



Evaluation of enzymatic activity and phenolic compounds during microbial transformation of *curcuma longa* to vanillin

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ABSTRACT

Aims: Evaluation of enzymes responsible for microbial transformation of *Curcuma longa* to vanillin and quantification of phenolic compounds present during the process.

Methodology and results: Yeast strains isolated from fermented beverages using curcumin-yeast extract agar were screened for their ability to transform curcumin to vanillin by evaluating ferulic acid esterase, ferulic acid decarboxylase, and vanillin dehydrogenase activities in growing cultures and cell-free supernatant. Based on bioconversion and enzymatic capabilities of the yeasts, *Pichia angusta* BT21 was selected for microbial transformation of *Curcuma longa* to vanillin using submerged fermentation technique under stationary mode. The metabolites were extracted from the transformation medium using solid-phase extraction technique and analyzed by thin-layer chromatography and gas chromatography-flame ionization detector. Phenolic compounds obtained in this research comprised of flavonoids (flavanols, flavones, isoflavones and flavonones), phenolic aldehyde (vanillin, vanillic acid, isoeugenol), simple phenolic (phenol) and phenolic acids (the hydroxybenzoic and the hydroxycinnamic acids) respectively. The research is quite profitable from an industrial point of view, considering the commercial price of vanillin and low cost and availability of *C. longa* which will eventually reduce the cost of industrial production of vanillin and increase supply.

Conclusion, significance and impact of study: Bioprocessing of *C. longa* by submerged fermentation technique under stationary mode reduces vanillin dehydrogenase activity of the *Pichia angusta* BT21, thus preventing the degradation of vanillin which subsequently leads to increase in vanillin concentration.

Keywords: Vanillin, ferulic acid esterase, vanillin dehydrogenase, *Curcuma longa*

INTRODUCTION

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is the chemical compound responsible for the vanilla aroma and is widely used as a flavoring agent in food and personal products. Most vanillin is produced via synthetic chemical routes starting from lignin, coniferin, the glucoside of coniferyl alcohol, guaiacol, or eugenol (Rao and Ravishankar, 2000). It is the major component of natural vanilla, which is one of the most widely used and important flavouring ingredient worldwide. Since current production of natural vanilla is not sufficient to meet the increasing demand for this flavour compound, vanillin has been a target for biotechnological production by several approaches: use of enzymes to release or generate vanillin from *Vanilla* and other plant material, development of tissue cultures, genetic modification and, finally, use of microbial cultures (Walton *et al.*, 2003).

Polyphenols of plant origin have been reported to have a variety of biological effects, including antioxidant,

anticarcinogenic, antiinflammatory and antimicrobial activities. Specifically some phenolic compounds such as resveratrol, hydroxytyrosol, oleuropein, quercetin and a number of phenolic acids have been reported to inhibit various pathogenic microorganisms (Dueñas *et al.*, 2005).

Antoniolli *et al.* (2015) evaluated the low molecular weight polyphenols (LMW-PPs) and anthocyanins present in grape pomace extract (GPE) of red grape. Galleano *et al.* (2012) also observed the action of polyphenols in controlling risk factors related to metabolic syndrome and several chronic diseases in aging humans. Turner *et al.* (2011) reported that polyphenols (caffeoyl derivatives and flavonoids) have antioxidant activity thus these compounds are protective agents against cardiovascular diseases, breast, gastrointestinal and skin cancers. Polyphenols have health-promoting effects and anti-aging properties due to its ability to interact with free radicals and others reactive oxygen species (ROS) involved in

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conditions such as inflammatory-immune injury, myocardial infarction and cancer (Fontana *et al.*, 2013). The powdered rhizome of *C. longa* has been used traditionally for alleviating the symptoms of gastrointestinal illnesses, motion sickness and cancer chemotherapy, thus its antioxidant action has been the mechanisms for the protective actions against toxicity (Trinidad *et al.*, 2012).

MATERIALS AND METHODS

Chemicals and growth media

Chemical reagents, curcumin and thiobarbituric acid (TBA, 99%), vanillin for the biotransformation medium was procured from Sigma and SD Fine Chemicals while phenolic acid standards were purchased from Aldrich (Aldrich Chemical Co., Milwaukee, WI).

Microorganism cultivation

Curcumin- biotransforming yeast was isolated from fermented beverages using Curcumin-Yeast Extract Agar (CYEA). Yeast extract agar was prepared according to manufacturer's instruction and 0.5% curcumin standard (membrane filtered) was added after sterilization and cooling to 45 °C.

Screening of vanillin synthesizing isolates

Ferulic acid esterase (FAE) activity

Sterile biotransformation medium which composed of Curcumin (10 g), maltose (0.24 g), Tween-80 (0.01 mL) and distilled water (100 mL) at pH 5.6 was inoculated separately with 1% v/v of 4 strains of Curcumin-biotransforming yeasts inoculum and incubated at 30 °C for 24 h. The released ferulic acid was extracted with equal volume of ethyl acetate. The organic fraction was separated and FAE activity was determined at 510 nm using JENWAY 6400 spectrophotometer (Kaur *et al.*, 2013) and ethyl acetate was used as blank. One nkat is defined as the amount of enzyme that catalyzes the release of 1 nM of free FA per second. (Hatfield *et al.*, 1999).

Ferulic acid decarboxylase (FDC) activity

FDC activity was assayed by harvesting cells through centrifugation; re-suspending in 70 mM sodium phosphate buffer pH 6.0 containing FA and incubated at 30 °C for 5 h. At an hour interval, the mixture was centrifuged and absorbance of the supernatant was determined within the UV range of 250 to 350 nm using JENWAY 6400 spectrophotometer (Kaur *et al.*, 2013).

Vanillin dehydrogenase (VDH) activity

VDH activity was determined by culturing curcumin-biotransforming yeast isolates in Erlenmeyer's flask containing Vanillin synthetic medium [Vanillin (0.001 g),

Tween-80 (0.001 mL), tri-ammonium citrate (0.002 g), sodium acetate (0.005 g), magnesium sulphate (0.0001 g), di-potassium hydrogen phosphate (0.0029 g) and distilled water (100 mL)] at 37 °C for 24 h. The supernatant (1 mL) from the synthetic medium was withdrawn at 8 h interval, thus mixture of 24% HCL (5 mL) solution and 1% thiobarbituric acid (1 mL) was added. The solution was heated in a water bath at 55 °C for 10 min and stored at room temperature for 20 min. The decrease in absorbance was read at 434 nm with JENWAY 6400 spectrophotometer (Kaur *et al.*, 2013).

Microbial transformation of *Curcuma Longa* to vanillin

Biotransforming medium comprised of mineral salt medium [Na₂HPO₄ (2.2 g), KH₂PO₄ (1.4 g), MgSO₄·7H₂O (0.6 g), FeSO₄·7H₂O (0.01 g), NaCl (0.05 g), CaCl₂ (0.02 g), yeast extract (0.02 g), distilled water (1000 mL)] supplemented with trace element [ZnSO₄·7H₂O (2.32 g), H₃BO₃ (0.56 g), CuSO₄·5H₂O (1.0 g), Na₂MnO₄·2H₂O (0.39 g), CoCl₂·6H₂O (0.42 g), EDTA (1.0 g), KI (0.66 g)] and *Curcuma longa* (1.5 g) was prepared in Erlenmeyer flask inoculated with 0.1% v/v of the best curcumin-biotransforming yeast isolate at 30 °C for 4 days.

Determination of biomass yield

Biomass yield of the biotransformation medium was determined at 24 h interval using JENWAY 6400 spectrophotometer at 600 nm.

Vanillin estimation using TBA reagent

Cell free supernatant obtained was diluted with equal volume of 70 mM phosphate buffer (500 µL, pH 7). The mixture was acidified with 24% HCl (5 mL) and 1% thiobarbituric acid (2 mL). Heated in a water bath at 55 °C for 10 min and kept at room temperature for 20 min as described by Rana *et al.* (2013). Absorbance was read at 434 nm using JENWAY 6400 spectrophotometer and the corresponding vanillin concentration was obtained from vanillin standard.

Qualitative analysis of metabolites using thin layer chromatography

The qualitative characteristics of vanillin produced was determined by TLC conducted on Silica gel plate using developing solvent system petroleum ether: ethyl acetate in the ratio 40:60 (v/v), and visualized by exposure to iodine (crystal) vapour thus vanillin was detected with 2,4-dinitrophenylhydrazine-HCl-reagent. The retention factor was calculated as distance travelled by the component divided by distance travelled by the solvent.

Phenolic compound extraction using Gas Chromatography- Flame Ionization Detector (GC-FID)

Two stage extraction procedures were used for the effective removal of the phenolic compounds as described

by Provan *et al.* (1994) and Kelley *et al.* (1994) respectively. Extraction was done by adding 5 mL of 1 M of NaOH to 50 mg of cell free-biotrasformation supernatant and incubating on a shaker at ambient temperatures for 16 h. The mixture was centrifuged (5000 rpm) twice, rinsed with water and the supernatant was heated at 90 °C for 2 h to release the conjugated phenolic compounds. The heated extract was cooled, titrated with 4 M HCL to pH<2.0, diluted to 10 mL with deionised water and centrifuged to remove the precipitate.

The supernatant was stored for subsequent purification; residue was extracted further by with 5 mL of 1 M of NaOH and heated to 160 °C in Teflon. After cooling, the supernatant was adjusted to pH <2.0 with 4 M HCL; filtered and the filtrate was purified by passing through a conditioned Varian (Varian Assoc., Harbor City, CA) Bond Elut PPL (3 mL size with 200 mg packing) solid-phase extraction tube at 5 mL/min attached to a Visiprep (Supelco, Bellefonte, PA). The GC condition for the analysis include GC (HP 6890 Powered with HP ChemStation Rev. A 09.01 [1206] Software), Injection temperature (Split injection), Split ratio (20:1), Carrier gas (Nitrogen), Inlet temperature (250 °C), Column type (HP-1, Capillary), Column dimensions (30 m x 0.25 mm x 0.25 µm), Oven program (Initial Temperature at 60 °C for 5 min; first rate at 15 °C/min for 15 min, maintained for 1 min; Second ramping at 10 °C/min for 4 min), Detector (FID), Hydrogen pressure (28 psi) and Compressed air (32 psi) respectively.

RESULTS

Identification of curcumin-biotransforming yeast

Based on their biochemical characteristics, curcumin-biotransforming yeasts namely *Candida utilis* B22, *Candida magnolia* BT20, *P. angusta* BT21 and *Candida utilis* PE11 were identified to the species level with API 20 C AUX kit (BIOMERIEUX).

Ferulic acid esterase activity (FAE)

Among the curcumin-biotransforming yeast screened for specific FAE activities; *P. angusta* BT21 expressed the highest enzymatic activity of 0.197 nkat while *C. magnolia* BT20, *C. utilis* BT22 and *C. utilis* PE11 expressed 0.140, 0.170 and 0.067 nkat respectively (Table 1). One nkat is defined as the amount of enzyme that catalyzes the release of 1 nM of free FA per second (Hatfield *et al.*, 1999).

Ferulic acid decarboxylase activity (FDC)

Pichia angusta BT21 was the best isolate because it expressed least FA degrading activity as observed by uniformity and minimal decrease in its absorbance between 280 and 320 nm compared to *C. utilis* PE11 degrading activity (Figures 1 and 2).

Table 1: Ferulic acid esterase activity of curcumin-biotransforming yeast.

Yeast isolates	Ferulic acid esterase activity (nkat/mg)
<i>C. magnoliae</i> BT20	0.140
<i>P. angusta</i> BT21	0.197
<i>C. utilis</i> BT22	0.170
<i>C. utilis</i> PE11	0.067

Vanillin dehydrogenase (VDH) activity

There was decrease in absorbance of vanillin medium between 0 and 8 h; and subsequently increase after the 8th hour for all curcumin-biotransforming yeasts utilized except *P. angusta* BT21 with gradual increase in absorbance between 0 and 8 h. Thus, as absorbance increases, vanillin concentration also increases and vice versa and *P. angusta* BT21 was found to be the best isolate based on its negative VDH property (Figure 3).

Microbial transformation of *Curcuma longa* to vanillin

Biomass yield

The biomass yield of *P. angusta* BT21 obtained from *Curcuma longa* biotransformation medium increased gradually from the 2.245 OD on the 1st day to 3.114 OD on the 4th day (Table 2).

Estimation of vanillin concentration using thiobarbituric acid (TBA) reagent

The absorbance of the cell-free supernatant was determined at 434 nm and the corresponding concentration of vanillin produced by *P. angusta* BT21 was extrapolated from the vanillin standard curve. The production is growth dependent because cell biomass increases as vanillin concentration increases (Table 2).

Table 2: Biomass yield and corresponding vanillin concentration during fermentation.

Fermentation period (days)	Experimental value		
	Biomass yield	Vanillin concentration (mg/mL)	Vanillin yield (%)
1	2.245	0.044	4.4
2	2.899	0.086	8.6
3	2.902	0.147	14.7
4	3.114	0.181	18.1

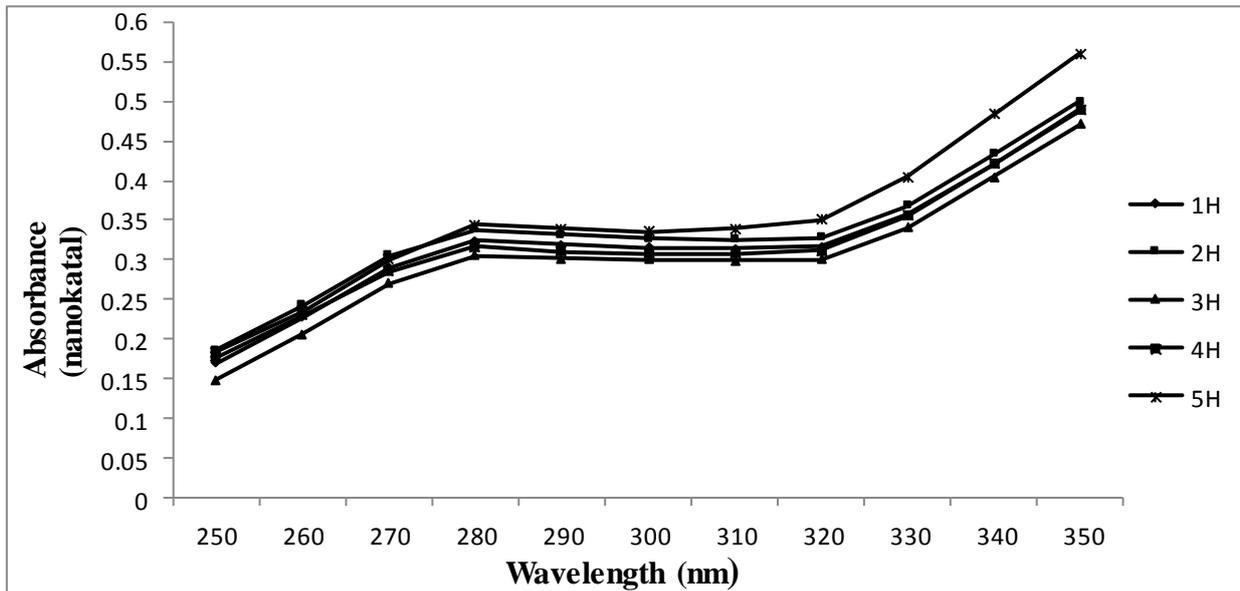


Figure 1: Ferulic acid decarboxylase activity of *P. angusta* BT21.

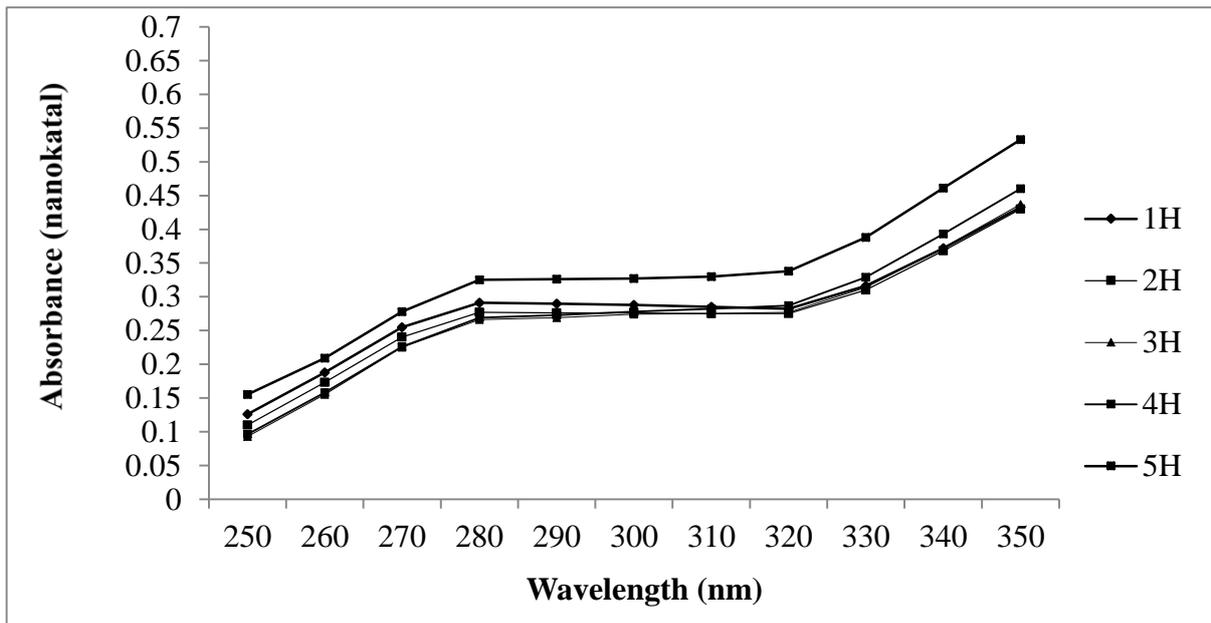


Figure 2: Ferulic acid decarboxylase activity of *C. utilis* PE11.

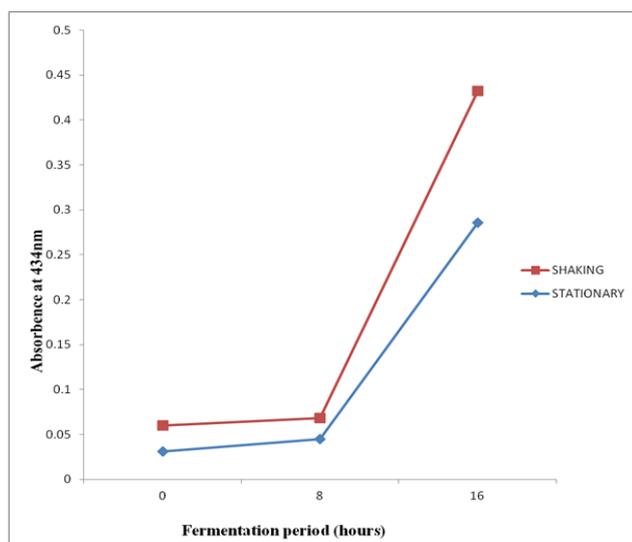


Figure 3: Vanillin dehydrogenase activity of *P. angusta* BT21 obtained from vanillin synthetic medium.

Qualitative analysis of metabolites using thin layer chromatography

Thin layer chromatography analysis revealed two different bands; the upper band had R_f value of 0.67 which corresponds with the R_f value of vanillin standard on silicon oxide TLC plate. Vanillin was further confirmed by occurrence of deep orange spot after spraying with 2,4-dinitrophenylhydrazine-HCL reagent.

Quantitative analysis of phenolic compounds using GC-FID

Pichia angusta BT21 and *C. utilis* PE11 biotransformation media were analysed in order to compare the vanillin concentration as well as other phenolic compounds present. Phenolic compounds obtained in this research comprised of flavonoids, phenolic aldehyde, simple phenolic and phenolic acids (Table 3). Flavonoids are categorized into major flavonoid classes such as flavanols (catechin, epicatechin, epigallocatechin and epigallocatechin gallate), flavonols (kaempferol, quercetin and myricetin), flavones (luteolin, rutin and apigenin), flavanones (naringenin), isoflavanones (genistein); phenolic aldehyde (vanillin, isoeugenol) simple phenolic (phenol) while phenolic acids are categorized into two subgroups according to their structure: hydroxybenzoic acids (gallic acid, protocatechuic acid, vanillic acid *p*-hydroxybenzoic acid, syringic acid, salicylic acid and gentistic acid) and hydroxycinnamic acids (ferulic acid, *p*-coumaric acid, sinapic acid cinnamic acid and chlorogenic acid) according to Silvia *et al.* (2011). The phenolic compounds concentrations with their corresponding retention time are as shown on Table 3. The chromatogram is shown on Figure 4.

DISCUSSION

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is one of the most important aromatic flavours used in food and cosmetics producing industries. Ferulic acid esterases (feruloyl esterases) are enzymes that catalyze the release of aromatic residues (e.g. ferulic acid) from plant cell wall polysaccharides and also essential for complete degradation of these polysaccharides to vanillin, vanillic acid and protocatechuic acid by bacteria and fungi (Ralet *et al.*, 1994). Among the four yeast isolates [*C. utilis* (2 strains), *C. magnolia* and *P. angusta* BT1] obtained in this research, *P. angusta* BT21 had maximum specific FAE activity of 0.197 nkat after 24 h (Table 1) culturing in curcumin medium which indicates the expression of feruloyl esterase-encoding gene (*faeA*) responsible for synthesis of FAE required for the release of ferulic acid from *C. longa* and sequential degradation to vanillin, vanillic acid, and protocatechuic acid. Slow and lesser FDC activity of *P. angusta* BT21 shows its ability to express *PAD1* gene and synthesize ferulic acid decarboxylase (FADase) enzyme which catalyzes the biotransformation of ferulic acid to 4-vinylguaiaicol and vanillin through non oxidative decarboxylation (Walton *et al.*, 2003). Thus, least minimum fall in absorbance between 280-320 nm (Figures 1 and 2) expressed by *P. angusta* BT21 corresponds with the report of Gu *et al.* (2011).

The physiological impact of vanillin dehydrogenase (VDH) on the catabolism of vanillin and related ferulic acid was investigated through stationary and shaking mode respectively (Figure 3). *Pichia angusta* BT21 was selected due to its least VDH activity during the biotransformation process. The gradual increase in vanillin concentration after 8 h may be due to the production of vanillin by the isolate rather than degrading/consuming it, which led to increase in its absorbance (Kaur *et al.*, 2013). Furthermore, significant decrease in vanillin concentration exhibited by the other yeast isolates is due to the inherent vanillin catabolism via vanillic alcohol (VAL) and vanillic acid (VA) as a result of presence of vanillin catabolism gene (*Vdh*) encoding the subunit of vanillin dehydrogenase (Fleige *et al.*, 2013; Priefert *et al.*, 1997).

Phenolic compounds also referred as polyphenols are natural antioxidants that represent an important group of bioactive compounds in foods (Dueñas *et al.*, 2005). Phenolic compounds obtained in this research comprised of flavonoids, phenolic aldehyde, phenolic acids and simple phenolics (Table 3). Thus, significant amount of phenolic compounds produced indicated the ability of *P. angusta* BT21 to express main extracellular enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase required in lignin degradation of *C. longa* and corresponding increase in microbial growth showed that the yeast utilizes the polysaccharides for grow and reproduce after lignin degradation (Philippoussis, 2009). Thus concurrent increase in biomass and production yield indicates that vanillin production is coupled to energy metabolism i.e. product will be formed whenever there is growth.

Table 3: Phenolic compounds concentrations obtained using GCFID.

Phenolic Compounds	Classes	Metabolites	Biotransformation Yeast Isolates		
			<i>P. angusta</i> BT21 (mg/100mL)	<i>C. utilis</i> PE11 (mg/100 mL)	
Flavonoid compounds	Flavanols	Catechin	11.47	9.28	
		Epicatechin	7.43	7.28	
	Flavanols	Epigallocatechin	2.30×10^{-2}	1.80×10^{-2}	
		Epigallocatechin gallate	1.46×10^{-4}	2.92×10^{-4}	
		Kaempferol	9.83	9.44	
		Quercetin	2.95	2.27	
		Myricetin	5.41×10^{-3}	2.53×10^{-2}	
		Luteolin,	1.48×10^{-2}	1.19×10^{-2}	
	Flavones	Rutin	1.27	1.24	
		Apigenin	5.95×10^{-2}	4.47×10^{-2}	
	Flavanones	Naringenin	1.33×10^{-4}	1.06×10^{-4}	
		Isoflavanones	Genistein	3.65×10^{-2}	3.08×10^{-2}
	Phenolic acids	Hydroxybenzoic acids	Gallic acid	6.38	4.63
			Protocatechuic acid	2.24×10^{-4}	1.20×10^{-4}
Hydroxycinnamic acids		Vanillic acid	5.14×10^{-2}	2.64×10^{-2}	
		<i>p</i> -hydroxybenzoic acid	3.78×10^{-1}	5.28×10^{-1}	
		Syringic acid	0.43	0.44	
		Salicylic acid	8.90×10^{-4}	2.75×10^{-4}	
		Gentistic acid	3.07×10^{-4}	1.43×10^{-4}	
		Vanillic acid	5.14×10^{-2}	2.64×10^{-2}	
		Ferulic acid	4.71	3.70	
		<i>p</i> -Coumaric acid	4.98	4.20	
		Sinapic acid	1.3×10^{-2}	1.2×10^{-2}	
		Cinnamic acid	2.2×10^{-4}	8.9×10^{-5}	
Phenolic aldehyde		Chlorogenic acid	16.69	11.68	
		Vanillin	6.62×10^{-1}	1.96×10^{-1}	
Simple Phenolic	Isoeugenol	2.39×10^{-4}	1.21×10^{-4}		
	Phenol	3.14×10^{-4}	5.91×10^{-4}		
Total (mg/100mL)			72.53	60.70	

Area (pA)

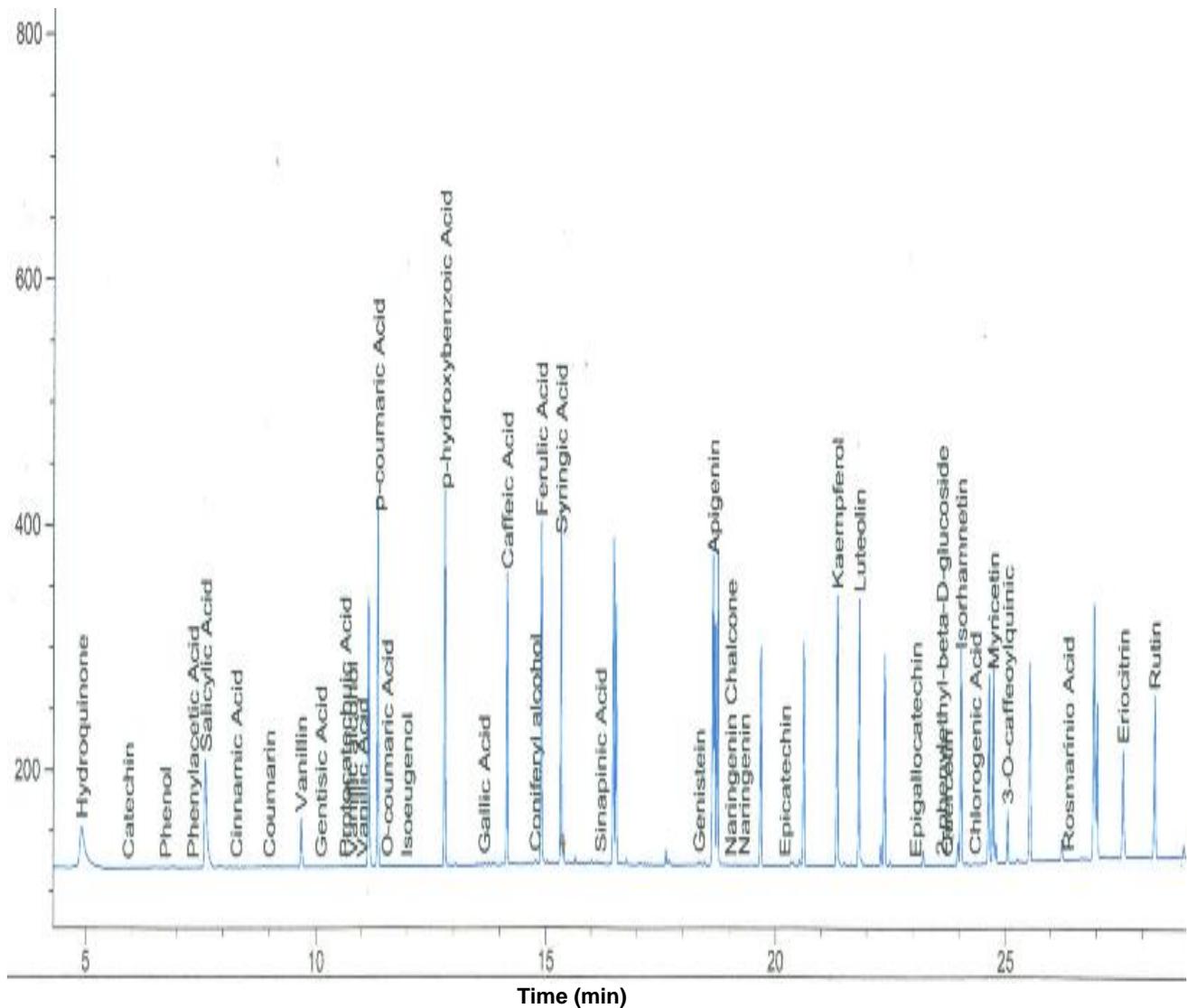


Figure 4: Phenolic compound profile of *P. angusta* BT21.

CONCLUSION

Phenolic compounds confer antioxidant, anti-mutagenic, anti-inflammatory and anticancer activities to the humans (Naveena *et al.*, 2008; Conforti *et al.*, 2009; Kim *et al.*, 2009). Although, these bioactive compounds are recovered from natural sources by solid-liquid extraction using organic solvents in heat-reflux systems; techniques involving use of supercritical fluids, high pressure processes, microwave-assisted extraction and ultrasound-assisted extraction according to Wang and Weller (2006) and Martins *et al.* (2010). Bioprocessing of *C. longa* by submerged fermentation technique under stationary mode

is recommended in order to reduce VDH activity of the *Pichia angusta* BT21 thus preventing the degradation of vanillin and subsequent increase of vanillin content, being an interesting alternative for the production of bioactive compounds and also serves as an alternative merit due to the potential of the yeast to produce high quality and high activity compounds while precluding any toxicity associated to the organic solvents. Thus, this process is quite profitable from an industrial point of view, considering the commercial price of the phenolic compounds and the low cost and availability of *C. longa*.

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