

Full Length Research Paper

Biotransformation of ferulic acid to 4-vinyl guaiacol by *Lactobacillus farciminis*

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Continuously growing demand for natural flavors has led to a tremendous increase in biotransformation process employing microorganisms of different genera using ferulic acid (FA) as the precursor. In this study, potential of *Lactobacillus farciminis* (ATCC 29644) for biotransformation of FA to 4-vinyl guaiacol (4VG) was investigated. 4-vinyl guaiacol is a volatile phenol, reported to have 40 fold higher economic value than FA and is biotransformable to acetovanillone, ethylguaiacol and vanillin. Biotransformation process started after 5 h incubation of *L. farciminis* with FA in Man Regosa and Sharpe (MRS) broth at 37°C under 5% CO₂. Production rate was observed at its maximum after 48 h. Formed 4VG was identified by GC-MS (QQQ) and quantification was done by HPLC UV-Vis. The impact of initial concentrations of FA and bacteria on the production of 4VG was studied. The results indicate that the production of 4VG is significantly affected by initial concentration of FA, and empirically 1, 15 and 50 mg/l of FA yielded 0, 3.34 and 10.26 mg/l of 4VG, respectively. The findings are a milestone towards safe high yielding means of biotransforming some common agro-industrial wastes to a value added product.

Key words: *Lactobacillus farciminis*, ferulic acid, 4-vinyl guaiacol, biotransformation.

INTRODUCTION

The biological impact of ferulic acid steryl esters, extracted from rice bran oil, brought ferulic acid (FA) into focus during the 1970s, which was later on found to be a potential anti-atherosclerotic agent (Zhao and Moghadasian, 2008). Other biological activities of FA encompass anticarcinogenetic and antimicrobial effects besides mutagenesis and chemoprevention of coronary heart diseases (Min et al., 2006; Max et al., 2009). Ferulic acid is a phenolic acid (Ghosh et al., 2006), which may be present either in free or bound form in plants (Zhao and Moghadasian, 2008). It is present in wheat, maize

and rice brans (Walton et al., 2000; Mariod et al., 2010). Ferulic acid can be made free by enzymatic and physical processing (Walton et al., 2000; Min et al., 2006). The utilization of FA as the primary source of carbon, by bacteria of assorted genera, has led to the production of catabolic intermediates such as 4VG (Couto et al., 2006), protocatechuic acid, vanillic acid and vanillin (Torres et al., 2009). There has been a continuous rise in demand for natural dietary materials because of the potential hazards associated with synthetic ones (Okeke and Venturi, 1999). Biotransformation has gained momentum during recent years as a vital means of renewing natural resources by conversion into commercially valuable products. Many fragrances and flavors have been prepared employing biotransformation technology so far (Tripathi et al., 2002) using microbial means (Brunati et al., 2004). 4-vinylguaiacol is a volatile phenol (Couto et al., 2006) and is reported to have 40 fold higher economic value than FA and can be biotransformed to

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Abbreviations: FA, Ferulic acid; 4VG, 4-vinyl guaiacol.

acetovanillone, ethylguaiacol and vanillin (Landete et al., 2010). It is most extensively used in food and alcoholic beverages for flavoring and in ophthalmic field too (Baqueiro-Pena et al., 2010). It is present in pods of *Hibiscus esculentus* (okra), cooked apples, grapefruit juice, wine, raw beans, celery, coffee, strawberry, roasted peanuts and white sesame seeds (IHBT, 2005). According to Bohlin (1993), 4VG isolated from *Ipomoea pescaprae* (beach morning glory) has been reported to inhibit prostaglandin synthesis. *Lactobacillus* species play major role in industrial processes due to their ability to bioconvert substrates coupled with their generally regarded as safe (GRAS) status hence their application as probiotics (Bhathena et al., 2007).

No report describing the potential of *Lactobacillus farciminis* 29644 for 4VG production has been presented so far. There have been reports on the production of 4VG from FA but with poor degradation rates and low yields of metabolites (Karmarkar et al., 2000). Thus, this study for the first time seeks to investigate the ability of *L. farciminis* (ATCC 29644) to biotransform FA to 4VG. Coupled to this condition that is, initial FA and bacterial concentrations have been optimized to improve the yield of product. The work is of high worth from an industrial point of view with wide economic potential.

MATERIALS AND METHODS

Chemicals

Ferulic and vanillic acids were purchased from Sigma-Aldrich (Germany), vanillin from MP Biomedicals (USA) and vanillyl alcohol from Merck (Germany) and liquid nitrogen from Malaysian Oxygen Berhad, Petaling Jaya, Selangor, Malaysia. Methanol, ethanol, acetic acid and acetonitrile were of HPLC grade and were procured from Fisher scientific (UK).

Microorganism

L. farciminis (ATCC 29644), was purchased from American Type Culture Collection (ATCC) and stored in Merck's MRS medium containing 30% (v/v) glycerol at -80°C.

Inoculum preparation and biotransformation

Colony count technique was used to determine total viable cell count. *L. farciminis* was observed to have a cell density of 1×10^8 cells/ml. *L. farciminis* was cultured following a method reported by Sabu et al. (2006). About 5 ml of 18 h culture was inoculated into 45 ml of MRS broth, contained in a 250 ml conical flask and incubated at 37°C under 5% CO₂ for 20 h. The inoculum was then inoculated into MRS broth supplemented with filter sterilized FA, which had been dissolved in 1M NaOH solution and made up to pH 8.5 using 6M HCl in a 100 ml total culture volume. This was then incubated under same conditions. About 3 ml of the sample was then withdrawn at intervals to determine the concentration of FA

degraded and that of 4VG formed. Ferulic acid conversion was expressed as:

$$\text{FA conversion (\%)} = \frac{\text{FA}_i - \text{FA}_f}{\text{FA}_i} \times 100$$

FA_i = ferulic acid initial concentration, FA_f = ferulic acid final concentration.

Analysis of spent media

Identification of 4VG by gas chromatography–mass spectrometry (GC-MS)

Samples were analyzed following the method described by Couto et al. (2006) with slight modifications. 1 ml mixture of ether and hexane (1:1 v/v) was used to extract the volatile phenol by vortexing 3 ml sample with the mixture of ether and hexane for 5 min and the organic layer obtained was concentrated under nitrogen to about one third of the initial volume. It was then injected into a gas chromatograph-mass spectrometer (Thermo scientific-TSQ Quantum, USA) with a thermo TR-5MS column (30 m × 0.25 mm ID × 0.25 μm) (USA) and analyzed using X-calibur software. Helium was used as carrier gas. Injection temperature was set at 250°C, temperature gradient was adjusted at 80°C for 2 min, 120°C for 4 min, 155°C for 4 min and heated at 250°C for 3 min, injection volume employed was 1 μl and flow rate was kept at 1 ml/min.

Quantification of 4VG by HPLC

Analysis of filtered spent media for quantification of FA and 4VG was done using HPLC system (Agilent 1200 series, Germany) using C18 reversed phase column (Zorbax) maintained at 22°C, UV-Vis detector set at 280 nm. A linear gradient of two solvents was chosen for the run: solvent A (4% acetic acid in distilled water, v/v) and solvent B (acetic acid: acetonitrile: methanol 1:5:94 v/v) from 0 to 52% of solvent B for 30 min at a flow rate of 1 ml/min. Identification was then carried out with respective standards, while peak area was used as the tool of quantification.

Experimental design

The impacts of initial concentrations of bacteria and FA on the production of 4VG were investigated as biotechnological processes are significantly influenced by initial concentrations of substrate and microbes (Bloem et al., 2006; Faveri et al., 2007). Microorganisms of different concentrations (1 to 5 ml) were grown in MRS media with various concentrations (1, 5, 15, 25, 35 and 50 mg/l) of FA.

Statistical analysis

All the results in this study were expressed as mean ± standard deviation (SD) of 3 replicate measurements. The significant differences (p < 0.05) among the means were determined by one way analysis of variance (ANOVA) using Minitab statistical software (Version 15.1.1.0, Minitab Inc, USA).

RESULTS AND DISCUSSION

L. farciminis (ATCC 29644) have earlier been reported in

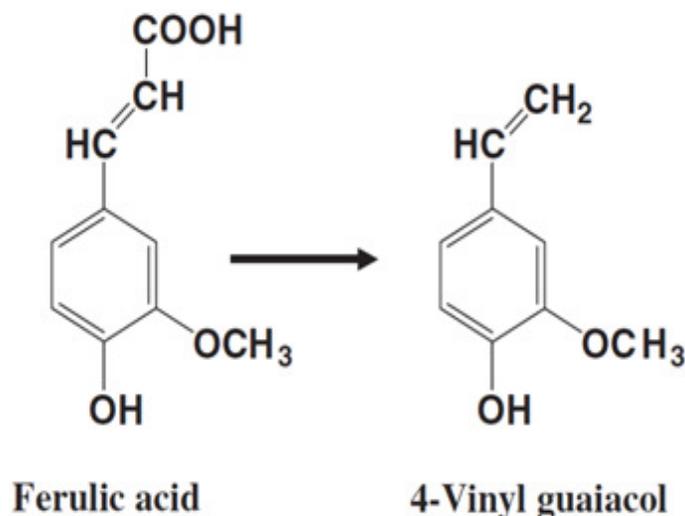


Figure 1. Proposed pathway of FA decarboxylation to 4VG by *Lactobacillus farciminis* ATCC 29644.

in vitro analysis, to have feruloyl esterase activity and has been identified as the enzyme responsible for microbial conversions of FA to vanillin (Bhathena et al., 2007) as it releases FA from plant cell walls making them available as substrates for phenolic acid decarboxylase, which transforms FA to 4VG (Landete et al., 2010). Numerous organisms such as *Aspergillus*, *Bacillus*, *Candida*, *Corynespora*, *Fusarium*, *Pseudomonas* are able to transform FA to a wide range of aromatic compounds. From our results, we do propose that the *L. farciminis* species used in this study utilizes the non-oxidative decarboxylation pathway for the production of 4VG from FA (Figure 1). To the best of our knowledge, the presence of phenolic acid decarboxylase in *L. farciminis* (ATCC 29644) has not been reported yet. Even though 4VG, as a breakdown product of FA was present in the culture medium, vanillin could not be detected. This may be due to the fact that vanillin is usually found at low concentration and is speedily metabolized, while the production of 4VG via decarboxylation of FA maybe a detoxification process in order to lower the concentration of inhibitory compounds (Baqueiro-Pena et al., 2010). The decarboxylation of FA due to one-carbon cleavage of FA has been chronicled for many lactic acid bacteria (Couto et al., 2006; Bloem et al., 2006). In this study, we report for the first time the production of high yields of 4VG from FA by non-oxidative decarboxylation using *L. farciminis*. The availability of agro-industrial wastes containing FA has been greatly highlighted in this work. Literature reports describe biotransformation of FA by different means including fungi, bacteria or genetically engineered microorganisms to other bioactives (Gosh et al., 2004; Li et al., 2008). *L. farciminis* in this study was able to biotransform FA to yield 4VG as the major degradation product; as detected by HPLC. The ability of lactic acid bacteria to degrade FA is in agreement with

the findings of Bloem et al. (2006), whereby wine associated lactobacillus namely: *Oenococcusoeni*, *L. hilgardii*, *L. brevis*, *L. plantarum* and *L. damnosus* were observed to degrade FA with the production of vanillin and traces of 4VG. Also in a study conducted by Couto et al. (2006), 4VG from FA was produced from thirty two strains of LAB out of the thirty five strains tested. In this study, the influences of initial concentrations of FA on the production of 4VG were also studied.

Identification using GC-MS-QQQ (Figure 2) and quantification with HPLC from plotted standard curves was done. Experiments were performed in triplicates. HPLC analysis of the culture supernatant showed FA with a retention time of 14.1 min (Figure 3) and 4VG at 24.1 min (Figure 4). Results reveal a proportionate increase in production of 4VG with an increase in initial FA concentration. An initial FA concentration of 50 mg/l yielded about 10 mg/l of 4VG, while an initial FA of 1 mg/l yielded no 4VG. This result is in line with the findings of Couto et al. (2006), whereby the higher the hydroxyl-cinnamic acid content, the higher the concentration of volatile phenols produced. Substrate inhibition could not be determined as precipitation occurred, when FA concentration exceeded 50 mg/l. It has been reported that the growth of lactic acid bacteria is inhibited by hydroxycinnamic acids at 500 mg/l (Couto et al., 2006). The initial concentration of FA also influenced the time for production of 4VG, as 4VG production was observed at about 5 h (Figure 5) after incubation in cultures containing 5, 15, 25, 35 and 50 mg/l of initial FA, while none was observed for 1 mg/l initial FA. The production rate of 4VG was maximum after 48 h (Figure 6) incubation irrespective of the initial amount of FA used, after which the rate of 4VG formation started to decline but was still detectable at day 10 of incubation. The bioconversion rate of FA at 48 h of incubation ranged over 41 to 87% for

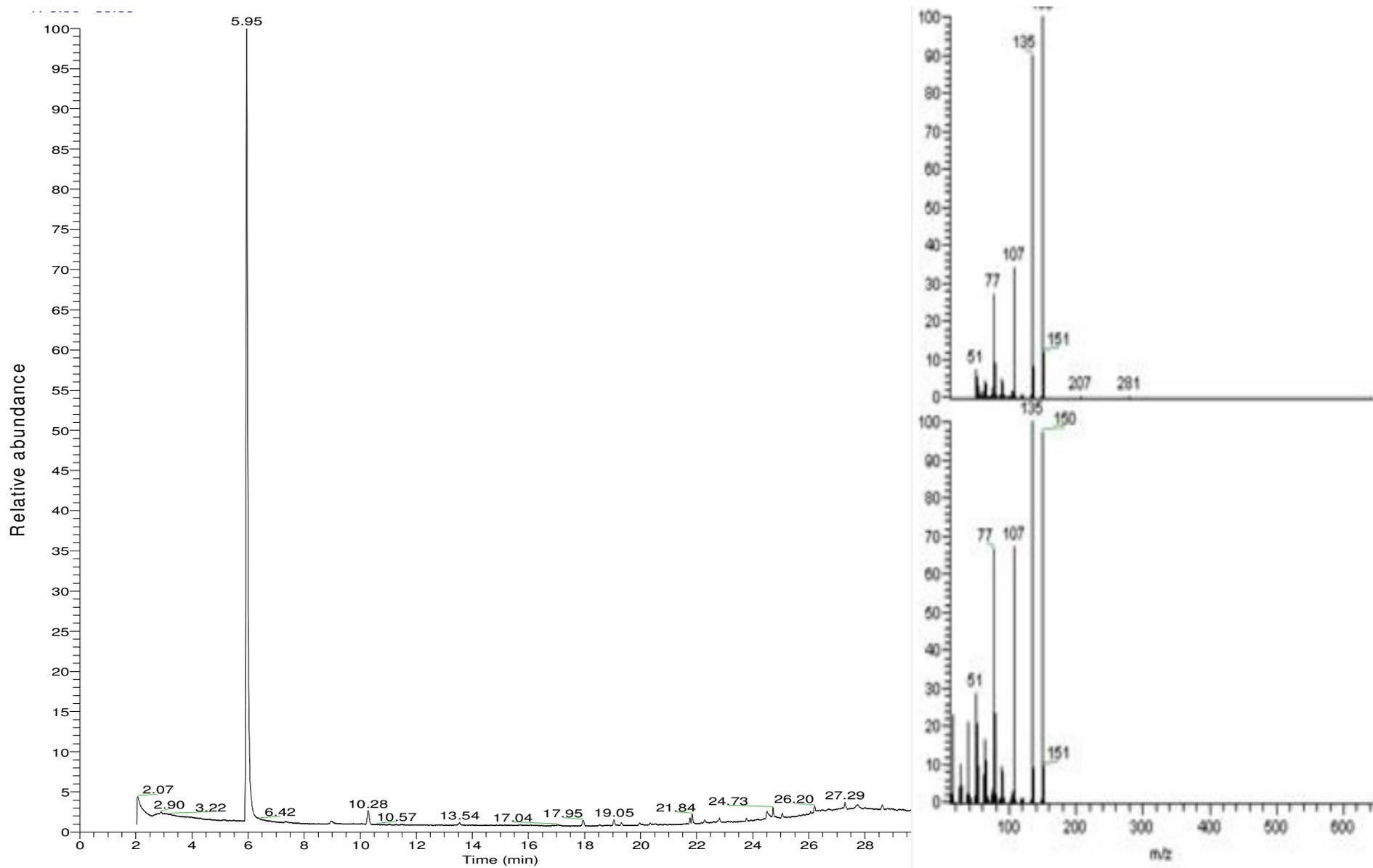


Figure 2. GC-MS chromatogram with retention time of 5.95 min and spectra of 4VG from culture supernatant after 48 h incubation.

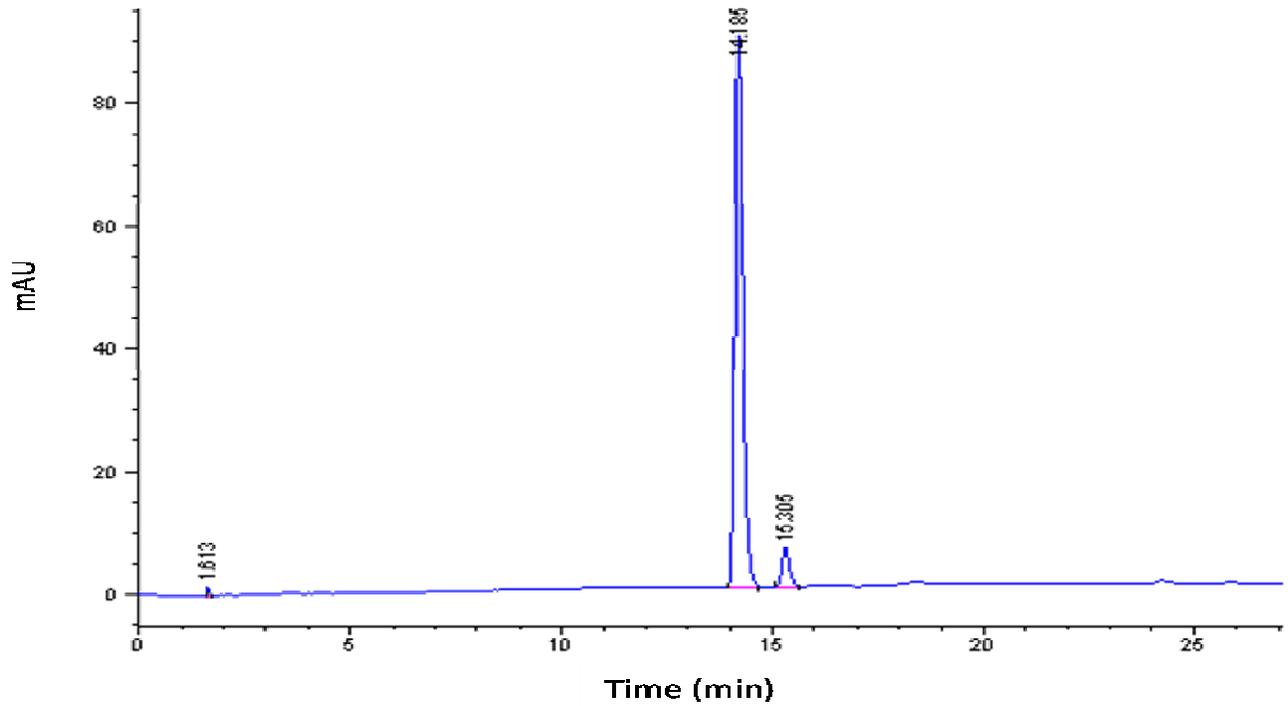


Figure 3. HPLC chromatogram of FA standard with retention time at 14.1 min.

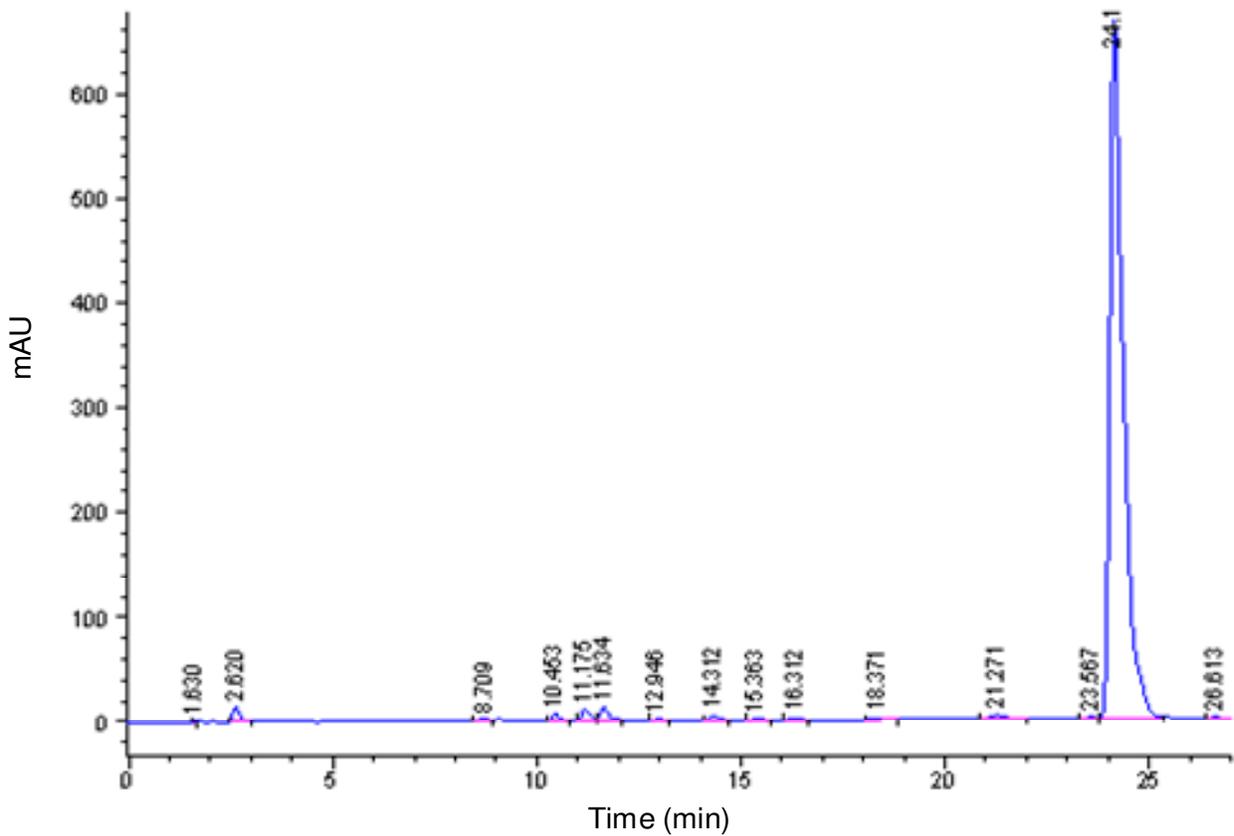


Figure 4. HPLC chromatogram of 4VG with retention time at 24.1 min.

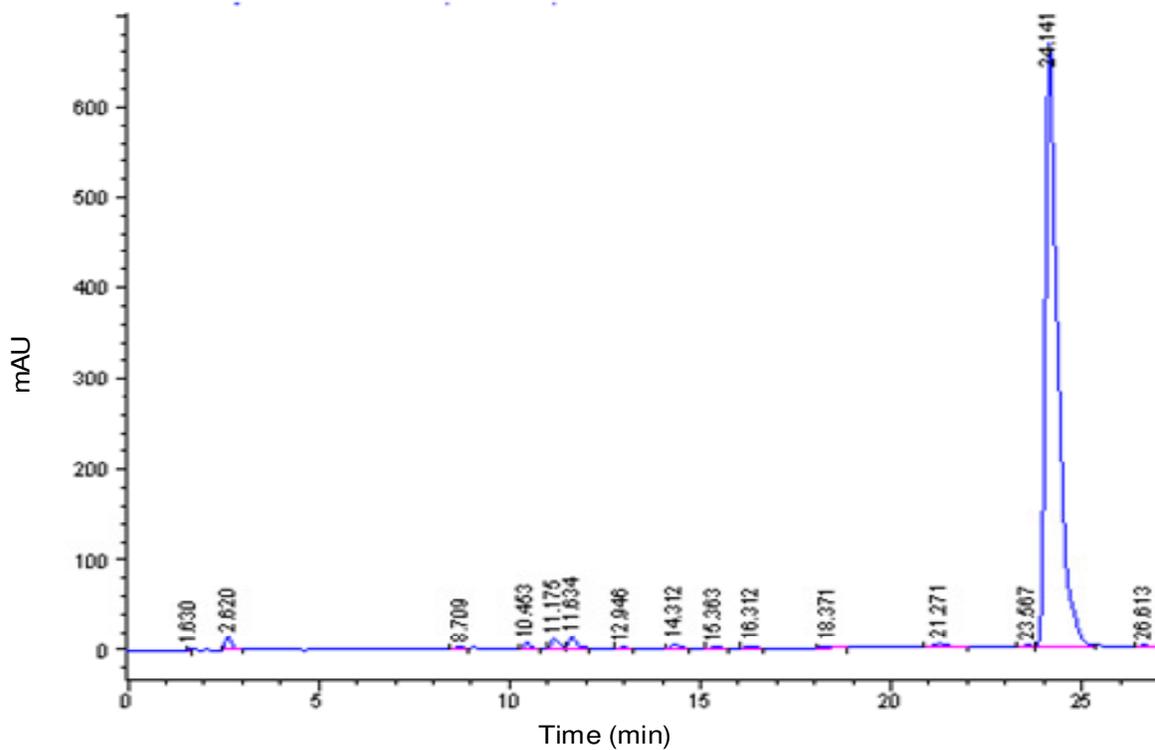


Figure 5. HPLC chromatogram of culture supernatant after 5 h of incubation.

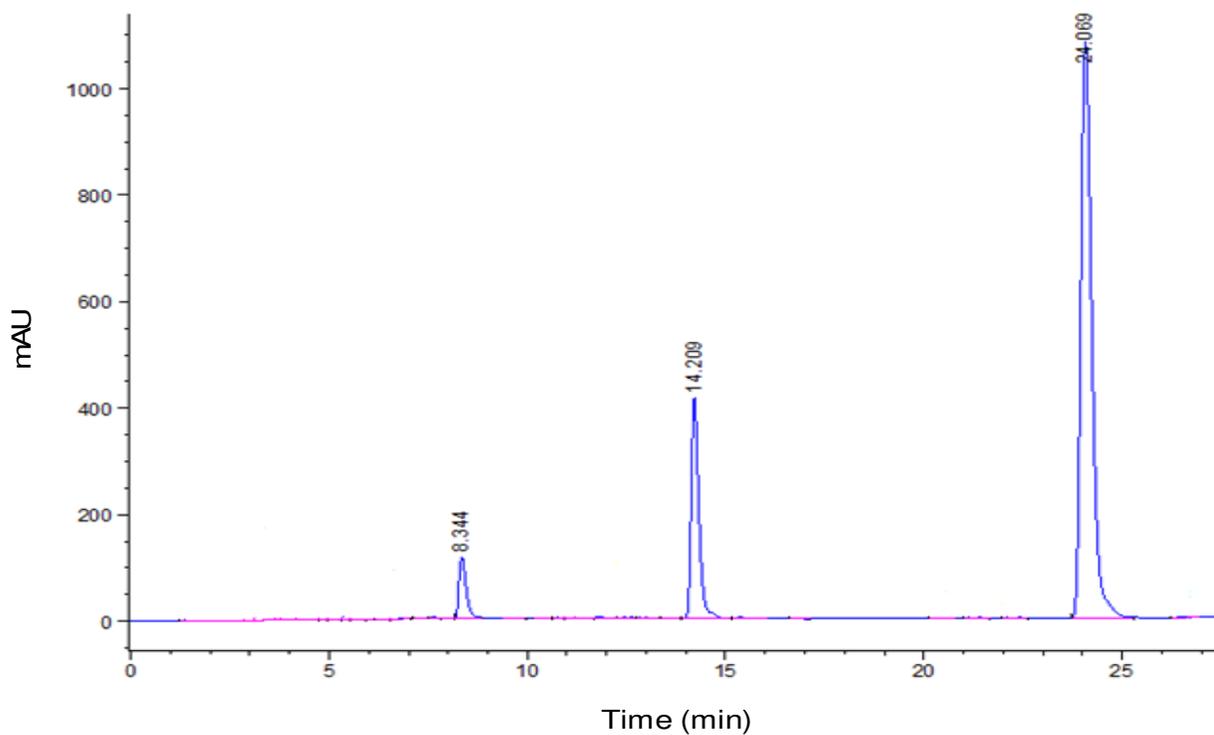


Figure 6. HPLC chromatogram of culture supernatant after 48 h of incubation.

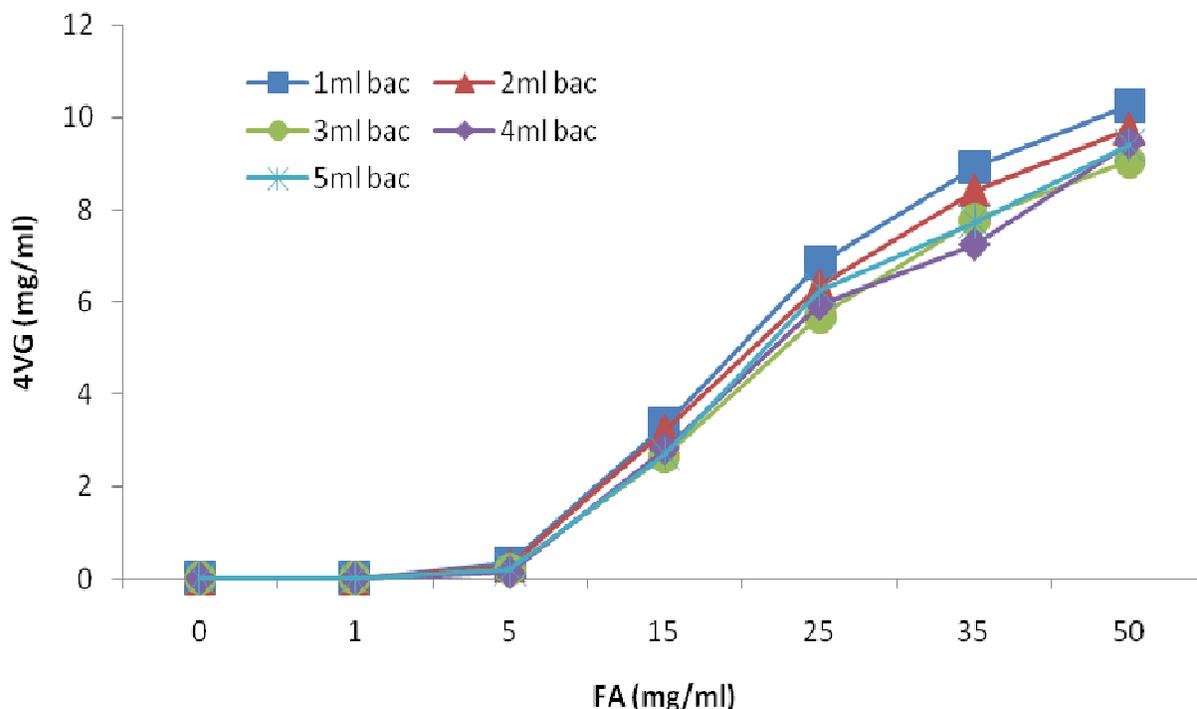


Figure 7. FA biodegradation and 4VG production by *Lactobacillus farcinis* after 48 h of incubation with varying concentrations of initial bacterial and FA. FA: Ferulic acid, 4VG: 4-vinyl guaiacol. The values are mean \pm standard deviation.

initial FA concentrations of 5, 15, 25, 35 and 50 mg/l; with the lowest being 24% at an initial concentration of 5 mg/l, which is also in agreement with Couto et al. (2006). Initial FA concentration significantly influenced the production of 4VG; as compared to initial bacterial concentration (Figure 7). On the basis of findings, 50 mg/l of initial FA concentration was taken as the optimum amount.

REFERENCES

- Barbosa ES, Perrone D, Vendramini AA, Leite SGF (2008). Vanillin production by *Phanerochaete chrysosporium* grown on green coconut agro-industrial husk in solid state fermentation. *Bioresources*, 3: 1042-1050.
- Baqueiro-Pena I, Rodriguez-Serrano G, Gonzalez-Zamora E, Augur C, Loera O, Saucedo-Castaneda G (2010). Biotransformation of FA to 4 Vinyl guaiacol by a wild and a diploid strain of *Aspergillus niger*. *Bioresour. Technol.* 101: 4721-4724.
- Bhathena J, Kulamarva A, Urbanska AM, Martoni C, Prakash S (2007). Microencapsulated bacterial cells can be used to produce the enzyme feruloyl esterase: preparation and *in-vitro* analysis. *Appl. Microbiol. Biotechnol.* 75: 1023-1029.
- Bloem A, Lonvaud A, Bertrand A, Revel G (2006). Ability of *Oenococcus oeni* to influence vanillin levels. *Flavour Sci.: Recent Ad.Trends.* 43: 137-140.
- Bohlin L (1993). Research on pharmacologically active natural products at the Department of Pharmacognosy, Uppsala University. *J. Ethnopharmacol.* 38: 225-231.
- Brunati M, Marinelli F, Bertolini C, Gandolfi R, Daffonchio D, Molinari F (2004). Biotransformations of cinnamic and Ferulic acid with actinomycetes. *Enzyme Microb. Technol.* 34: 3-9.
- Couto JA, Campos FM, Figueiredo AA, Hogg TA (2006). Ability of lactic acid bacteria to produce volatile phenols. *Am. J. Enol. Vitic.* 57: 166-171.
- Faveri D, Torre P, Aliabarian B, Dominguez JM, Perego P, Converti A (2007). Response surface modeling of vanillin production by *E. coli* JM109pBB1. *Biochem. Eng. J.* 36: 268-275.
- Ghosh S, Sacham A, Sen SK, Mitra A (2007). Microbial transformation of Ferulic acid to vanillic acid by *Streptomyces sannensis* MTCC 6637. *J. Ind. Microbiol. Biotechnol.* 34: 131-138.
- IHBT (2005). Vinyl guaiacol: An economic and ecofriendly process for a FEMA approved flavouring agent. <http://www.ihbt.res.in/vinyl.pdf>. Accessed 28 October 2010.
- Karmarkar B, Vohra RM, Nandarwar H, Sharma P, Gupta KG, Sopti RC (2000). Rapid degradation of Ferulic acid via 4-vinylguaiacol and vanillin by a newly isolated strain of *Bacillus coagulans*. *J. Biotechnol.* 80: 195-202.
- Landete JM, Rodriguez H, Curiel JA, Rivas B, Mancheno JM, Munoz R (2010). Gene cloning, expression and characterization of phenolic acid decarboxylase from *Lactobacillus brevis* RM84. *J. Ind. Microbiol. Biotechnol.* Doi.10.1007/S 10295-010-0709-6.
- Li X, Yang J, Li X, Gu W, Huang J, Zhang K (2008). The metabolism of Ferulic acid via 4-vinylguaiacol to vanillin by *Enterobacter* sp. PX6-4 isolated from vanilla root. *Process Biochem.* 43: 1132-1137.
- Mabinya LV, Mafunga T, Brand JM (2010). Bioconversion of ferulic acid and 4-vinylguaiacol by a white-rot fungus isolated from decaying wood. *Afr. J. Biotechnol.* 9: 1955-1958.
- Mariod AM, Adamu HA, Ismail M, Ismail N (2010). Antioxidative effects of stabilized and unstabilized defatted rice bran methanolic extracts on the stability of rice bran oil under accelerated conditions. *Grasas Aceites*, 61: 409-415.
- Max B, Torrado AM, Moldes AB, Converti A, Dominguez JM (2009). Ferulic acid and p-coumaric acid solubilization by alkaline hydrolysis of the solid state residue obtained after acid prehydrolysis of vine shoot prunings: Effect of the hydroxide pH. *Biochem. Eng. J.* 43: 129-134.
- Min J, Kang S, Park D, Kim Y, Jung H, Yang J, Seo W, Kim S, Karigar CS, Choi M (2006). Enzymatic release of Ferulic acid from *Ipomoea*

- batatas* L. (Sweet Potato) stem. Biotechnol. Bioprocess Eng.11: 372-376.
- Okeke B, Venturi V (1999). Construction of recombinants *Pseudomonas putida* B014 and *E. coli* QEFCA8 for Ferulic acid biotransformation to vanillin. J. Biosci. Bioeng. 88: 103-106.
- Torres BR, Aliabarian B, Torre P, Perego P, Dominguez MZ, Converti AS (2009). Vanillin bioproduction from alkaline hydrolyzate of corn cob by *E. coli* JM109/pBB1. Enzyme Microb. Technol. 44: 154-158.
- Tripathi U, Rao SR, Ravishankar, GA (2002). Biotransformation of phenylpropanoid compounds to vanilla flavour metabolites in cultures of *Haematococcus pluvialis*. Process Biochem. 38: 419-426.
- Walton NJ, Narbad A, Faulds CB, Williamson G (2000). Novel approaches to the biosynthesis of vanillin. Curr. Opin. Biotechnol.11: 490-496.
- Zhao Z, Moghadasian MH (2008). Chemistry, natural sources, dietary intake and pharmacokinetic properties of Ferulic acid: A review. Food Chem.109: 691-702.