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Fortification of yogurt containing isolated *Lactococcus lactis cremoris* with Fenugreek Seeds extract

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Abstract

People in their everyday life in midst the bustling schedule have developed taste for go-to nutritious food products such as, nutrition bars, nutrition cookies, flavored yogurts, protein shakes, sugar-free juices, fruit juices with additional vitamins, probiotic pickle, and flavored teas. With emergence of fortified food items, increase in health awareness and changed outlook of consumers, has made food industry to adopt fortification of beverages and common food like cheese, bread, and yogurt with herbal extracts. Plant extract are known to have antioxidant, anti-diabetic, antimicrobial, and anti-cancer properties. Fenugreek, for instance, are rich in polyphenols and this class of secondary metabolites have strong antioxidant capacity in comparison to vitamins and carotenoids. Current study aims at extracting polyphenols from fenugreek seeds (PFFS). Total phenol content of PFFS was estimated using Folin Ciocalteau Method and it was calculated to be 182 µg of GAE/ 5 mg of PFFS. Lactic acid bacteria (LAB), *Lactococcus lactis cremoris* was isolated from homemade yogurt. Isolated *L. lactis cremoris* culture was used to prepare yogurt fortified with polyphenol fraction extracted from fenugreek seeds. The antioxidant activity of fortified yogurt was determined qualitatively using DPPH assay which showed immediate change in color from purple to yellow which is indicative of free radical scavenging activity.

Keywords: *Lactococcus lactis cremoris*, fenugreek, flavonoid, antioxidant

Introduction

Consumption of natural antioxidants through dietary supplements have shown to reduce cardiovascular and cancer risks. Naturally occurring antioxidants that are present in the vegetables are grouped into three categories, vitamins, carotenoids and phenolics^[1]. Research, characterization and application of natural antioxidants is a mainstay of numerous research teams all over the world^[2]. Phenols, particularly, have the ability to scavenge reactive oxygen species because of their electron donating property. In literature, phenols are shown to demonstrate higher antioxidant activity than vitamins and carotenoids^[3].

Fenugreek or *Trigonella foenum-graecum*, is a popularly known and variedly used plant, in day to day life^[4]. Fenugreek constitutes of flavonoids, alkaloids, coumarins, vitamins and saponins. Fenugreek has been known to contain a great percentage of polyphenols that contribute to the antioxidant's property. Fenugreek is used as a traditional remedy for many disorders eg. Diabetes^[5, 6], arthritis, irritation^[7], hypercholesterolemia^[8, 9], among others. Fenugreek seeds are also known to contain gum, fiber, saponin and volatile content. Its high fiber content makes it suitable as an emulsifier, stabilizer or adhesive agent which have implications in food industry^[10]. Human history mentions use of fenugreek seeds in flavors, essence and medicines^[4].

Yogurt has been in increasing demand between the masses mainly because it's a "to go" food product and also nutritious. Yogurt is different from curd and is made by fermenting the milk with a yogurt starter culture (*Lactobacillus*, *Lactococcus*, *Streptococcus*) which helps in maintenance of gut microflora and flavoring agents to enhance texture and taste. Yogurt helps in maintaining cholesterol levels, blood pressure and aids digestion^[11]. Long since it has been known that fruits, vegetables, and dairy products are healthy components of the diet according to food guides and nutritional guidelines. Recently it has been investigated that whole plant-based foods, probiotics, and prebiotics can modulate the microbiota, leading to cardiac health^[12]. With emergence of fortified food items, increase in health awareness and changed outlook of consumers has made food industry to adopt fortification of beverages and common food like cheese, bread, and yogurt with herbal extracts^[13].

Current study focuses on extraction of polyphenol content from fenugreek seed and its role as an antioxidant in fortified yogurt containing *L. lactis cremoris* as a starter culture.

A part of the work also involves isolation and characterization of probiotic microbe from preformed yogurt as an already available Yogurt can be a good source for isolation of *Lactobacillus* and *Lactococcus* species. This isolated and characterized probiotic microbe and polyphenol fraction of fenugreek seed (PFFS) will be utilized to produce a fortified yogurt with antioxidant property.

Material and Methods

MRS Broth and Agar (Himedia), UV-Visible Spectrophotometer (Cary 50), all chemicals used were of analytical grade, DPPH (Himedia), all microbiological media, chemicals and broth used was procured from Himedia.

Extraction of polyphenols from fenugreek

Fenugreek seed were obtained from local market store from Mumbai, Maharashtra in the month of November, 2018. 50 g of the seeds were ground using a mechanical grinder to obtain uniform powder. The powdered seeds were then soaked in 80% methanol and kept at room temperature for 5 days. After incubation, the suspension was filtered and residue was collected after solvent evaporation. Water was added to the residue, heated for 2 min and reduced to form a viscous solution. The residue was washed by centrifugation at 5000 rpm for 10 min using deionized distilled water. The supernatant was collected and filtered through Whatman's Filter Paper No.1. The filtered aqueous layer was washed several times with petroleum ether several times and aqueous layer collected was again washed with ethyl acetate containing glacial acetic (10%). The extract was kept in oven for 36 hours. This served as a polyphenol fraction (PFFS) obtained from fenugreek seeds and was stored at 4°C until further use [14].

Total phenol content

The total phenolic content (TPC) of PFFS extract was determined using a modified procedure mentioned by Kakodkar *et al*, 2019. [15] 120 µL of PFFS extract was mixed with 3 mL of Folin-Ciocalteu reagent and incubated for 10 mins. The solution was then neutralized with 3mL of 2% Na₂CO₃ solution and incubated in dark for 30 min for color development. Similar procedure was carried for gallic acid (Standard) with a range of concentration from 100-1000 µg/mL. The reaction mixture containing PFFS was measured at 765nm along with gallic acid. The absorbance was read for all the tubes. A standard graph was plotted using Gallic acid and calibration curve was obtained. The total phenolic content of PFFS was expressed as Gallic acid equivalent (GAE) in mg/g of dry extract.

Isolation of probiotic microbe from yogurt

Homemade Yogurt was used to isolate probiotic microorganism. 5g of curd was taken in a sterile flask and was diluted using saline. A loopful of solution was streaked onto Sterile MRS agar plate. The plates were incubated for 24hrs at 37 °C. A typical colony was selected which was further streaked on MRS agar and after incubation the plate was stored at 4 °C for further tests. Bacterial colonies were purified by sub-culturing in St. MRS broth.

Colony characterization for final identification

Colony characterization, gram staining, and biochemical tests were carried out and the identification of the strain was done by Bergey's Manual of determinative bacteriology [16].

Biochemical tests performed were test for carbohydrate metabolism, test for specific breakdown of products, utilization of specific substrate test, test for enzymes and metabolism of nitrate and amino acids.

Starter Culture

Isolated colony was enriched in St. MRS broth overnight. Approximately 10⁷ cells were inoculated in the 10mL of pasteurized full cream milk in a sterile test tube. The inoculated milk cream was incubated in the water bath at 41 °C for 12 hrs. Yogurt formed was stored at 4 °C and used as a starter culture within 7 days.

Preparation of PFFS Yogurt

This included fermentation of milk with PFFS along with starter culture. Pre-warmed pasteurized milk (85mL) was taken in to a beaker. 10 mL of 20% fenugreek extract was added along with 5mg of starter culture. The mixture was incubated in water bath at 41 °C until the pH is dropped to 4.5. The prepared yogurt was stored at 4 °C for 14 days.

Qualitative antioxidant profile of PFFS yogurt using DPPH assay

Antioxidant activity of PFFS yogurt was determined using stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) by the slightly modified method.¹⁷ The solution of DPPH in methanol 6 × 10⁻⁵ M was prepared freshly. 10 ml of PFFS yogurt was distributed in 1.5mL eppendorf tubes and centrifuged at 10000 rpm for 10 min. The supernatant was collected and was used as a test solution for determining free radical scavenging activity. 100 µL of test was added to 100 µL of DPPH solution. Color blank was maintained to give a solution of 100 µL of test and 100 µL of methanol. Negative control used was of 100 µL of DPPH and 100 µL of Methanol. The test was conducted in 96 well plates. Color change was used to determine the antioxidant activity present in PFFS Yogurt.

Results and Discussions

Extraction of polyphenol fraction (PFFS) from Fenugreek Seeds

Methanol was selected as a solvent for extraction of Polyphenol Fraction (PF) as it shows better affinity towards polyphenols. Later water was added to the solution as polyphenol will get solubilized in aqueous fraction leaving residues to be absorbed by methanol. 50% water tends to increase the solubility of polyphenols lowering the viscosity. The centrifugation step concentrated the polyphenol [18]. Filtration removes polyphenols from other unwanted residues and petroleum ether was added to dissolve mixture of hydrocarbons. Petroleum also separates oils and fats from polyphenols in aqueous layer [19]. 0.02% Glacial acetic acid in Ethyl acetate finally extracted polyphenols from aqueous solution as ethyl acetate is a strong hydrogen bond acceptor and glacial acetic acid prevents oxidation of phenols [20]. Finally 1.42% of polyphenols were extracted from 100g of Fenugreek Seeds.

Total phenolic content of PFFS

Gallic acid was used as a standard for deriving calibration curve. From the curve the linear equation was observed and was found to be $y = 0.0008x + 0.0944$, $R^2 = 0.9869$. Based on this equation the TPC in PFFS was found to be 182 µg of GAE/ 5 mg of PFFS.

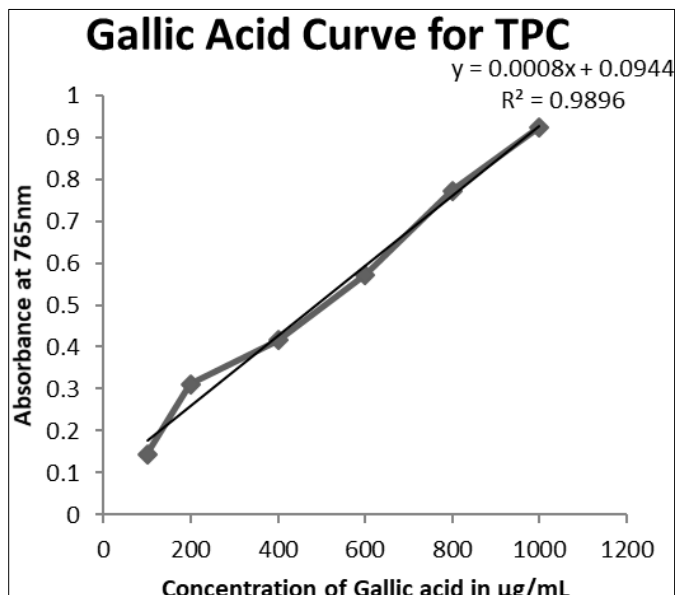


Fig 1: Standard Calibration Curve of Gallic Acid for TPC

Characterization of Probiotic Microbe isolated from Homemade Yogurt

Colony characterization and biochemical test results are given below as table 1 and table 2 respectively.

Table 1: Colony Characterization of Isolated Bacterial Colonies

Colony Characteristics	Results
Shape	Circular
Elevation	Raised
Margin	Entire
Color	Creamy white
Consistency	Mucoid
Surface	Glistening
Density	Opaque
Gram's Nature	Purple Cocobacilli

Table 2: Biochemical test for isolated colony from Homemade Yogurt (+): Positive (-): Negative

	Biochemical test	Results
1	Glucose	Acid (+) Gas (-)
2	sucrose	Acid (+) Gas (-)
3	lactose	Acid (+) Gas (-)
4	maltose	Acid (+) Gas (-)
5	cellobiose	Acid (-) Gas (-)
6	xylose	Acid (-) Gas (+)
7	indole	(-)
8	methyl red	(+)
9	voges Proskauer	(-)
10	citrate utilization	(-)
11	catalase test	(-)
12	oxidase test	(-)
13	growth in 4% NaCl	(-)
14	growth in 6.5% NaCl	(-)
15	nitrate reduction test	(-)

Selected colony was further sub-cultured and enriched in MRS broth and Bacteriological tests were carried out with respect to the Bergey's Manual of systemic bacteriology and Identification was done accordingly. Table 1. enlists the colony characteristics of isolated bacterial species and Table 2. gives results of biochemical tests. The obtained results were studied, compared and concluded using the Bergey's manual of systemic bacteriology. The bacterial strain is a coccobacilli, non motile, non spore forming, gram positive,

sucrose, glucose, lactose and variably a maltose fermenter. It is a catalase, oxidase, voges Proskauer, indole, nitrate reduction test and Citrate test negative, although Methyl Red positive. the salt tolerance is very low too, since it ceased to grow in 4% NaCl. Thus the strain was identified to be a firmicute belonging to the family of Streptococcaceae, genus Lactococcus, species *L. lactis*. and is a sub specie of *L. lactis* called *L. lactis cremoris*

Preparation of Yogurt

L. lactis cremoris isolated from homemade yogurt was used as a starter culture prepare PFFS yogurt. Addition of starter culture helped to obtain a presumably characteristic yogurt. The phenol in the extract was added to influence the aroma and flavor of the yogurt. (Fig.1). The antioxidant activity of yogurt, also, maybe attributed to phenol content of fenugreek seeds.



Fig 1: Yogurt with *Lactococcus lactis cremoris* fortified with Polyphenols of Fenugreek Seeds

Qualitative DPPH Activity of PFFS Yogurt

10 ml of PFFS yogurt was distributed in 1.5mL eppendorf tubes and centrifuged at 10000 rpm for 10 min. The supernatant was collected and evaluated for DPPH activity. Upon addition of DPPH to supernatant there was immediate color change from purple to yellow (Fig.2) suggesting antioxidant activity of PFFS yogurt [21].

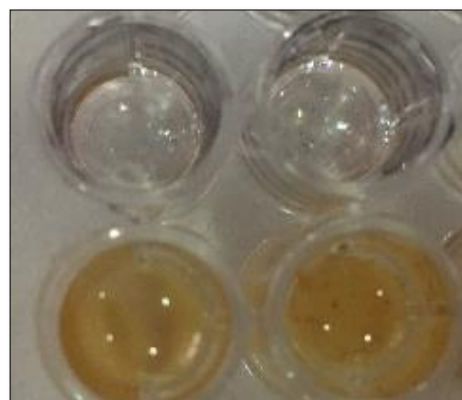


Fig 2: Qualitative DPPH activity of PFFS Yogurt

Conclusion

The polyphenol content from fenugreek seeds was used to prepare homemade yogurt with potent antioxidant properties. Polyphenol fraction from fenugreek seeds showed 182 µg of GAE/ 5 mg of FPPS phenol content. The isolation, characterization and identification of the LAB, revealed the

presence of *Lactococcus lactis cremoris*. The *L. lactis cremoris* was used to prepare as a starter culture to prepare yogurt. Polyphenolic extract of Fenugreek seeds was utilized to add flavor and aroma to the yogurt. The phenolic extract influenced the yogurt's antioxidant activity.²² 20% of seed extract was used which gave immediate antioxidant activity upon addition of DPPH. In addition to the flavor, the fenugreek seed extract is also a potent candidate for extraction of polyphenols, its related antioxidant activity and its use in preparation of Yogurt.

Conflict of Interest

The authors declare no conflict of interest.

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