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Bioconversion of ferulic acid to vanillin by combined action of *Aspergillus niger* K8 and *Phanerochaete crysosporium* ATCC 24725

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Ability of 10 fungi strains for the degradation of ferulic acid and production of vanillic acid was examined. The findings suggested that all the fungi were able to degrade ferulic acid via different pathways producing variety of products. Vanillic acid was the main bioconversion product for all the fungi strains. *Aspergillus niger* K8 was chosen as a more suitable fungus for conversion of ferulic acid to vanillic acid, due to its highest potential to produce high concentration of vanilic acid (116 mg/l) compared to other fungi. Bioconversion proves was further carried out with *Phanerochaetechrysosporium* ATCC 24725 for production of vanillin from vanillic acid produced by *A. niger* K8. The vanillin production (44.8 mg/l) was significant from the economical aspect, due to the cheapness and available source of ferulic acid as a substrate and short time for bioconversion of ferulic acid to vanillic acid.

Key words: *Aspergillus niger* K8, biotransformation, ferulic acid, *Phanerochaetechrysosporium* ATCC 24725, vanillic acid, vanillin.

INTRODUCTION

Vanillin (3-methoxy-4-hydroxybenzaldehyde) is a flavoring compound of high economic value. On the basis of its origin, it may be classified as natural or synthetic. Natural vanillin covers less than 1% of its total demand (Priefert et al., 2001) and is obtained from vanilla pods, whereas the synthetic vanillin is prepared chemically. Limited availability of vanilla beans has resulted in high prices of natural vanillin ($4000/kg), but still this yield is less than what is needed (Feron et al., 1996). To fulfill this demand, many biotechnological methods to produce natural vanillin from ferulic acid using fungi have been proposed. For example *Schizophyllum commune* degraded ferulic acid to 4-vinylguaiacol, then it was oxidized to vanillin and vanillic acid (Tsujiyama and Ueno, 2008). Biotransformation of ferulic acid using *Enterobacter* sp. Px6- has been studied by Li et al. (2008). During the bioconversion process, 4-vinylguaiacol and vanillin were produced; ferulic acid was decarboxylated to 4-vinyl guaiacol and converted to vanillin. Muheim and Lerch (1999) have screened two microorganisms (*Streptomyces setonii* and *Pseudomonas putida*) for testing their ability to accumulate vanillin from ferulic acid. They found that *P. putida* is the major producer of vanillic acid; whereas *S. setonii* accumulated vanillin. Falconnier et al. (1994)

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produced vanillin from ferulic acid by *Pycnoporus cinnabarinus*. Review of the related scientific literature indicates that natural ferulic acid exist indefinitely on the graminaceous plant cell walls as well as residues of agricultural wastes such as sugar beet pulp, cereal bran and rice bran oil (Harris and Hartley, 1980; Zheng et al., 2007) is the most appropriate precursor for production of vanillin because of its chemical resemblance to vanillin (Bonnin Estelle et al., 2002), abundance, being replenishable, capability to form fine substrate from agricultural feed supplies and low toxicity as compared to eugenol (Muheim and Lerch, 1999).

In this study, different fungi strains were screened, for their ability to bioconvert ferulic acid, extracted from waste residue of rice bran oil, to yield higher amount of vanillic acid. One of them is *Aspergillus niger* k8 which is a local isolated and is explored for the first time to biotransform ferulic acid to vanillic acid. Vanillic acid was used subsequently for vanillin production.

MATERIALS AND METHODS

Chemicals and reagents

Ferulic Acid (FA), vanillic acid, vanillin, vanilly alcohol, 4-vinyl guaiacol was purchased from Sigma Chemical Co. (St. Louis, USA). Waste residue of rice bran oil was provided by the Laboratory of Biomoloecular Science, Institute of Bioscience, University Putra Malaysia. Potato dextrose agar (PDA) and polystyrene resin HZ816 were purchased from Huachang polymer (Shanghai, China) and Merck (Germany), respectively. PDA medium was prepared according to the manufacturer's instructions. The used solvents were of high-performance liquid chromatography (HPLC) grade. All other chemicals were obtained from Fisher Scientific and Merck.

Microorganisms

Different fungi strains were used for the biotransformation of ferulic acid to vanillic acid. Original freeze-dried strains of the *A*. *niger* ATCC 200345, *Aspergillus terreus* ATCC 74135, *Aspergillus terreus* ATCC 10029, *Ceriporiopsis subvermispora* ATCC 90467, *Fusarium oxysporum* ATCC 11137, *Penicilliumdigitatum* ATCC 201167, *Phanerochaete chrysosporium* ATCC 24725 and *Trametes versicolor* ATCC 20869 were obtained from the American Type Culture Collection (ATCC). *Penicillium purpurogenum* PTCC 5212 was obtained from the Persian Type Culture Collection (PTCC). *Fusarium oxysporum* CBS 620.87 was obtained from Centraal bureau voor Schimmel cultures (CBS). *A*. *niger* K8 was isolated by the Laboratory of Industrial Biotechnology, Institute of Bioscience, University Putra Malaysia. The strains were kept at 4°C on potato agar slants.

Growth and bioconversion of ferulic acid to vanillic acid

The composition of basal medium used for the production of fungal cultures was according to the study of Zheng et al. (2007). Bioconversion of ferulic acid to vanillic acid was performed using, at least, one fungal culture. To carryout bioconversion, inoculation was performed with spores (10^6 spores/ml medium) or mycelia fragment into 100 ml basal medium of 250 ml flask at 30°C and agitated at 150 rpm for 48 h. The culture of fungus was filtered with filter paper whatman paper No. 1. Mycelium mass of the fungus was inoculated to flask containing 120 ml basal medium (yeast extract 4 g/l) at 30°C, 150 rpm (Zheng et al., 2007), until a biomass was obtained possessing the capacities needed for the bioconversion (biomass of at least 0.2 g/l). After 24 to 96 h, the cultures for biotransformation stage were ready. Ferulic acid solution (300 mg/l) was added to the cultures for bioconversion to vanillic acid. Three replicates were used for each time interval.

Growth and bioconversion of vanillic to vanillin

Mycelium of *P. chrysosporium* ATCC *24725* was collected after growth on basal medium for 48 h and inoculated into conical flask containing 120 ml medium containing (g/l): Cellobiose 2.5, beef extract 8, $MgSO_4 \cdot 7H_2O$ 0.5, K_2HPO_4 0.2, $CaCl_2$ 1.3 mg, $VB_2$2.5 mg, pH 5. Incubation was then carried out at 30°C for 48 h, followed by suspension of mycelium of *P*. *chrysosporium* ATCC 24725 in vanillic acid solution, which was produced by selected fungus. Bioconversion of vanillic acid to vanillin by *P*. *chrysosporium* ATCC 24725 was carried out at 35°C and 120 rpm (Zheng et al., 2007). Suitable amount of adsorbent resin HZ816 was applied to biotransformation system, 1 h, after adding fungi biomass to bioconversion of vanillic acid to vanillin. The molar yield of vanillic acid and vanillin formation to ferulic acid and vanillic acid consumed respectively was expressed as described by Baqueiro-Pe a et al. (2010).

Analytical procedures

Samples of bioconversion were withdrawn at different incubation times and applied to high performance liquid chromatography (HPLC) for analysis of bioconversion products. Each sample was centrifuged at 4000 rpm for 10 min, the supernatant was filtered through 0.2 µm Whatman nylon filter, acidified to pH = 2 and extracted with an equal volume of ethyl acetate. Ethyl acetate fractions were evaporated to dryness under reduced pressure. These were resuspended in 1 ml of methanol (50% v/v). The HPLC (Agilent 1200 series, Germany) analysis was done in order to detect any metabolites or byproducts. The C18 column (ZORBAX SB, 5 µm, 150 mm × 4.6 mm) was used and maintained at 22°C. Compounds were eluted with a gradient A 100:1 water: acetic acid solution and gradient B 95:5:1 methanol: aceonitrile: acetic acid mixture as used as: 0 to 2 min 5%, 2 to 10 min 5 to 25%, 10 to 20 min 25 to 40%, 20 to 30 min 40 to 50%, 30 to 40 min 50 to 100%, 40 to 45 min 100%. The flow rate was 1 ml/min. Sample detection was achieved with UV-Vis detector at 280 to 360 nm and injection volume was 20 µL. Quantitative data were obtained using external standards which were ferulic acid, vanillic acid, vanillin, vanilly alcohol and 4-vinyl guaiacol. Cell growth was measured by dry cell weight (DCW) measurement using filtration and drying method. In this method, filter paper was put at 70°C for 24 h, then weight and recorded. Ingredients in conical flask were filtered and remaining fungal mycelium was put on filter paper at 70°C for 24 h followed by weighting the filter paper with fungus. The difference between weights of the filter paper with and without fungus was taken as weight of dried mycelium.

The mean values of all the data obtained from the experiment were analyzed by using statistical analysis software (SAS) program (release 6.12, 1988.SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

Screening of fungi for bioconversion of ferulic acid to vanillic acid

The objective here is to compare the production of vanillic

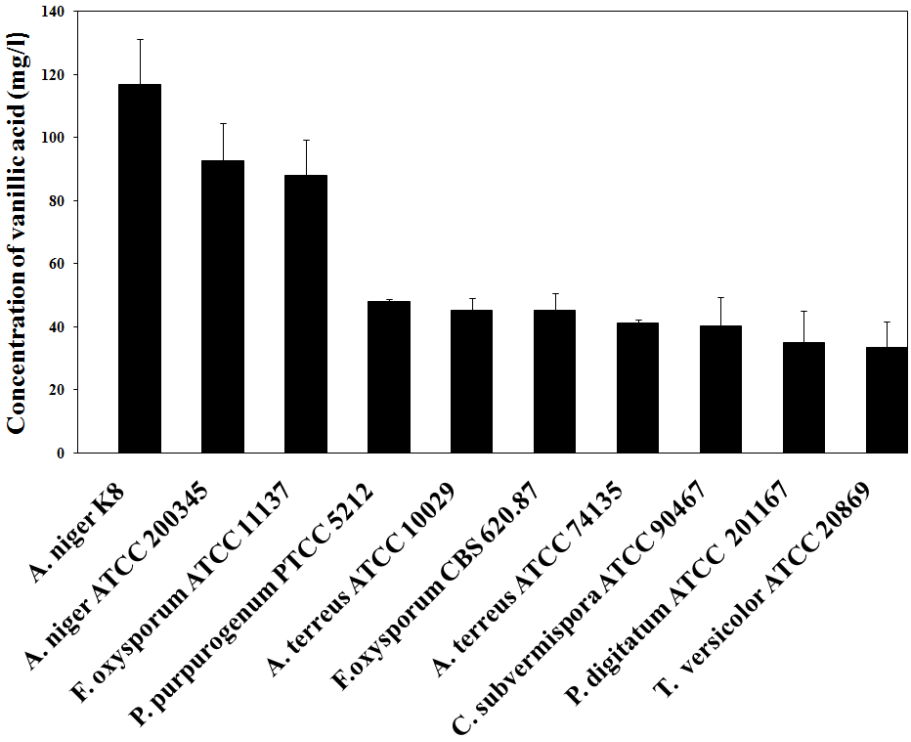


Figure 1. Vanillic acid concentration (mg/l) produced from ferulic acid through bioconversion process at 30°C with shaking speed of 150 rpm using 10 various strains fungi. Triplicate assay were carried out and the error bars represent standard deviations.

acid from ferulic acid using different fungi. The fungus, which yielded the highest concentration of vanillic acid, was used in fermentation process for the production of vanillin. The bioconversion results of vanillic acid produced by different fungi are depicted in Figure 1. The results suggest that the concentration of vanillic acid is different among fungi maybe due to different fungi having various pathways to degrade ferulic acid (Priefert et al., 2001). Ligninolytic enzyme activity also may be have effect on vaniilic acid formation whereas different fungi have various lignolytic enzyme for example *Trametes versicolor* have laccase, lignin peroxidase and manganese peroxidase and *Ceriporiopsis subvermispora* have laccase and manganese peroxidase (Johansson and Nyman, 1992; Lobos et al., 1994; Maciel et al., 2010) which play critical role on degradation of ferulic acid. The highest vanillic acid concentration (116.9 mg/l) was obtained using *A. niger* K8. The bioconversion time course of ferulic acid by *A. niger* K8 is shown in Figure 2; at the beginning of bioconversion process, the concentration of ferulic acid was 300 mg/l, while no vanillic acid could be detected. During bioconversion process, ferulic acid was converted to vanillic acid and concentration of vanillic acid reached 76.33 mg/l within 12 h. Meanwhile, ferulic acid concentration decreased to 92 mg/l, the formation of vanillic acid increased finally to 116.9 mg/l, within 36 h and subsequently decreased. In this case, ferulic acid conversion was found to be 69.16% with molar yield of 64.56%, which indicated it used most of ferulic acid to produce vanillic acid; however, in other fungi due to the production of unwanted byproduct, the final yield (vanillic acid) is lower compared to that of *A. niger* K8.

During the biotransformation of ferulic acid by *A. niger* K8, no coniferyl alcohol and vanillin was observed therefore from our result we do propose that reaction to be occurring via a propenoic chain degradation to vanillic acid as described for *A. niger* (Lesage-Meessen et al., 1996). A similar route for ferulic acid degradation was reported using *A*. *niger* C28B25 and 57% yield of vanillic acid was obtained by Baqueiro-Pea et al. (2010).

Effect of ferulic acid concentration on vanillic acid yield

Different concentrations of ferulic acid, that is, 100 to 1200 mg/l were used based on previous work (Lesage-Meessen et al., 1999). Initial ferulic acid concentration of 100 mg/l resulted in formation of 29.96 mg/l vanillic acid with molar yield of 33.3%. By using 300 mg/l ferulic acid as an initial substrate concentration of vanillic acid was found to be 116.9 mg/l with a molar yield of 64.56%. With increasing concentration of ferulci acid to 600 mg/l, molar yield of vanillic acid decreased to 40.6% (128.4 mg/l) was

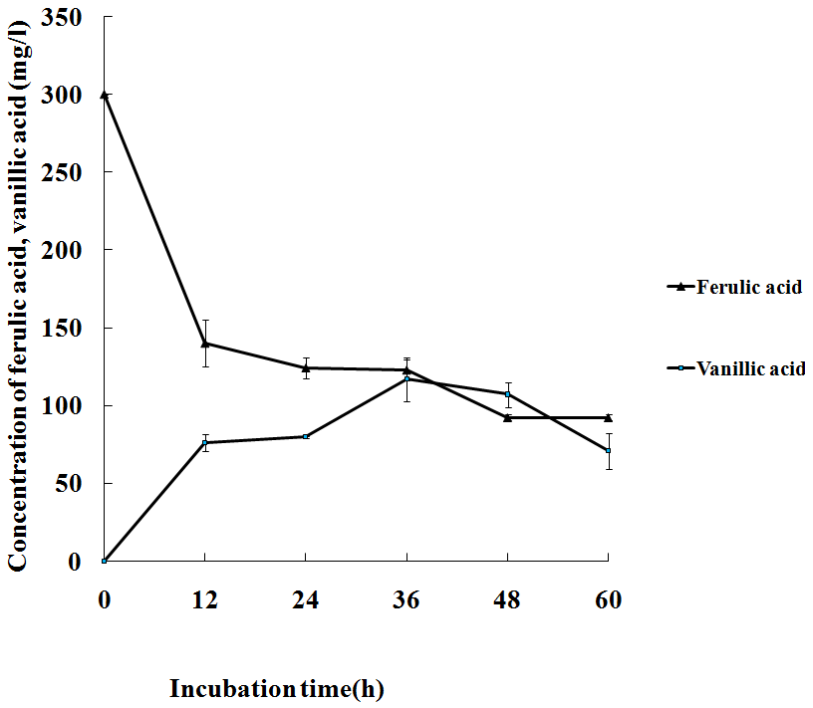


Figure 2. The bioconversion time course of ferulic to vanillic acids using *A. niger* K8 under condition of 30°C and 150 rpm. Triplicate assay were carried out and the error bars represent standard deviations.

observed. With the increase in concentration of ferulci acid, a proportionate decrease in yield of vanillic acid was observed. Higher ferulic acid concentration that is, 1200 mg/l resulted only in 11.34% yield of vanillic acid and 832.8 mg/l of ferulic acid remained unreacted (Figure 3). This maybe assumed due to substrate toxicity at high concentration, which indicated inhibitory of ferulic acid to mycelail growth and fungal metabolism (Gross-Falconnier, 1991). Conclusively, the best initial concentration of ferulic acid was decided to be 300 mg/l with 64.56% yield of vanillic acid. The toxicity of substrate at certain concentration was also reported by Allouche and Sayadi (2005). Ghosh et al. (2005) reported that increase in the yield of vanillic acid is not linearly correlated to increase in the concentration of ferulic acid.

Bioconversion process of vanillic acid to vanillin by *P. chrysosporium* ATCC 24725

Prior to bioconversion, the mycelium of *A. niger* K8 was filtered and *P. chrysosporium* ATCC 24725 was added to the filtrate medium. As shown in Figure 4, concentration of residual ferulic acid in filtrate medium decreased negligibly after 60 h. This result showed that *P. chrysosporium* ATCC *24725* had a slight degradation role on ferulic acid in our study therefore it preferentially used vanillic acid to produce vanillin. During the bioconversion process, maximum concentration of vanillin (44.8 mg/l) was produced by *P. chrysosporium* ATCC 24725 at 60 h of bioconversion time (Figure 4) with added HZ816 to this medium. The molar yield of vanillin was 42.6% and very low amount of vanilly alcohol was determined in bioconversion medium. According to the study of Falconnier et al. (1994), *P. chrysosporium* ATCC 24725 matabolized vanillic acid from two pathways; reduction pathway leading to the formation of vanillin and oxidative decarboxilated leading to formation of methoxyhydroquinone. In this study, the presence of cellobiose methoxyhydroquinone was not produced therefore *P. chrysosporium* ATCC 24725 metabolized vanillic acid from first step. Proposed pathways for the metabolism of ferulic acid to vanillin by *A. niger* K8 and *P. crysosporium* ATCC 24725 is shown in Figure 5. The bioconversion route of vanillic acid to vanillin by *P. chrysosporium* ATCC 24725 is in agreement with the study of Stentelaire et al. (1998), who showed the same metabolic pathway obtained using *P. chrysosporium* I-1471.

Lesage-Meessen et al. (1997) found cellobiose in culture medium was able to inhibit the production of methoxy-hydroquinone from vanillic acid. Bonnin et al. (1999) reported by adding cellobiose in culture medium of *Pycnoporus cinabarinus* vanillin production increased. In order to improve vanillin production and inhibited from transformation to vanilly alcohol (Lesage-Meessen et al., 2002), HZ816 resin was added to adsorb vanillin. Increase in the yield of vanillin in the fermentation liquor with adding resin has been reported elsewhere (Stentelaire et

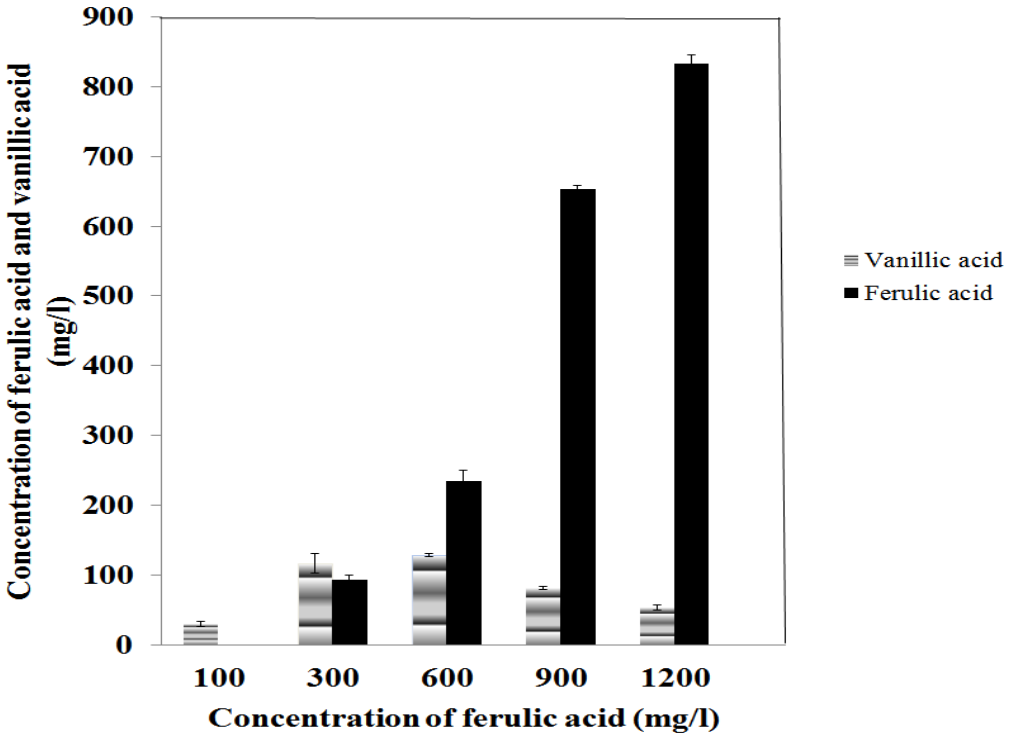


Figure 3. Effect of different concentarations of ferulic acid (100 to 1200 mg/l) on vanillic acid formation during the bioconversion process at 30°C and 150 rpm by *A. niger* K8. Triplicate assay were carried out and the error bars represent standard deviations.

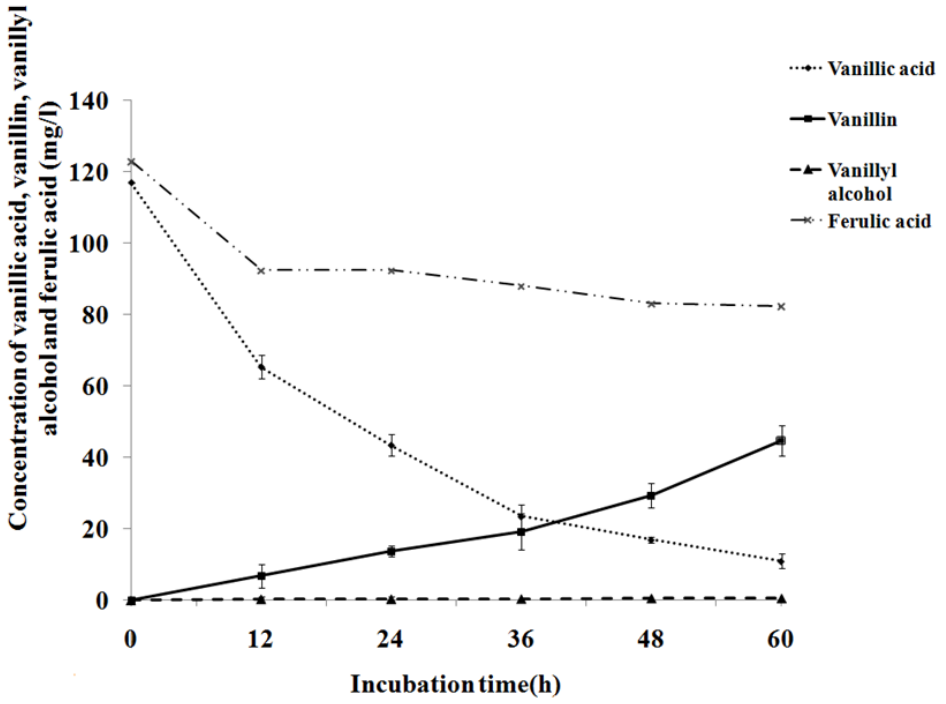
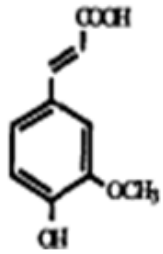


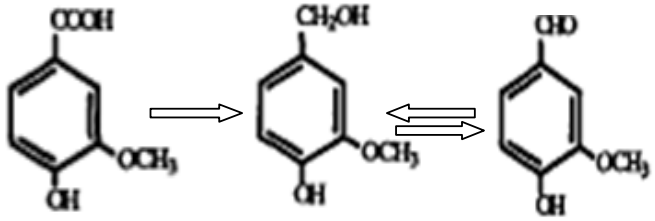
Figure 4. The bioconversion time course of vanillin production using *P. chrysosporium* ATCC 24725 from vanillic acid produced by *A. niger* K8 and residual ferulic acid. Experiment was performed at 35°C and 120 rpm with adding 5% HZ816 resin. Triplicate assay were carried out and the error bars represent standard deviations.

Pathway 1: Ferulic acid propenoic chain degradation



Ferulic acid

Pathway 2: Vanillic acid reduction



Vanillic acid Vanillin Vanillyl alcohol

Figure 5. Proposed pathways for the metabolism of ferulic acid to vanillin by *A. niger* K8 and *P. crysosporium* ATCC 24725.

al., 2000; Nilvebrant et al., 2001).

Optimization of adsorbent resin amount for vanillin production

This part of study is conducted to determine the significant amount of adsorbent resin for highest vanillin production. Different concentrations of HZ816 resin ranging from 0 to 6% were investigated. As shown in Table 1, no vanillyl alcohol was absorbed by resin. By adding resin up to 5%, the vanillin concentration got increased to 39 mg/l, while without resin, its concentration was low. However, subsequent increase in the amount of resin to 6% did not result in any improved sorption of vanillin showing that 5 and 6% of resin had almost same adsorption ability. So, 5% resin was chosen because of high ability to absorb vanillin and economy reason. The resin for adsorption of vanillin in the bioconversion of ferulic acid to vanillin was also applied by Hua et al. (2007), who reported that 8 and 10% (wet w/v) concentration of DM11 resin had same ability and upon increasing concentration of resin to 15%, its adsorption ability was decreased. It can be concluded that, the increasing demand for natural vanillin in the food industry highlights the significance of this study on the production of vanillin from ferulic acid by use of microorganisms. Screening of microorganisms was done using different fungal strains to select the best microorganism that may produce the highest concentration of vanillic acid. A local strain of *A. niger* K8 was chosen to cotablenvert ferulic acid to vanillic acid, where the yield of vanillic acid was 64.56%. Next, an experiment was conducted to identify the best concentration of substrate. The results showed 300 mg/l ferulic acid to be most suitable for the bioconversion process. *P. chrysosporium* ATCC 24725 converted vanilic acid broth to vanillin. Trace amounts of vanillin were obtained from the bioconversion process of vanillic acid to vanillin; therefore, application of resin was critical for the bioconversion of vanillic acid to vanillin by *P. chrysosporium* ATCC 24725. The amount of resin was optimized and 5% of resin HZ816 was found to be the best for bioconversion process. With the addition of resin, the molar yield of vanillin increased to 42.6% as compared to vanillin

Table 1. Effect of HZ816 resin on vanillin yield.

Percentage (%) HZ816 resin (w/v)	Residual in medium (mg/l)		Adsorbed onto HZ816 (mg/l)	
	Vanillin	Vanillyl alcohol	Vanillin	Vanillyl alcohol
2	18.9^{a}±1.2	0.81^{a}±0.02	25.8^{c} ± 2.1	-
3	11.5^{b}±1.2	0.71^{a}±0.01	29.3^{c}± 1.9	-
4	8.7^{c}±1.0	0.59^{b}±0.08	34.2^{b} ± 4.0	-
5	5.7^{d}±0.2	0.59^{b}±0.06	39.0^{a} ± 1.0	-
6	5.7^{d}±0.1	0.59^{b}±0.06	39.1^{a} ±0.9	-

production without resin. The present study obtained some findings regarding improvement in the biotechnological process for the production of vanillin, but there is still room for further investigation. Genetic modification of *A. niger* K8 and *P. chrysosporium* ATCC 24725 may also be employed in bioconversion process for increase vanillin production.

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