

Microbiology

Journal

ISSN 2153-0696



Academic
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Evaluation of Total Phenolic Content in Ghee Residue: Contribution to Higher Laccase Production

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ABSTRACT

Phenolic compounds are the major group of secondary metabolites produced in plants. As the herbivores feed on plants, the milk obtained from such animals was supposed to contain Phenolic compounds. The aim of this study was to evaluate the total Phenolic content expected in dairy waste "Ghee residue" and its optimization in the production medium for laccase production. The fungal strain used in this project was an improved strain of *Trametes hirsuta* (Accession No. 20503), named as PH2. When compared to the previously published article reporting maximum production in presence of 1% Ghee residue, approximately 28.14% increase in laccase production was obtained by the improved strain in the best production medium with optimum concentration of Ghee residue (2% w/v) at optimum temperature (35°C), optimum pH (3.0) in 9 days of incubation. In this study the procedure for obtaining ghee residue, extraction of the Phenolics and evaluation of its concentration was described.

Key words: Ghee residue, phenolic compounds, laccase, *Trametes hirsuta mutant* (PH2)

INTRODUCTION

Phenolic Compounds (PCS), a diverse group of chemicals having at least one aryl ring with minimum one attached hydroxyl group, are the secondary metabolites produced by most plants. These have various applications including role as natural antimicrobial agents, as natural deterrents to grazing animals or as inhibitors of pre-harvest seed germination (Haslam and Lilley, 1988; Bravo, 1998; Haslam, 1998).

PCS extracts from hawthorn, raspberry and strawberry have been used to treat burns, frostbite, scurvy, polio (Singleton, 1981). Polyphenols are still referred as vitamin C2 in France because of the synergism between polyphenols and dehydroascorbic acid (vitamin C) in the treatment of scurvy and related conditions (Singleton, 1981). Reduction in the incidence of pasture bloat which results in respiratory failure and death of the cattle caused due to high pressure applied on lungs formed by extensive foam in the reticulorumen was reported when the cattle or sheep were fed with high level of PCS (Haslam, 1998). Gossypol-rich diet for cattle and proanthocyanidins in the diet of sheep resulted in increased milk yield, fat content and non-casein nitrogen content (Blauwiekel *et al.*, 1979; Wang *et al.*, 1996).

Though there are reports mentioning the antioxidant, anticarcinogenic, antimutagenic properties of PCS and as agents in the treatment of Rh-factor-threatened pregnancies and in problems encountered during parturition (Singleton, 1981; Haslam and Lilley, 1988; Mukhtar *et al.*, 1992; Chang *et al.*, 1998), a number of evil effects on health has also been reported such as their ability to increase susceptibility to oesophageal cancer, hepatic necrosis and

bladder carcinomas (Chang *et al.*, 1998). Cattle fed with snakeroot, siam weed, mist weed were reported to suffer with liver cirrhosis, ataxia, anorexia, photosensitization and respiratory problems mainly due to the phenolics present in it (Sharma *et al.*, 1998). The physiological detoxification processes in humans was performed by the gut microflora (Kelly *et al.*, 1993) and excreted after methylation or conversion to phosphate or sulphate conjugates in the liver and kidney, thereby reducing the health risk to humans (Singleton, 1981).

The olfactory properties of the smoked food products and fruit juices are also due to the PCS like guaiacol, eugenol, syringol, cresol and phenol, present in wood smoke (Kornreich and Issenberg, 1972; Maga, 1988; Singleton, 1981). Protein-PC interactions lead to formation of a haze in beer (Delcour *et al.*, 1984). Considerable concentrations of the principal PCS found in bovine, caprine and ovine milk. The variations in the Phenolics in the milk sample are based on the variations in feed of cattle or sheep (Lopez and Lindsay, 1993). High level of ptaquiloside and shikimic acid in bovine milk, was reported to be partially responsible for the high incidence of gastric cancer (Alonso-Amelot *et al.*, 1996; Austwick and Mattocks, 1979).

Reports regarding the influence of Phenolic compounds on laccase production are available. Assessment of the influence of Phenolic compounds on laccase production is another area of interest of several researchers (De Souza *et al.*, 2004; Sampoorna Laxmi and Mazharuddin Khan, 2010).

Induction of higher laccases production using various Phenolic compounds was performed among which a mixture of ferulic acid and vanillin was more efficient than the isolated phenolics by the white rot fungus *P. pulmonarius* (De Souza *et al.*, 2004).

As the reports in the literature confirmed the presence of Phenolic compounds in milk, in this article efforts were put forward to evaluate such PCS in a dairy waste "Ghee residue". Thus the influence of this dairy waste on laccase production by the fungus under investigation was reported.

MATERIALS AND METHODS

Test fungus: The strain of white rot fungus *Trametes hirsuta* was obtained from Hosur, Tamilnadu, India, improved by using physical and chemical mutagens and given an Accession No. 20503 by NCBI BLAST.

Laccase production medium: The best laccase production medium was formulated by using agricultural and industrial waste (Khanam *et al.*, 2012).

Preparation of ghee residue: Butter is manufactured by the churning of cream obtained from cow/buffalo or mixed milk. For making ghee, butter out of any coloring matter or preservative or common salt is heated to remove its moisture by evaporation. Usually the equipment called a steam jacketed kettle is employed by the dairy-processing plant for heating butter. After heating, the contents of the kettle are allowed to stand undisturbed for a while. Figure 1 shows the different stages from milk to ghee residue. The yield of ghee by this method is about 92% of the milk fat in the original milk. And fat, losses are, about 0.8% in the buttermilk, 1.4% in the ghee residue and 4.5% in handling of that milk (Chandy, 2011).

Properties of ghee residue: Physical properties like color, taste, texture and solubility and the chemical properties like phenolic compounds were examined. As the effect of phenolic compounds on laccases production was found to be good (Elisashvili *et al.*, 2010; Krastanov *et al.*, 2007), the tests for extraction of phenolic compounds from Ghee residue and its quantitative estimation was performed with an aim to show the presence of phenolics encouraging laccases production.



Fig. 1(a-f): Preparation of Ghee residue, (a) Milk, (b) Cream, (c) Butter, (d) Ghee with ghee residue, (e) Pure Ghee and (f) Ghee residue

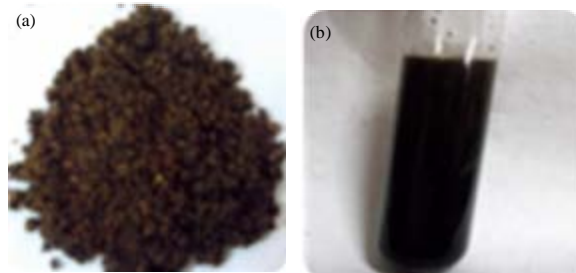


Fig. 2(a-b): Extracted Ghee residue, (a) Solid Ghee residue and (b) Ghee residue dissolved in hot con. H₂SO₄

Optimization of ghee residue concentration for laccases production: Since the presence of ghee residue in the production medium was influencing the laccase production, it was necessary to optimize its concentration to formulate the production medium.

The best laccases production medium was indulged with different concentrations of ghee residue. A set of Erlenmeyer flasks in triplicates containing 0.05 to 5.0% of Ghee residue was added in the best production medium, adjusted to optimum pH 3.0 and incubated at optimum temperature 35°C in an orbital shaking incubator at 120 rpm for optimum 9 days.

Extraction of phenolic compounds from ghee residue: 0.5 g of Ghee residue was dissolved in 5 mL of hot concentrated H₂SO₄ (Fig. 2). To this mixture 50 mL of methanol was added and mixed well and then 50 mL of water was added and allowed to stand for 5-10 min at room temperature. The resulting mixture is separated into two layers of a methanol-water layer and an oil layer. Phenolic compounds in ghee residue were extracted into the methanol-water layer (Fig. 3). After 10 min, the methanol-water layer was separated from the oil layer and evaporated to remove methanol. The aqueous phase obtained was extracted with 50 mL of dichloromethane.

It resulted in organic layer with phenolics and a water layer. The organic layer was separated and the extracts were dried over sodium sulfate (Fig. 4). Filtration and removal of the solvent resulted in a mixture of phenolic compounds as brown oil (Kodera *et al.*, 1989).

Quantitative estimation of total phenolic compounds: The concentration of total phenolic compounds in methanolic extracts of Ghee residue was determined by the Folin-Ciocalteu method. An aliquot (100 μL) of the methanolic extract was taken in a test tube and made up to 1 mL with distilled water. Diluted Folin-Ciocalteu reagent (0.5 mL) was added. After 3 min, saturated sodium carbonate (1 mL) was added and incubated for 30 min. After 30 min, absorbance at 765 nm against a reagent blank was measured using a UV-vis spectrophotometer (Hismath *et al.*, 2011). The quantitative estimation of total Phenolics was obtained from the standard curve of Gallic acid (1 mg mL^{-1}) (Fig. 5).

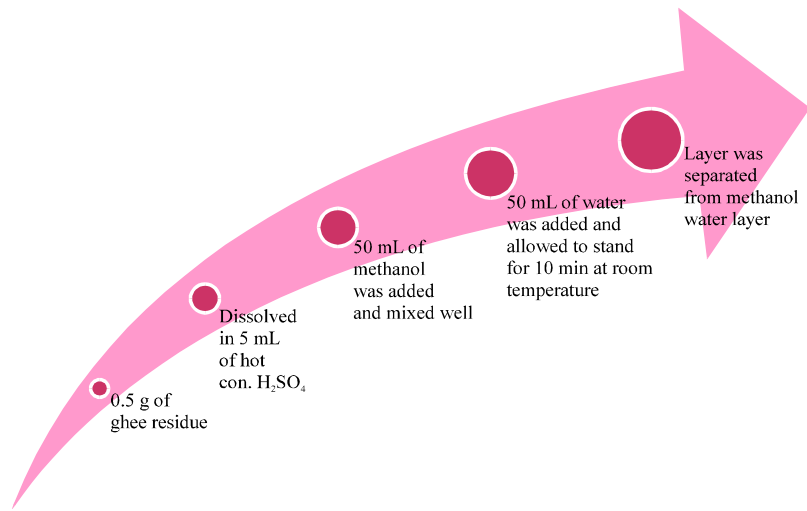


Fig. 3: Protocol for extraction of phenolics

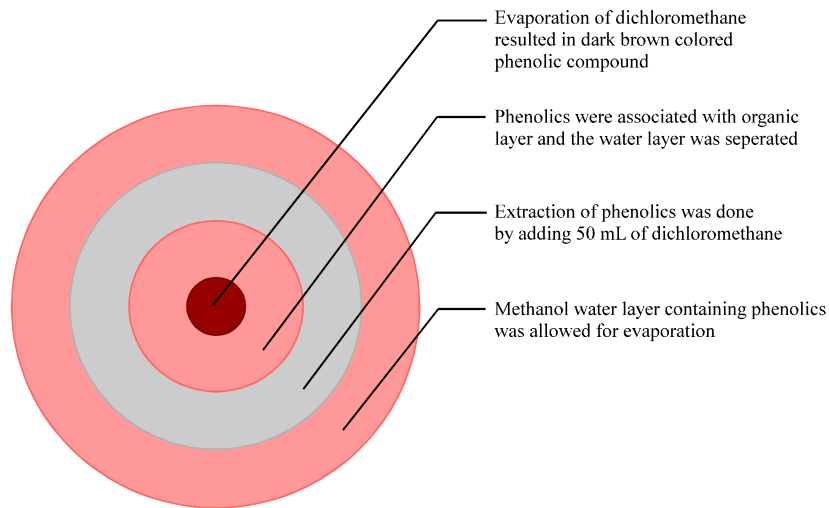


Fig. 4: Strengthening of phenolics from ghee residue

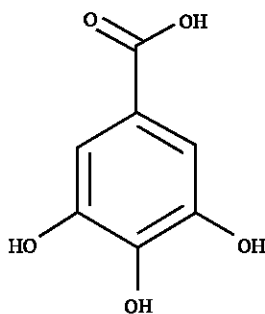


Fig. 5: Structure of gallic acid

RESULTS AND DISCUSSION

The improved strain of *T. hirsuta* was established by using various physical mutagens (X-rays and UV-rays) and chemical mutagens (Colchicine, hydrogen peroxide and Ethidium bromide) among which a mutant strain obtained by Hydrogen peroxide (2 μ g) treatment was beneficial for laccases production. It was mentioned as PH2 and was given an Accession No. 20503 (Fig. 6).

Ghee residue as an inducer: The dark brown colored residue obtained in Ghee manufacturing process has minimum use in future. Thus the it was found to be advantageous if added in the production medium (2% w/v) as an inducer for producing industrially important enzyme laccases using improved strain of *T. hirsuta*.

Physical properties of ghee residue:

- Color:** Dark brown (Fig. 2a)
Texture: Soft earlier, hard and granular on storage
Odor: Pleasant
Taste: Slightly sweet
Solubility: Soluble in hot concentrated H₂SO₄ (Fig. 2b)

Optimum concentration of Ghee residue for laccases production: Laccases production increases with increasing concentration of ghee residue reaching maximum at 2.0% (116.85 U/mL/min). Beyond this concentration the production was neither positively nor negatively effected (Fig. 7). The presence of ghee residue in the medium is meant for the presence of Phenolic compounds which was supposed to encourage the laccase production.

Laccase production by *T. hirsuta*

Total phenolic compounds in ghee residue: The concentration of total phenolics in 0.5 g of ghee residue (concentrated to 1 mL after a series of treatment for the separation of Phenolics) was calculated in mg of Gallic acid and was estimated to be 2.52 mg g⁻¹ of ghee residue (Table 1).

Sequences producing significant alignment:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
Z5E03		1067	1067	98%	0.0	99%	

Alignments
Id: Z5E03
Length=610
 Score = 1067 bits (1182), Expect = 0.0
 Identities = 597/601 (99%), Gaps = 0/601 (0%)
 Strand=Plus/Minus

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PR 1      TCAGACGGGTCTGTTTAAAGCCATTATGCCGATCTAAGCACGAGGTAGGGTAAACC 60
PH2 60L    TCAGACGGGTCTGTTTAAAGCCATTATGCCGATCTAAGCACGAGGTAGGGTAAACC 542

PR 61     CCCCCTACGAGGCGTCTGCGTTCCCTCGATCCCAAACGCCGTATGCCACTGGAGGCTAT 120
PH2 54L    CCCCCTACGAGGCGTCTGCGTTCCCTCGATCCCAAACGCCGTATGCCACTGGAGGCTAT 482

PR 121    AACACACCCGAGGCTGCCACATTCCTCCAGCCCTTTTCGCGGTCAAAATCGATGCTGG 180
PH2 48L    AACACACCCGAGGCTGCCACATTCCTCCAGCCCTTTTCGCGGTCAAAATCGATGCTGG 422

PR 181    CCGTCTATCGGAAAGTGCACCAAGCCGAAAGCAAGGCTGAGTCCCGGACAACTCGACTGA 240
PH2 42L    CCGTCTATCGGAAAGTGCACCAAGCCGAAAGCAAGGCTGAGTCCCGGACAACTCGACTGA 362

PR 241    CTTCAAGCGTTTCCTTTCAAGCAATTTCAAGTACTGTTTAACTCTCTTTTCCAAAGTGC 300
PH2 26L    CTTCAAGCGTTTCCTTTCAAGCAATTTCAAGTACTGTTTAACTCTCTTTTCCAAAGTGC 302

PR 301    TTCATCTTTCCCTCACGGTACTTGTTCCTATCGGTCTCTCGCAATATTTAGCTTAAAG 360
PH2 30L    TTCATCTTTCCCTCACGGTACTTGTTCCTATCGGTCTCTCGCAATATTTAGCTTAAAG 242

PR 361    TGGAAATTCACCACTTTTGAAGTGCATTCACAAAACAACCTCGACTCTTTGAGAGCGCAT 420
PH2 24L    TGGAAATTCACCACTTTTGAAGTGCATTCACAAAACAACCTCGACTCTTTGAGAGCGCAT 182

PR 421    CACAAAACAATGGTAGTCCTGTCCAAAGACGGGATTCCTCACCTCTATGACGCTCTGTTC 480
PH2 18L    CACAAAACAATGGTAGTCCTGTCCAAAGACGGGATTCCTCACCTCTATGACGCTCTGTTC 122

PR 481    CAGGAGACTTATACAGGTCAGCGCGGAAAGACGTTCTCCAGACTACAACTCGAGCGGC 540
PH2 12L    CAGGAGACTTATACAGGTCAGCGCGGAAAGACGTTCTCCAGACTACAACTCGAGCGGC 62

PR 541    CAAAGACCGCCAGATTTAAATTTGAGCTTTTCCTGCTTCACTCGCAGTTACTAGGGGAA 600
PH2 6L     CAAAGACCGCCAGATTTAAATTTGAGCTTTTCCTGCTTCACTCGCAGTTACTAGGGGAA 2

PR 60L    T 60L
PH2 1     T 1
    
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Fig. 6: Comparative sequence alignment of *Trametes hirsuta*-NCBI BLAST, PR: Wild, PH2: Mutant obtained by treating with 2 µg Hydrogen peroxide

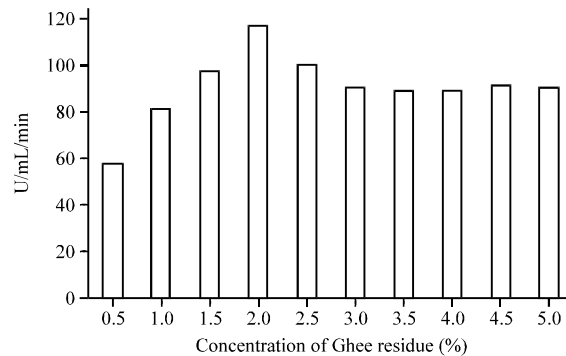


Fig. 7: Effect of ghee residue concentration on laccase production by *T. hirsuta*

Table 1: Concentration of total phenolics in ghee residue

Variable	No. of trials (N)	Mean (mg gm ⁻¹)	Standard deviation	Variance	p-value
Conc. of total Phenolics	8	2.52125	0.0264	0.000698	0.075

CONCLUSION

Phenolic compounds were found to be naturally available in embryophytes like in vascular plants, in non-vascular plants or bryophytes [Dihydrostilbenoids and bis(bibenzyls)], in ferns (kaempferol), in green algae like *Botryococcus braunii* (protocatechuic, p-hydroxybenzoic, 2,3-dihydroxybenzoic, chlorogenic, vanillic, caffeic, p-coumaric and salicylic acid, cinnamic acid, in brown algae like *Alaria marginata* (Phlorotannins), basidiomycetes fungi (protocatechuic acid, pyrocatechol), in lichens (Gyrophoric acid), in insects (protocatechuic acid, Acetosyringone, Guaiacol, Orcinol), in mammals (phenol, m-cresol and p-cresol), in prokaryotes *Streptomyces neyagawaensis* (Orobol).

In this article phenolic compounds were also found to be present in dairy waste “Ghee residue”. By the induction of 2% (w/v) ghee residue in the production medium, the test fungus was provided with approximately 10 mg of phenolic compounds resulting higher enzyme production (116.85 U/mL/min). The aforementioned results in this article are in synchronization with those reported by De Souza *et al.* (2004) and Sampoorna Laxmi and Mazharuddin Khan (2010).

Ghee residue, by virtue of its chemical composition, nutritional quality, physical characteristics, bulk of production and long shelf life permitting its collection and centralized handling has great potential and is more amenable to exploit its utilization. Ghee residue can be utilized in a number of products like chocolate burfi, samosa filling, chapatis etc. However, most dairy plants in India have not been utilizing ghee residue profitably except for fat extraction. Most of the ghee residue goes to waste ([vedyadhara.ignou.ac.in/wiki/images/BPVI-16-Block_4-Unit-14.pd.Unit14 Buttermilk and Ghee Residue](http://vedyadhara.ignou.ac.in/wiki/images/BPVI-16-Block_4-Unit-14.pd.Unit14%20Buttermilk%20and%20Ghee%20Residue)).

Beside the available literature, the survey reports gathered from the dairy industries also enlightened that a minimum value is given to the dairy waste “Ghee residue” with least commercial value or most of the end product is dumped out without any commercial value.

Every dairy industrialist is looking forward for getting marketable value to the waste.

In presence of ghee residue, the production medium favored laccase production by the basidiomycete fungus under investigation. Ghee residue was expected to be an inducer for laccase production due to the presence of phenolic compounds in it. In order to utilize whole ghee residue commercially it can be used as an important component in the production medium for laccase production to put it on the market.

Though literature was available on the composition of ghee residue, it is still under investigation to confirm whether any other compound in ghee residue is encouraging the laccases production.

ACKNOWLEDGMENTS

The authors are thankful to Prof M.A.Singara charya, Department of Microbiology, Kakatiya University, for the preservation of the collected fungal cultures and a moral support. We also thank the co-workers in the laboratory of Microbiology department at A.Q.J Degree and PG College for their cooperation during the tenure of the study.

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