth during bioconversion of ferulic acid using *B. aryabhattai* NCIM 5503

4. Conclusion

The present study was carried out to optimize vanillin production from various carbon and nitrogen sources at various pH using *B. aryabhattai* NCIM 5503. This study showed a maximum vanillin concentration of 0.87 g l^{-1} with fructose (carbon source) and beef extract (nitrogen source) at pH 9 as optimal conditions. The HPLC and spectral analysis revealed the presence of vanillin in the fermentation broth.

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6. Conflict of Interest

The authors declare no conflict of interest. The authors have mutually agreed to submit the manuscript in Applied Food Biotechnology.

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فرآیند بهینه سازی و تعیین مشخصات وانیلین با بازده افزایش یلفته با استفاده از *باسیلوس آریب‌هاتایی* NCIM 5503

وینا پال، ابهیشک دات تریپتی*، دینش چاندرا رای

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- تخمیر غوطه‌ور
- وانیلین

***نویسنده مسئول**

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چکیده

سابقه و هدف: وانیلین طعم دهنده ای قوی است که به‌طور گسترده در صنایع غذایی مورد استفاده قرار می‌گیرد، اما کیفیت این ترکیب به‌دست آمده از منابع گیاهی پایین است. در مطالعه حاضر، وانیلین با استفاده از روش‌های زیست فناوری به‌عنوان وانیلین زیستی تولید شد و به منظور افزایش غلظت وانیلین با استفاده از *باسیلوس آریب هاتایی* NCIM 5503، اثرات عوامل موثر بر فرایند (منبع کربن، منبع ازت و pH) بر زی‌تبدیل[1] فرولیک اسید به وانیلین مورد مطالعه قرار گرفت.

مواد و روش ها: به‌طور خلاصه، محیط کشت حاوی ۵ گرم بر لیتر منبع کربن (گلوکز، سوکروز، فروکتوز، سوربیتول، لاکتوز، گزیلیتول و مانیتول) و ازت (آمونیوم سولفات، پپتون، عصاره گوشت گاو، عصاره مخمر و اوره) در آب مقطر حاوی فرولیک اسید (۵ w v^{-1} ٪) و تلقیح شده با *باسیلوس آریب هاتایی* NCIM 5503 (۱ w v^{-1} ٪) درمحدوده pH، ۱۲-۴/۵ بود. آبگوشت تخمیر عصارهبا استفاده از سانتریفوژ عصاره‌گیری و سپس میزان وانیلین در آن با استفاده از طیف سنجی[2] و کروماتوگرافی مایع با کارایی بالا[3] تعیین شد.

یافته‌ها و نتیجه‌گیری: این مطالعه نشان داد در شرایط بهینه (محیط کشت حاوی فروکتوز و عصاره گوشت گاو در pH برابر ۹)، درجه حرارت ۳۰ °C و ۱۵۰ rpm)، بیشینه غلظت وانیلین ۰/۸۷ گرم بر لیتر بود. علاوه‌براین، مشخصات وانیلین به‌دست آمده از آبگوشت تخمیر با استفاده از کروماتوگرافی مایع با کارایی بالا و طیف سنجی با سنجش تیوباربیتوریک اسید نگهداری شده به‌مدت ده دقیقه در ۵۵°C ، پس از ۲۰ دقیقه گرم‌خانه‌گذاری[4] در درجه حرارت اتاق تعیین شد.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

[1] bioconversion
[2] spectrophotometry
[3] high-performance liquid chromatography (HPLC)
[4] incubation