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Quorum quenching activity of Green tea polyphenols: An *in-vitro* study to control enzyme-based food spoilage

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Abstract
Food, being nutritionally rich, can harbor a variety of food spoilage causing microorganisms. Food spoilage microorganisms involve quorum sensing (QS) mechanism for expression of spoilage causing enzymes. Therefore, disruption of quorum sensing could be an effective strategy to control microbial food spoilage. Green tea polyphenols (GTP) are reported to have quorum quenching activity against various microorganisms. Hence, the present study was aimed at exploring the potential of GTP as a quorum quencher against the extracellular protease and lipase enzymes of one of the representatives of food spoilage microorganisms (*Bacillus subtilis*). Quorum quenching property of GTP was confirmed using biosensor strain of *Chromobacterium violaceum* MCC 2290. These polyphenols were observed to inhibit QS controlled protease and lipase enzyme activity of *B. subtilis* at sub-inhibitory concentrations suggesting the role of GTP as a novel biopreservative or active packaging ingredient to improve the shelf life of the food.

Keywords: green tea polyphenols, enzymes, food spoilage, quorum quencher, shelf life

1. Introduction
Food spoilage is a serious global problem manifested as visible growth of microbes, changes in food texture, taste, and odour which results into rejection of food [1]. Spoilage due to microbial interaction is a major concern in various food industries as it causes significant economic losses and could have serious public health consequences. Several spoilage causing microorganisms secrete extracellular enzymes which could be proteolytic, pectinolytic, and lipolytic resulting in metabolic end products linked with food spoilage. Therefore, control of enzyme based microbial food spoilage is considered to be of great importance for maintaining the quality of food.
In the current scenario, various chemical preservatives which are being used to increase the shelf life of food have been banned due to the emergence of resistance, potential toxicity and adverse health effects [2, 3, 4, 5]. This has prompted researchers to search for novel greener alternatives. On the other hand, in recent years, Quorum sensing (QS) signaling molecules have been detected in spoiled food products which have paved a new way to study the process of food spoilage.
QS is a cell density dependent mechanism widely employed by a diverse group of bacteria including food spoilage causing microorganisms to modulate the gene expression essential for survival and virulence [6]. Several proteolytic and lipolytic activities which are associated with food deterioration have been reported to be regulated by QS. Hence, disrupting the QS circuit can be an effective strategy to control spoilage causing microbial gene expression and improving the shelf life of food [7, 8]. Quorum Quenching (QQ) compounds can be used as novel biopreservatives or active packaging materials which could hamper the virulence of food spoilage microbes to maintain the quality of all types of food and food products. The present study is therefore aimed at exploring alternative sources of safe, Effective and acceptable natural biopreservatives as novel greener alternatives to chemical preservatives.
Reports on QQ property of clove oil, *Mangifera indica L.*, grape juice, cinnamon etc. are already available in literature [9]. Green tea mainly contains polyphenols which have been shown to possess anti-inflammatory, antiarthritic, antiangiogenic, antioxidative, neuroprotective as well as antimicrobial [10]. Extract of green tea can modulate the QS of *Pseudomonas aeruginosa* [11]. However there are hardly any reports stating the effects of QQ of polyphenols from green tea on other food spoilage causing organisms. Hence this research aims at evaluating the potential of polyphenolic content of Green tea as a QQ in context of *Bacillus subtilis*, one of the major food spoilage causing organism in order to increase the shelf life of food. Furthermore, in the present study we have also made an attempt to explore the quorum quenching property of Green tea polyphenols against enzymes produced by food spoilage causing microorganisms. The findings of this research work will enable us to unravel a novel application of polyphenols as biopreservative as well as active packaging material in turn enhancing the shelf life of food but, with lesser side effects as compared to chemical preservatives.

2. Materials and Methods
All media, chemicals (AR grade) and reagents were purchased from HiMedia®, Laboratories, Mumbai.

2.1 Bacterial strains and culturing conditions
Biosensor strain (*Chromobacterium violaceum MCC 2290*) was purchased from NCCS and maintained on Nutrient agar at 26± 1℃. *B. subtilis* was used as one of the representatives of food spoilage microorganisms, obtained from Microbiology laboratory culture collection, The Institute of Science, Mumbai and maintained on Nutrient Agar at 26± 1℃.

2.2 Extraction of total polyphenols from Green tea

Green tea was purchased from a local supermarket in Mumbai. Leaves of Green Tea were washed with sterile distilled water, dried and pulverized to a fine powder. Polyphenols were extracted using two solvent systems viz; system A containing only 80% (v/v) methanol whereas system B containing a mixture of 80% (v/v) each of methanol, acetone and ethanol (2:1:1). Dried powder of green tea (10 g) was mixed with both the solvent systems respectively and the polyphenols were extracted using maceration technique [12]. The resulting mixture was filtered using Whatman filter paper no.1 and the filtrate was used to determine the total polyphenol content. Total polyphenolic content determined using the two solvent systems was compared and analyzed.

2.3 Quantitative assay of GTP

The total polyphenolic content of the extracts was analyzed spectrophotometrically using Folin-Ciocalteu (FC) Method[13] with slight modification. Gallic acid was used as standard; linear range of calibration curve being 10-100 µg/ml. GTP (0.01% (v/v) in D/W) mixed with FC reagent and 7% (w/v) Na_2CO_3 was used for constructing the calibration curve. The absorbance was measured after 30 min at a wavelength 765 nm. The total phenolic content of the extract was expressed in terms of mg of Gallic acid equivalent/ml of sample. The extract showing a higher polyphenolic content among the two systems used for study was for further analysis.

2.4 FTIR analysis of GTP:

FTIR analysis of GTP was carried out using the KBr Pellet Method and the spectra was further analyzed.

2.5 Antimicrobial assay for GTP

Antimicrobial activity of GTP was investigated against *B. subtilis*, and *C. violaceum* MCC2290 using Agar Well Diffusion Method [14]. Overnight grown (10^8 cells/ml) culture of test microorganisms was separately seeded into sterile molten Mueller Hinton Agar taken in sterile petri plates. Wells (diameter 7mm) were bored into the solidified agar medium using a sterile cork borer and approximately 50- 100 µl of undiluted extracts were added to the wells. The plates were incubated at 26± 1℃ for 24 h. The antibacterial activity was assessed by measuring the diameter of the inhibition zone formed around the wells.

2.6 Determination of Minimum Inhibitory Concentration (MIC) of GTP (Macrobroth Dilution Method)

For determining MIC value of GTP, 3.2 % (w/v) of GTP was taken as an initial concentration (calculated after extraction) to make two-fold dilutions of the GTP (up to 0.003% (w/v)) using St. Nutrient broth as a diluent to give a total volume of 2 ml. The test culture suspension was adjusted to 1×10^8 cells/ml. To the test tubes containing St. twofold diluted extract, test microorganisms were added and incubated at 26± 1℃ for 24 h. Tube containing St. Nutrient broth without the extract but, with the test organism was included as a positive control whereas a tube containing St. Nutrient broth with the extract but without the test organism was included as a negative control. The results were interpreted to determine the MIC in terms of the absorbance observed at a wavelength of 620 nm in comparison with the controls. The sub-MIC concentration was selected to estimate anti-quorum sensing activity.

2.7 Biosensor assay using *C. violaceum* MCC 2290 as a sensor organism

Standard Agar Well Diffusion Assay was used to detect anti-QS activity of polyphenolic extract using an overnight grown biomonitor strain of *C. violaceum* MCC 2290. Molten St. Nutrient Agar was seeded with overnight culture of *C. violaceum* MCC 2290 and overlaid over the surface of a pre-solidified basal St. Nutrient Agar plate. Wells (diameter 7mm) were bored into the solidified agar medium using a sterile cork borer and GTP (0.01% w/v) was added to the wells. Plates were incubated overnight at 26± 1°C and examined for violacein production. QS inhibition was detected in form of a colorless, opaque halo around the well.

2.8 Protease assay

Proteolytic enzymes act on peptide linkages which results in the release of free amino acids as determined by the Folin Lowry method. To investigate the effect of GTP on enzyme activity, protease assay was performed [15] with slight modification. St. casein broth with GTP (0.01% (w/v)) was inoculated with *B. subtilis*. The tube was centrifuged at 5000 x g for 10 min and the supernatant was used as a crude enzyme source. St. casein broth without GTP inoculated with *B. subtilis* was maintained as positive control.

Reaction mixture was setup by adding different dilutions of crude enzyme (1% (v/v), 0.1% (v/v) and 0.001% (v/v) respectively in st. Phosphate Buffered Saline; pH 7.2) to casein (1% (w/v)) and incubating the tubes at 50°C for 30 min followed by addition of 5% TCA to precipitate the undigested protein and centrifugation at 5000 x g for 5 min. The supernatant so obtained was used to estimate the concentration of free amino acids with reference to a standard curve of tyrosine (range 20-200 µg/ml) using the Folin Lowry method [16]. The absorbance was recorded at a wavelength of 660 nm. The standard curve of tyrosine so obtained was used to calculate the protease units.

2.9 Lipase assay

GTP (0.01% (w/v)) was added to the mineral medium inoculated with *B. subtilis* and incubated at 26± 1℃ for 24 h on shaker. A control containing phosphate buffered saline (pH 7.2) in place of the GTP was maintained and all the tubes were centrifuged at 5000 rpm for 10 min. The resulting supernatant was used as a crude enzyme.

For enzyme assay, titration cocktail containing 95% (v/v) ethanol with added phenolphthalein indicator was used to quench the reactivity of subsamples of the reaction mixture. Olive oil- Tween 80 emulsion (5% (v/v) in st. Phosphate Buffered Saline pH 7.2) was used as substrate for lipase enzyme and pre-incubated at 37ºC for 15 min. To this crude enzyme was added to initiate lipase activity. Aliquot of this reaction mixture was taken at 0, 5, 10, 15, 20 and 25 min respectively and transferred into titration cocktail and titrated with 0.05N NaOH until appearance of pink colour. In an Erlenmeyer flask containing titration cocktail, phosphate buffered olive oil- Tween 80 emulsion substrate titrated with 0.05 N NaOH served as a reagent blank. Reaction progress curve was plotted as quantity of fatty acid liberated vs time of reaction.

3. Results & Discussion

3.1 Extraction of GTP

Since the polyphenols are heat sensitive, degrading at high temperatures approximately above 50°C [17], a simple maceration technique was carried out in order to prevent loss of polyphenols during the extraction process. As there is no information available in literature regarding the most suitable solvent to be used for extraction of Green tea, three different organic solvents (viz. Methanol, ethanol, and acetone) were selected for this study.

Polyphenols have a low solubility in absolute organic solvents due to strengthening of the hydrogen bonds between polyphenols and protein. Addition of water has been found to weaken such interaction thereby increasing the solubility of polyphenols. Therefore, a minimum of 20% (v/v) water was added to each solvent system [18]. Furthermore, as polyphenols are extracted more efficiently in 80% (v/v) methanol and mixture of solvents [19, 20, 21] in the present study, two solvent systems were used for extraction as mentioned earlier.

On extraction, a comparative analysis of the polyphenolic content determined using both the solvent systems was carried out.

3.2 Quantitative estimation of GTP

Total Polyphenolic Content (TPC) of Green tea extract in solvent system 'A' consisting of mixture of 80% (v/v) each of methanol, ethanol and acetone (2:1:1) allowed better extraction of polyphenolic compounds than the solvent system 'B' consisting of only 80% (v/v) methanol (Table 1). More yield of GTP viz. 3.2% (w/v) was obtained using solvent system 'A' in comparison with that using solvent system 'B' viz. (1.24% (w/v)). There are reports indicating methanol to be more efficient in extraction of lower molecular weight polyphenols, whereas aqueous acetone is found to be good for extraction of higher molecular weight flavanols. Ethanol has been known as a good solvent for polyphenol extraction and is safe for human consumption [22, 23]. However, there is hardly any literature available indicating a use of a mixture of solvents for effective extraction. Therefore, we made an attempt to exploit the properties of these solvents in combination and check whether the synergistic action of these could enable better extraction and higher yields of polyphenols. As per our expectation, using solvent system 'A', a synergistic action of methanol, ethanol and acetone did enable a better extraction of all lower as well as higher molecular weight polyphenols from the extracts resulting in a high yield of total polyphenolic content. Whereas, solvent system 'B' consisting of only methanol must have enabled extraction of only lower molecular weight polyphenols, resulting in lower observed yield of total polyphenols.

Since solvent system 'A' resulted in a better extraction of polyphenols from leaves of Green tea as compared to solvent system 'B', further analysis was carried out using total polyphenols extracted using solvent system 'A'.

Table 1: Total Polyphenolic content of Green tea extract

Sample	Solvent system used for extraction	Total polyphenolic content in Gallic acid equivalent (% GAE(w/v)
Green tea	A. Mixture of Methanol, Ethanol & Acetone (80% v/v each) in 2:1:1 ratio	3.2%
	B. 80% v/v Methanol	1.24%

3.3 FTIR spectra of GTP

Fourier Transform Infrared Spectrophotometer (FTIR) is perhaps the most powerful tool that identifies the types of chemical bonds present in compounds [24]. Chemical compounds were identified by analyzing the functional groups using peak value from the Green tea extract (Table 2). FTIR spectra showed characteristics of O-H as well as C=O stretch near 3300 cm^{-1}, 1637.70 cm^{-1} which confirmed the presence of polyphenolic compounds (Figure 1).

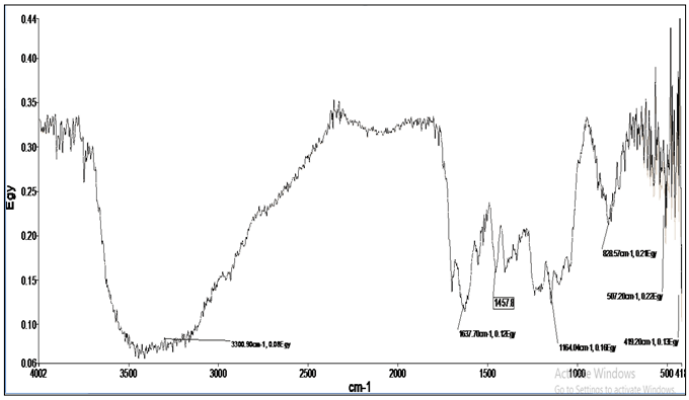


Fig 1: FTIR spectra of Green Tea extract.

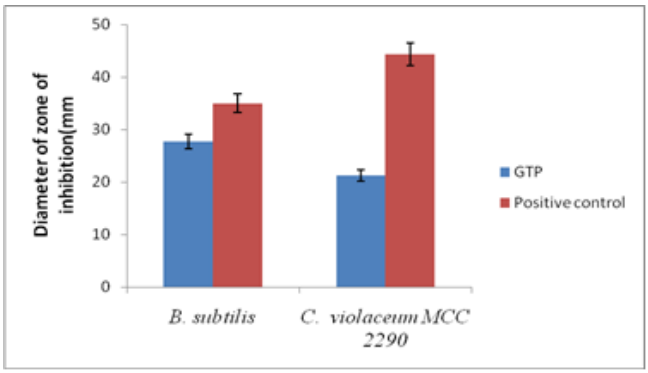
Table 2: FTIR spectral peak values and functional groups obtained for the Green Tea extract.

Peak values (wave number cm^{-1})	Functional groups	Chemical compound
3300	O-H stretch, H- Bonded	Phenols, alcohols
1637.70	C=O stretch	Flavonoids, polyphenols, catechin
1164.04	C-O-C stretch	Esters
828.57	C-H stretch	Alkenes

3.4 Antimicrobial activity of GTP

It has been known that in order to evaluate QS inhibitory potential of any compound, it needs to be tested at sub inhibitory concentration against test microorganism/s. Green tea contains polyphenols, flavonoids, and tannins, which are well known antimicrobial compounds [25, 26]. In the present study an attempt was made to determine the antimicrobial activity of GTP against test microorganisms viz. *B. subtilis, and C. violaceum* MCC 2290 using Agar Well Diffusion Method.

Results of antimicrobial activity against test microorganisms were measured in terms of diameter of the inhibition zone formed around the well containing the extracts. The antimicrobial studies showed that GTP had inhibitory effect against both test microorganisms. Maximum Inhibitory effect was observed for *B. subtilis* (29 mm) followed by; *C. violaceum* MCC 2290 (21 mm) (Figure 2). Observed antimicrobial activity of GTP was close to the activity recorded for control antibiotic (ampicillin 100 µg/ml).



Key: GTP- Green Tea Polyphenols; Positive control – ampicillin 100µg/ml.

Fig 2: Antimicrobial activity of extracts by Agar Well Method.

3.5 MIC analysis of GTP:

QS inhibitory effects occur below the MIC (if the inhibitor exhibits antimicrobial property) [27]. Hence, the MIC of the GTP for the test microorganisms was determined using Broth Microdilution Assay.

MIC of GTP for *B. subtilis* and *C. violaceum* MCC 2290, was found to be 0.025% (w/v) of total polyphenol (Table 3). Therefore, the sub-inhibitory concentration of GTP was selected as 0.01% (w/v) for ease of experimentation.

Table 3: Minimum inhibitory concentrations of GTP against selected microorganisms

Concentration of GTP %(w/v)	*Bacillus subtilis*	*Chromobacterium violaceum* MCC 2290
3.2	-	-
1.6	-	-
0.8	-	-
0.4	-	-
0.2	-	-
0.1	-	-
0.05	-	-
0.025	-	-
0.0125	+	+
0.00625	+	+
0.00313	+	+

Key: no growth; +: growth in the form of turbidity.

3.6 Inhibition of violacein production in *C. violaceum* MCC2290

C. violaceum, a Gram-negative bacterium produces the pigment violacein in response to QS regulated gene expression [28]. Hence in the present study *C. violaceum* MCC 2290 (purchased from NCCS, Pune) used as an indicator organism to detect the QQ property of GTP. QS inhibition is generally detected by colorless, opaque halo around the well containing QS inhibitors.

It was found that GTP at its sub-inhibitory concentration (0.01% (w/v)) showed a turbid zone of violacein inhibition around the wells, circumscribed by purple pigmented bacterial growth in the remaining part of the plate indicating anti-QS activity of GTP (Figure 3).

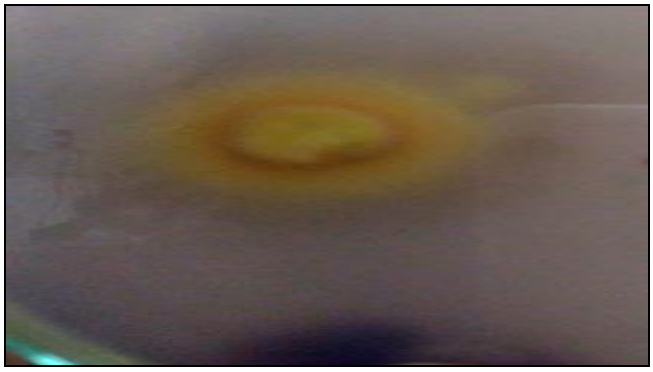


Fig 3: Screening of quorum quenching property of GTP

3.7 GTP inhibits Proteolytic activity of *B. subtilis*

Protease, an enzyme produced by most of the food spoilage microorganisms hydrolyses the proteins leading to release of amino acid and small peptides which cause bitter flavor, reduction of shelf life and over tenderness in foods (such as in eggs, fish, and flour)[29]. Research has indicated that in many microorganisms, genes that code for several enzymes are quorum sensing regulated. Hence, blocking microbial QS systems could help prevent QS controlled food spoilage causing enzyme production.

Therefore, in the present study, an attempt was made to find out the QS inhibitory effect of sub-inhibitory concentration of (0.01% (w/v)) GTP on the production of protease enzyme. *B. subtilis* which is known to produce protease enzyme, is QS regulated hence, it is used as test microorganism in the present study [30].

In total protease assay, the amount of amino acid released was determined from the calibration curve of tyrosine (range: 20-100 µg/ml) and enzyme activity was expressed as µg of amino acid released/ml/min. An inhibition in protease production was observed for *B. subtilis* when treated with GTP which showed 70.55% inhibition of protease production as compared with positive control (Figure 4). A reduction in enzyme activity is an indication of quorum sensing inhibition. In fact, it can be concluded that QS inhibitors has a direct effect on the expression of spoilage causing phenotype.

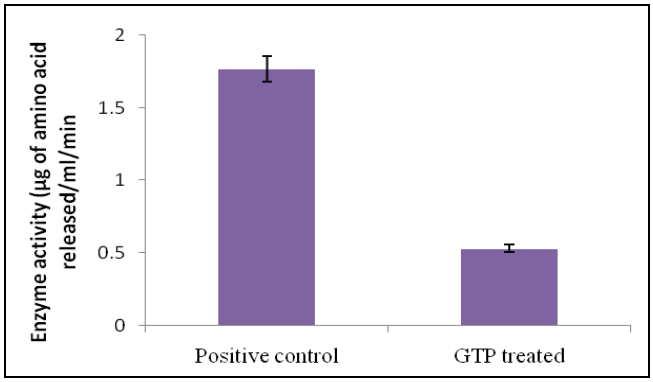


Fig 4: Effect of GTP (0.01% (w/v)) on protease activity of *Bacillus subtilis.*

3.8 GTP inhibits lipolytic activity of *B. subtilis*

As stated earlier various enzymes play an important role in food deterioration. Lipase is one such enzyme produced by various spoilage causing microorganisms. Lipase hydrolyzes fats present in various food types such as milk, meat and so on, to release short-chain fatty acids and cause rancidity and off-flavors. In many cases lipase production is also QS regulated as in case of *B. subtilis* [30].

Hence, in present study, in order to further confirm effect of GTP as a QS inhibitors on QS controlled enzyme production, lipase assay of *B. subtilis* which is known to have QS regulated lipase production was performed with treatment of GTP at sub inhibitory concentration (0.01% (w/v)), and compared with control without treatment. Titrimetric method was performed to estimate lipase activity. In titrimetric procedure, the native substance i.e. triacylglycerols are hydrolyzed to yield fatty acids. After quenching the reactivity of the subsamples withdrawn at regular intervals, by addition of ethanol, the amount of fatty acids released therein can be determined by direct titration with NaOH using phenolphthalein indicator. Reaction progress curve was plotted as the quantity of fatty acid liberated vs time of reaction [31]. The results revealed that lipase enzyme activity of *B. subtilis* was significantly reduced when treated with GTP as compared to control (Figure 5). This investigation further confirms that QS inhibitors can be useful in reducing enzyme production which is under control of the QS system, which in turn helps in delaying food spoilage.

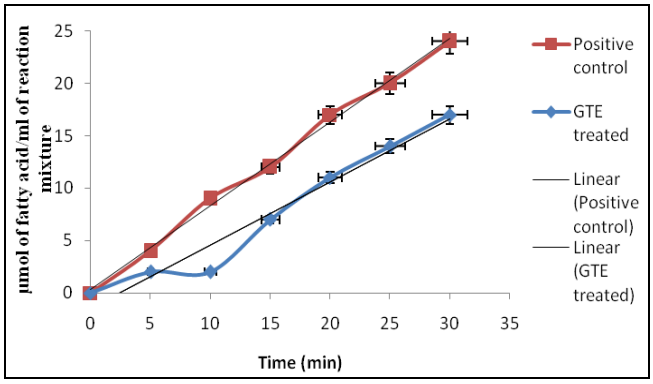


Fig 5: Effect of GTP (0.01% (w/v)) on Lipase activity of Bacillus subtilis.

4. Conclusion

The present study demonstrates the QS mediated enzyme inhibitory potential of Green Tea Polyphenols. These polyphenols significantly reduces the protease as well as lipase activities of *Bacillus subtilis* which positively confirms their use as a Quorum quencher to mitigate enzyme based food spoilage. Though further studies to explore stability parameters of GTP as well as in vivo effect of GTP as a QQ may be required to be carried out, they would help to pave the way for Green Tea Polyphenols as a novel biopreservative for prevention of food spoilage.

5. Acknowledgment

We would like to express our sincere gratitude to the National Centre for Microbial Resources, Pune for providing *Chromobacterium violaceum MCC 2290* culture.

6. Conflict of interest

The authors have declared no conflict of interest.

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