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Original Article

Production of vanillin by a novel bacterium from waste residues of rice bran oil

Bushra Mazhar[1], Nusrat Jahan[1], Nazish Mazhar Ali[1], Saiqa Andleeb[2], Shaukat Ali[2*]

[1] *Department of Zoology, Government College University Lahore, Pakistan*
[2] *Microbial Biotechnology and Toxicology Laboratory, Department of Zoology, University of Azad Jammu and Kashmir, Muzaffarabad, 13100, Pakistan.*

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Abstract
Major aroma used in food, cosmetics, and pharmaceutical industries is chemically synthesized vanillin. The current research was carried out to explore local bacterial isolates for vanillin production using waste residues of rice bran oil. After the screening of different bacterial isolates, a novel strain *Enterobacter hormaechei* showed significant production of vanillin when inoculated in waste residues of rice bran based media. *E. hormaechei* produced 5.2 g/l of vanillin in culture media containing ferulic acid as a substrate. Optimum pH for *E. hormaechei* was 7 and the temperature was 30°C. Using optimum growth conditions and suitable fermentation medium, *E. hormaechei* can be used at an industrial level as producer of vanillin. Bioconversion of waste residues of rice bran oil to vanillin by bacterial strains is of greater interest nowadays. Bioproduction of vanillin is not only considered natural but also safe for human health.
Keywords: Vanillin production; Ferulic acid; vanillin bioconversion; bacterial production; *Enterobacter hormaechei*

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INTRODUCTION

One of the most important flavor being used in the world is Vanillin (3-methoxy-4-hydroxybenzaldehyde). Due to its use in food, feed, cosmetics and pharmaceutical industries, it is considered as the second largest aroma of the world (Priefert *et al.,* 2001). Naturally, vanillin is obtained from the seed pods of *Vanilla planifolia*, and in fewer amounts from *Vanilla tahitiensis* and *Vanilla pompona*, commonly named as vanilla. Today, the world's need for vanillin consumption is about 12,000 tons/year. Naturally, 50 tons vanillin is produced annually and rest of vanillin is produced synthetically from lignin-containing waste and a petroleum based raw material guaicol (Li and Rosazza, 2000).

Chemically synthesized vanillin can cause health hazards and raised consumers demand to produce natural vanillin. So, biotechnological methods are considered natural as per U.S and European legislation (Dignum *et al.,* 2001). Today bacterial transformation of natural vanillin from agro-industrial wastes is of greater interest. Ferulic acid is considered as a natural substrate for vanillin synthesis (Krishna *et al.,* 2001) and is the most abundant as the hydroxycinnamic acid in plant's cell wall. Ferulic acid is covalently bonded to lignin with help of ether bond. It is the very important to the structure of plant cell wall as it is covalently linked with polysaccharides of the wall. Wastes of agro-industrial such as wheat bran, rice bran, and pulp of sugar beet contain a high ferulic acid amount. Therefore, agro-industrial waste can be degraded by bacteria to produce vanillin.

It has been seen that fungi *A. niger* I-1472 and *P. cinnabarinus* MUCL3953 produced 105mg l^{-1} vanillin from the pulp of sugar beet and maize bran (Lesage-Meessen *et al.,* 2002). A bacterial strain *Staphylococcus aureus* has been reported to produce 275mgl^{-1}ferulic acid from wheat bran (Sarangi and Saho, 2010). Agro-industrial wastes such as waste residues of rice bran contain a large quantity of ferulic acid which can be used by bacteria as a precursor to transforming into vanillin (Shimoni *et al.,* 2002; Ryu *et al.,* 2005). The main aim of present study was to produce vanillin by using the waste substrates.

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*Corresponding author: shaukatali134@yahoo.com

MATERIALS AND METHODS

Chemicals

Standard vanillin, ferulic acid, and 2-thiobarbituric acid were purchased from Sigma-Aldrich. All other chemicals were obtained from the Department of Zoology of Zoology, GC University, Lahore, Pakistan. Waste residues of rice brans oil were obtained from Season Canola Oil Company, the first in Pakistan to manufacture and market manufacture and market rice bran oil.

Synthesis of ferulic acid from rice bran oil's waste residues

Waste residues (55 g) of rice bran oil was dissolved in ethanol solution and pH adjusted to 8.0 with 0.4 M NaOH. This mixture was heated to 135˚C and hydrolyzed for 5h. After cooling the plant sterols, such as b-Sitosterol, and campesterol etc. were precipitated and ferulic acid enriched fraction was obtained in form of solution. Enriched fraction of ferulic acid was filter sterilized and used for the process of bioconversion.

Enrichment culture

For the isolation of ferulic acid degrading bacteria, enrichment culture was carried out. Strain E9 was isolated from soil of Industrial Estate Faisalabad, Pakistan. Ten g of each wet soil sample was suspended in 90 mL of sterile distilled water and this soil suspension was used as inoculum for enrichment cultures. Each soil suspension was added separately in 100ml of nutrient broth (NB) medium containing 0.3 g bees extract, 0.5 g peptone and 0.5% NaCl. Ferulic acid (1%) was added to the medium. Flask was incubated at 30°C at 150rpm for 24 h. Hundred µl of enrichment culture was spread on already prepared nutrient agar plates and again incubated at 30°C for 24 h. Visible colonies (CFUs) were streaked on a fresh agar plate to obtain pure colonies.

Screening for transforming strain

For primary screening, isolated bacterial strains were separately grown till growth phase in 100 ml of modified M9 medium (MM9), containing Glucose (0.5 g), $(NH_4)_2SO_4$(0.2 g), $CaCl_2.6H_2O$ (0.032 g), $MgSO_4.7H_2O$ (0.5), KH_2PO_4 (0.03 g), and $Na_2HPO_4.12H_2O$ (0.15 g) for 24 h at 30°C, and then 1% (1 ml) ferulic acid was added. A loop full of isolated bacterial strains approximately having 100-120 CFUs/ml was inoculated in each of the MM9 media with pH 7. Simultaneously, a control experiment was carried out with 1% (v/v) ferulic acid. After an additional 48 h of incubation at 30°C, potential biotransformation products were separated by acidifying with 10 N H_2SO_4 and extracted with equal volumes of ethyl acetate. After centrifugation (3,000 rpm for 1 min) organic layer was separated and used for paper chromatography analysis; 10 µl samples were spotted on plates. Ferulic acid degradation and vanillin accumulation were noted.

Bioconversion of ferulic acid

Ten ml growth culture of screened strain E9 was inoculated into 500 ml flask containing 200 ml basal medium with (g/l) {(20 g) glucose, (8 g) beef extract, (0.5 g) $MgSO_4.7H_2O$, (0.2 g) K_2HPO_4, (1.3 mg) $CaCl_2$. The pH of the media was adjusted to 6.5. Bacterial strain was incubated at 130 rpm at 30˚C for 24 h. After incubation, ferulic acid rich fraction was added to medium and further incubated for 48 h at 130 rpm at 30˚C for bioconversion of ferulic acid into vanillin.

Analytical methods

Both qualitative and quantitative analyses of vanillin production were done. For qualitative analysis of vanillin, paper chromatography method was used (Lederer and Lederer, 1957). A 0.03M standard solution of vanillin was prepared. Fifty µl of standard vanillin and samples were added to the chromatographic paper (Whatmann I/Desaga No. 2045). The chromatographic tank paper was irrigated vertically for 2-3 h in a solvent system of Hexane: Ethyl acetate (3:4 v/v) until distance on filter paper up to a certain limit was traveled by the solvent. Then the paper was dried and 0.1% 2-thiobarbituric acid (0.1g/100 ml of 2N HCL) was sprayed to get yellow-orange spots. Under these conditions, vanillin appeared and gave R_f value of 0.93. Quantification of vanillin was performed by an acid colorimetric method as described by He *et al.* (2008). Fifty µl of culture supernatant was added to 900µl of 2-thiobarbituric acid solution (500 µl, 24% HCL, 200 µl, 1% thiobarbituric acid, 250 µl distilled water). Standard solutions of vanillin of known concentrations were prepared in a same way. The tubes were heated on a water bath for 1 h at 55ºC. Then tubes were cooled at room temperature for 20 min. Optical density was recorded at 434 nm. A standard curve was prepared by taking a known concentration of

vanillin and determining their OD values as described above.

Biochemical and Molecular Characterization of bacterial strain E9

Upon analyzing qualitative and quantitative values, the strain which gave highest vanillin yield was selected and characterized biochemically and at molecular level. By using Berges manual, strain E9 was physically and biochemically characterized (Gram test, motility, color and colony shape, catalase, oxidase, nitrate reduction, indole, starch, and urease, *etc*). Genomic DNA was isolated by phenol-chloroform method and 16S rRNA gene was amplified by using 27F and 1522R universal primers. PCR product was sequenced from First Base Laboratories, Malaysia.

Determination of Optimum Growth Conditions

Determination of optimum growth conditions was done. For bacterial strain E9 were two parameters *i.e.*, temperature and pH were studied.

Determination of optimum temperature

For optimum temperature, a set of 55test tubes for strain E9 were prepared. Five ml of LB broth was taken in each test tube and then inoculated with 25µl of fresh bacterial culture. The test tubes were incubated at different temperatures like 25°C, 30°C, 37°C and 40°C for 24 h. After incubation, OD was recorded at 600nm. For determination of optimum temperature, the graph was constructed between temperature and optical density.

Determination of optimum pH

For optimum pH, a set of five test tubes for strain E9 were prepared. Five ml of LB broth was added to each test tube and then inoculated with 25µl of fresh bacterial culture. The test tubes were incubated at pH values of 5.5, 6.5, 7 and 8 for 24 h. After incubation OD was taken at 600nm. For determination of optimum pH, the graph was plotted between pH and optical density.

RESULTS

Screening of vanillin producers

In the recent work, the aim was to isolate bacteria capable of converting ferulic acid to vanillin. To isolate significant vanillin producer, an enrichment culture was used. Based on morphology 72 bacterial strains were selected and tested for vanillin production. In qualitative (paper chromatography) strain E9 showed the highest amount of vanillin production (Figure 1).

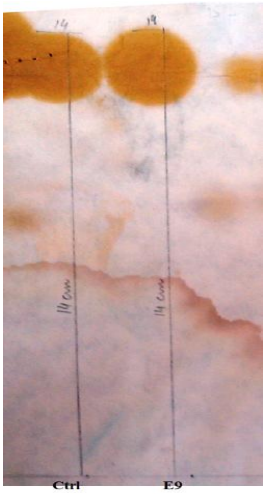

Figure 1: Paper chromatography for qualitative analysis of vanillin production from bacterial isolates.

Identification of Strain E9

Physiological, morphological, biochemical and molecular characterization was used to identify the E9 strain. This bacterium was isolated from a soil sample of an industrial estate, Faisalabad. E9 strain is a gram negative non-spore-forming rod. Morphological and physiochemical characters of E9 are given in Table I. Strain E9 was 100% identical to *E. hormaechei*. Based on morphological, physicochemical and molecular characterization strain E9 is identified as *Enterobacter hormaechei*. Accession no. obtained from NCBI, is KT385666.

Formation of ferulic acid

Waste residues of rice bran oil contain about 70% of ferulic acid which is esterified and almost 25% ferulic acid content is present in it. Approximately 5.6g ferulic acid was released when 55g of waste residues of rice bran oil was hydrolyzed as described above. It has been reported that fungal strains *A. niger* CGMCC0774 can directly degrade waste

residues of rice bran oil in broth a media (Sun *et al.,* 2005).

Bioconversion of ferulic acid to vanillin

Different concentrations of ferulic acid from enriched fraction were used. Initially 8g ferulic acid was used and it gave 2.7g l^{-1}vanillin after 72h in the broth medium. But when the initial concentration of ferulic acid was reduced to 6g the vanillin production reached to 5.2g l^{-1} after 72h. Increased initial concentration caused substrate inhibition.

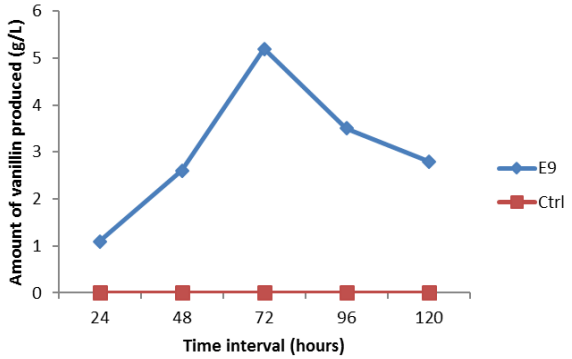

Figure 2: Amount of Vanillin produced during 24 to 96 hours

Table I: Physiochemical characterization of strain E9

Tests		Results
Staining techniques	Gram Staining	Negative
	Spore formation	Negative
	Motility	Positive
Biochemical tests	Catalase test	Positive
	Oxidase	Negative
	Starch hydrolysis	Positive
	Nitrate reduction	Positive
	Citrate hydrolysis	Positive
	Indole test	Negative
Fermentation tests	Glucose fermentation	Positive
	Sucrose fermentation	Positive
	Mannitol fermentation	Positive

Table II: Production of vanillin in gL^{-1}

Incubation period (h) →	24	48	72	96
Vanillin production (gL^{-1})	1.3	3.6	5.2	2.5

When incubation time increased from 24h to 72h the production of vanillin increased but further increased in incubation time declined the vanillin production, indicating that ferulic acid was consumed completely (Fig. 2). Since ferulic acid was present in cereals, so waste residues of rice bran oil were used as a substrate for production of vanillin. Bacterial strain was grown in a 500ml flask containing 100ml basal medium at 30˚C and after 24h of incubation waste residues of rice bran was added. Sample from the fermentation medium was withdrawn after 12h to check the amount of vanillin production. Vanillin production was at its peak after 72h of incubation (Table II). However, the amount of production did not increase further. This may be due to the utilization of substrate (ferulic acid).

Spectrophotometric analysis

Vanillin was analyzed by spectrophotometric method (Morahem*et al.,* 2008). One ml standard vanillin solution was prepared as described in materials and methods. Unknown vanillin and standard vanillin absorbance were detected at 434nm wavelength by using a PD-303S spectrophotometer. Yellow orange color appears when a thiobarbituric acid reacts with vanillin. In culture medium yellow orange color appeared when thiobarbituric acid was added which confirmed the presence of vanillin. A standard curve of vanillin was prepared by spectrophotometric absorbance of known concentrations of vanillin. The range of standard vanillin solutions was from 100-1000 µg/50 µl (Fig. 3). In culture media, the concentration of vanillin was calculated. *E. hormaechei* (KT385666) produced 5.5 gl^{-1} of vanillin in culture media containing ferulic acid as a substrate.

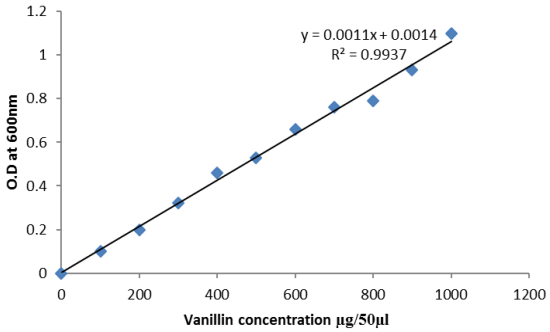

Figure 3: Standard Curve of Standard Vanillin Solutions (100-1000µg/50µl). The correlation coefficient is 0.994.

Optimum Temperature and pH

The optimum temperature of strain E9 *E. hormaechei* (KT385666) was 30˚C as shown

in Fig. 4. The optimum pH of strain E9 *E. hormaechei* (KT385666) was 7 (Fig. 5).

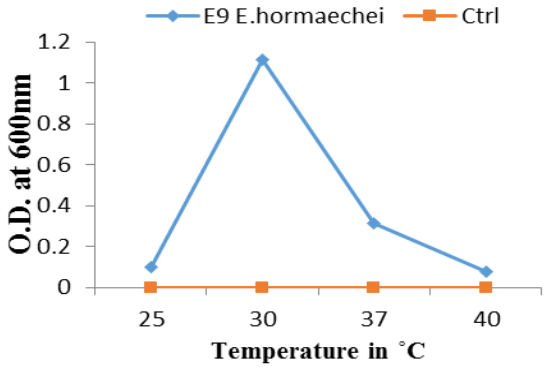

Figure 4: Optimum temperature of bacterial strain E9 *E. hormaechei* (KT385666)

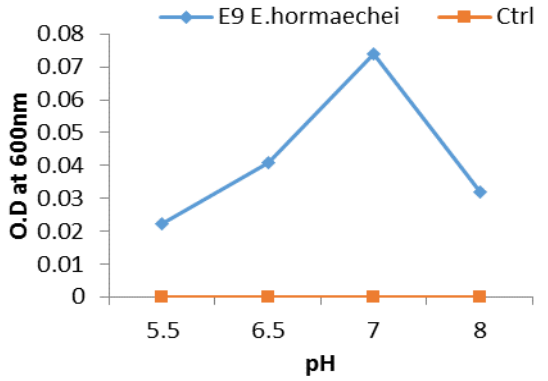

Figure 5: Optimum pH of bacterial strain E9 *E. hormaechei* (KT385666)

DISCUSSION

Vanillin (C8H8O3) is the major aroma used in food, cosmetics, and pharmaceutical industries. Natural vanillin is obtained from the seed of *Vanilla planifolia* and is very expensive (about 1200 to 4000 US$ per kg). Whereas, chemically synthesized vanillin cost is 15US $ per kg and it is synthesized by sulphite pulping and petroleum waste. As per US and European legislation, chemically synthesized vanillin is not considered natural because of its method of synthesis. Nowadays there is an increasing interest to produce natural vanillin by bioconversion methods. Bioconversion of waste residues of rice bran oil to vanillin by bacterial strains is now days of greater interest (Rabenhorst and Hopp, 1997; Müller et al., 1998). Bioproduction of vanillin is not only considered “natural” but also safe for human health. The price of vanillin produced by bioconversion of ferulic acid is approximately 12US $ per kg. In this study, we described the isolation and characterization of bacterial strain E9 *E. hormaechei* (KT385666) capable of utilizing ferulic acid and synthesize vanillin. The source of ferulic acid was waste residues of rice bran oil which is a cheap method to obtain natural vanillin. Initially, the ferulic acid rich fraction was obtained was hydrolyzing waste residues of rice bran oil, then this ferulic acid rich fraction was used in culture media to release vanillin by *E. hormaechei* (KT385666). Vanillin production from waste residues of rice bran oil was 5.2gl^{-1} with a molar yield of 86.6%. However, it should be noticed that this molar yield was accomplished in the non-optimized process, indicating the greater potential of strain *E. hormaechei* (KT385666) to produce vanillin. The methodology used in this study to isolate and screen bacterial strain capable of transforming waste ferulic acid to vanillin proved useful. In further studies, vanillin production can be enhanced using *E. hormaechei* (KT385666) E9 strain by optimization (varying nutrient conditions and mutation) as well as purification and use of enzymes which catalyzed these reactions.

Conclusion

A novel strain *E. hormaechei* (KT385666) E9 was isolated from a soil sample of an industrial estate, Faisalabad which transformed ferulic acid to vanillin obtained from waste residues of rice bran oil. Many other bacterial strains including *Pseudomonas aeruginosa, Bacillus subtilis* and *E. coli* showed production of vanillin from ferulic acid obtained from waste residues of rice bran oil but *E. hormaechei* (KT385666) E9 showed maximum production of vanillin. 5.2g vanillin was synthesized from 6g/L ferulic acid after 72 hours of incubation with pH of 7 at 30 °C. Vanillin produced by *E. hormaechei* (KT385666) E9 from ferulic acid obtained from waste residues of rice bran oil was higher than reported by any bacterial strain. So it is recommended that utilization of already available producers and exploration of more native bacterial strains should be focused.

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