SPENT GREEN TEA EXTRACT BASED FUNGAL GROWTH MEDIA DEVELOPMENT

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Abstract. We devised a media from Spent Green Tea Extract (SGTE) and tested its proficiency using two yeast strains; *Saccharomyces cerevisiae* ATCC 9763 and *Candida albicans* ATCC 10231, and two filamentous fungal strains *Aspergillus brasilensis* ATCC 16404 and *Penicillium chrysogenum* MTCC 5108. No morphological and microscopic differences were observed in SGTE agar medium, when compared with Pottato Dextrose Agar (PDA) media. The absorbance for cell densities in SGTE broth was found to similar in comparison to PDA. Crude SGTE was evaluated for its Minimal Inhibitory Concentration (MIC) and a concentration of 1 mg/mL was found to be effective for *Bacillus* sp. PVMX4 and a concentration of 7 mg/mL was effective for *E. coli* ATCC 8739. Bactericidal assay of SGTEA revealed that the medium selectively supported the growth of fungi, wherein PDA failed to inhibit the growth of heterotrophic bacteria from soil samples.

Key words: Spent green tea extract; fungal growth media; caffeine; MIC; PDA.

INTRODUCTION

In laboratory, fungal strains are isolated and maintained on specific culture medium for further microscopic, biochemical and physiological characterization [27]. To carry out laboratory based fungal studies, suitable growth media has been devised previously, such as commonly used media including modified Malt Extract Agar-(MEA) [28], Potato Dextrose Agar-PDA, modified Sabouraud dextrose Agar-SDA [2] from Sabouraud agar [23].

PDA is widely used in the examination of yeast and molds present in foods and for performing plate count technique for fungal identification and also used for differentiating dermatophytes and maintenance of their stock cultures [31]. Griffith et al. (2007) [11] reported reduction in pigmentation of fungi, when grown in batch cultures of Potato Dextrose Broth (PDB) due to copper deficiency and recommended the addition of 1000 ng/mL of copper to the PDA. Moreover, rising prices of potato and the possible reduction of potato for the non-edible based usages has forced us to look for other possible alternatives.

We devised a medium using Spent Green Tea Extract (SGTE) for supporting as an alternative to the PDA, to support fungal and to inhibit the bacterial growth.In our research, we surveyed for a suitable compound that could replenish our needs for devising a selective fungal media. For this we come with an idea that in restaurants and houses, we noted that the garbage containing used green tea bags were surrounded by fungal spores and hyphae. Green tea leaves contain polyphenols (36%), methyxanthines(3.5%), amino acids (4%), organic acids (1.3%), carotenoids (<0.1%), volatiles (0.1%), Carbohydrates (2.5%), protein (15%), lignin (6.5%), lipids (25), ash (5%), chlorophyll and other substances (0.5%) [10]. Xu et al. (2011) [32] reported that the dominant genera *Eurotium, Debaryomyces* and *Aspergillus* are beneficial fungi associated with the fermentation of Fuzhuan brick-tea. Green tea has a variety of secondary metabolites such as catechin, caffeine, theanine and saponin that are important for human welfare [33, 34] . These compounds in green tea are well known for their broad spectrum of biological activities such as antibacterial, antioxidant, antifungal, and antitumor functions [8, 24].

MATERIALS AND METHODS

Fungal strains and growth conditions

Two yeast strains (*Saccharomyces cerevisiae* ATCC 9763 and *Candida albicans* ATCC 10231) and two filamentous fungi [*Aspergillus brasilensis** ATCC 16404 (*syn *A. niger*) and *Penicillium chrysogenum* MTCC 5108] were used for the present study were obtained from the Stock was made for the cultures stated according to the revised methods of McGinnis et al. (1974) [16]. ATCC strains were obtained from the American Type Culture Collection, USA and MTCC strains were obtained from Microbial Type Culture Collection, Chandigarh, India.

SGTE Preparation

Spent green leaves were ground in a blender to fine powder and approximately 2 g of the dried spent green tea powder pepared by drying the filterd aquous tea extract in vaccum dryer at 50 °C, 2 g of Dextrose, 1.5 g of agar was added to a sterile conical flask and suspended in 100 mL distilled water. No manual pH adjustment for medium was required since the pH of the medium at this stage was found to be 5.6 ± 0.2. The medium was sterilized by autoclaving at 1.5 kg/cm², 121 °C for 15 min and no significant changes in the medium pH was observed at this stage and the medium was plated on sterile Petri dishes.

Stock culture preparation

From stock culture, yeasts were streaked on SGTEA (spent green tea extract agar) and PDA (potato

dextrose agar) (Himedia, India) containing ($g \cdot L^{-1}$) Potatoes, infusion from 200, Dextrose 20, Agar 15 and final pH 5.6±0.2 (at 25°C) plates aseptically, whereas filamentous fungi were inoculated aseptically on the SGTEA and PDA plates by stabbing using sterile L-shaped nichrome inoculation loop and the plates were incubated at 25 °C for 48-72 h. Filamentous fungi containing plates were incubated at for two to three days. Macroscopic and microscopic characteristics of the all the four strains were recorded using a compound binocular microscope at 40× magnification.

The method described by Chapman et al. (2015) [3] was used for obtaining yeast cell densities. After incubation in media for 16 h, the optical densities of the yeast cultures were observed using a plate reader (Biorad) at 600 nm to compare the cell growth in each medium.

Growth in SGTEB

Growth of filamentous fungi in SGTEB (spent green tea broth) and PDB with the same constituents, but without agar was carried out according to the methods of Meletiadis et al. (2001) [17] with required modifications. Briefly, spores were collected using a cotton swab from the fungal stock cultures and suspended in 0.1% Tween 80. The suspensions were adjusted to $2x10^4$ spores/mL by counting the cells using a haemocytometer. Fungal suspension of 45 µL containing 0.1% Tween 80 was inoculated into 45 µL of double strength SGTEB and PDB media in 96-well microtitre plates. The plates were sealed and incubated at 30±2 °C for 36 h inside a plate reader. The optical density (O.D.) was recorded at 450 nm for each well at 12 h, 24 h and 36 h without shaking.

MIC for SGTE

Minimum inhibitory concentration (MIC) for SGTE was carried out according to the methods of Elshikh et al. (2016) [7]. The activity of SGTE over gram-positive bacteria (*Bacillus* sp. PVMX4; Gen Bank Acc No. KJ921622) and a gram-negative bacterium (*E. coli* ATCC 8739) were studied.

The resazurin (7-hydroxy-10-oxidophenoxazin-10-ium-3-one) solution at 0.04 % concentration was prepared by dissolving 40 mg of resazurin in 100 mL of sterile distilled water and the contents were filter sterilized. Plates for MIC assay were prepared under aseptic conditions. Two sterile 96 well plates were labeled separately for MIC assay for *Bacillus* sp. and *E. coli* strains and the experiments were performed individually. Test material of 100 µL volume (a concentration of 0.05-10 mg/mL of crude extract in sterile water) was pipetted to the first row of wells denoted as A-E (replicates). Plates were wrapped loosely with a film (to prevent dehydration of bacteria) and were incubated at 37 °C for 12-18 h. After incubation, the plates were observed for color change, and the pink color indicated growth and blue color shows growth inhibition. The lowest concentration at which color change occurred was taken as the MIC value.

Soil sample from Garden soil belonging to fertile clay-loam soil samples with pH 5.8 and organic matter content of 2.86% was collected, and one gram of the sample was weighed, and corresponding serial dilutions were performed in a sterile phosphate buffered saline-PBS (pH-7.4) up to 10^{-6} dilution.

RESULTS

Growth comparison- macroscopic and microscopic Observations

In our present study growth of fungal strains observed in SGTEA was comparable to that of PDA plates and no observable morphological variations could be noticed among these two media (Fig. 1, 2). The colony characters of all the four strains on SGTEA medium are discussed below, which was equivalent to the colonies developed on PDA medium. At the optimal temperature of 37 °C, colonies of *S. cerevisiae* appear medium sized, flat, smooth, moist, and creamy in color. Microscopic morphology based on wet mount reveals ellipsoidal budding that reveals yeast-like cells (blastoconidia) are unicellular and simple staining with safranin revealed pink-colored cells. For *C. albicans* macroscopic and morphological observation of at 37 °C, reveal colonies are medium sized, white with soft consistency and surface and margins of the colonies are observed to be smooth. Microscopic morphology of wet mount revealed budding of cells of ellipsoidal in shape. Simple staining with methylene blue revealed purple-colored cells (data not shown).

Macroscopic morphological observation of *A. brasilensis* colonies grown at 25 °C reveal that these colonies were initially white, and changed to black with conidial production. Microscopic observation of *A. brasilensis* reveal that the conidiophores are long, smooth, hyaline, and dark at the apex and terminating in a globose vesicle. Conidia are brown to black, rough, and globose. Macroscopic morphology of *P. chrysogenum* grown at 25 °C, reveal that the colonies are rapid in growth, flat, filamentous and velvet in texture. Colonies are grey green to olive grey in color. Microscopic morphology of *P. chrysogenum* reveals hyphae are septate, with branched conidiophores. Metulae and phialides are visible on the tip of conidiophores.

The optical densities for yeast and filamentous fungal cultures in two different media were observed at OD 600 nm and at 450 nm, respectively. From the triplicates, the mean values of outcomes were noted and are plotted as bar graph (Fig. 3), to show the absorbance peak for four different cultures grown on SGTEB and PDB, respectively. From the Fig. 3, it was notable that the absorbance value for *S. cerevisiae* (0.9:0.7 for SGTEB: PDB), *C. albicans* (1.3:1.6 for SGTEB: PDB), *A. brasilensis* (1.30:1.1 for SGTEB: PDB) and *P. chrysogenum* (1.0:1.2 for SGTEB:PDB) were found to be statistically indifferentiable. From the

graph O.D values plotted for yeast and filamentous fungi, it was clear that there was no significant difference in growth in SGTEB, when compared with PDB; which was generally used as a culture medium for fungi. Therefore, SGTEB supported the growth of fungal cultures just as same as PDB.

In the present study MIC of SGTE against *Bacillus* sp. PVMX4 and *E. coli* ATCC 8739 strains in microtitre plates containing the media along with resazurin was evaluated with the color changes observed at 12 h. It was observed that a concentration of 1 mg/mL of the SGTE crude extract is necessary to kill gram positive *Bacillus* sp. and a concentration of 7 mg/mL is required to kill gram negative *E. coli* (Data not shown). Growth was observed on PDA plates; cultured with fertile clay-loam soil samples with pH 5.8 and organic matter content of 2.86% showed diverse bacterial colonies in addition to the fungal colonies in these plates (Fig. 4). Different bacterial colonies were appeared on PDA plates in which we did not add the antibiotics. Only fungal colonies are observed in SGTEA plates and bacterial growth was not observed on these plates. From this, SGTEA medium shows its selectiveness as a fungal growth medium.

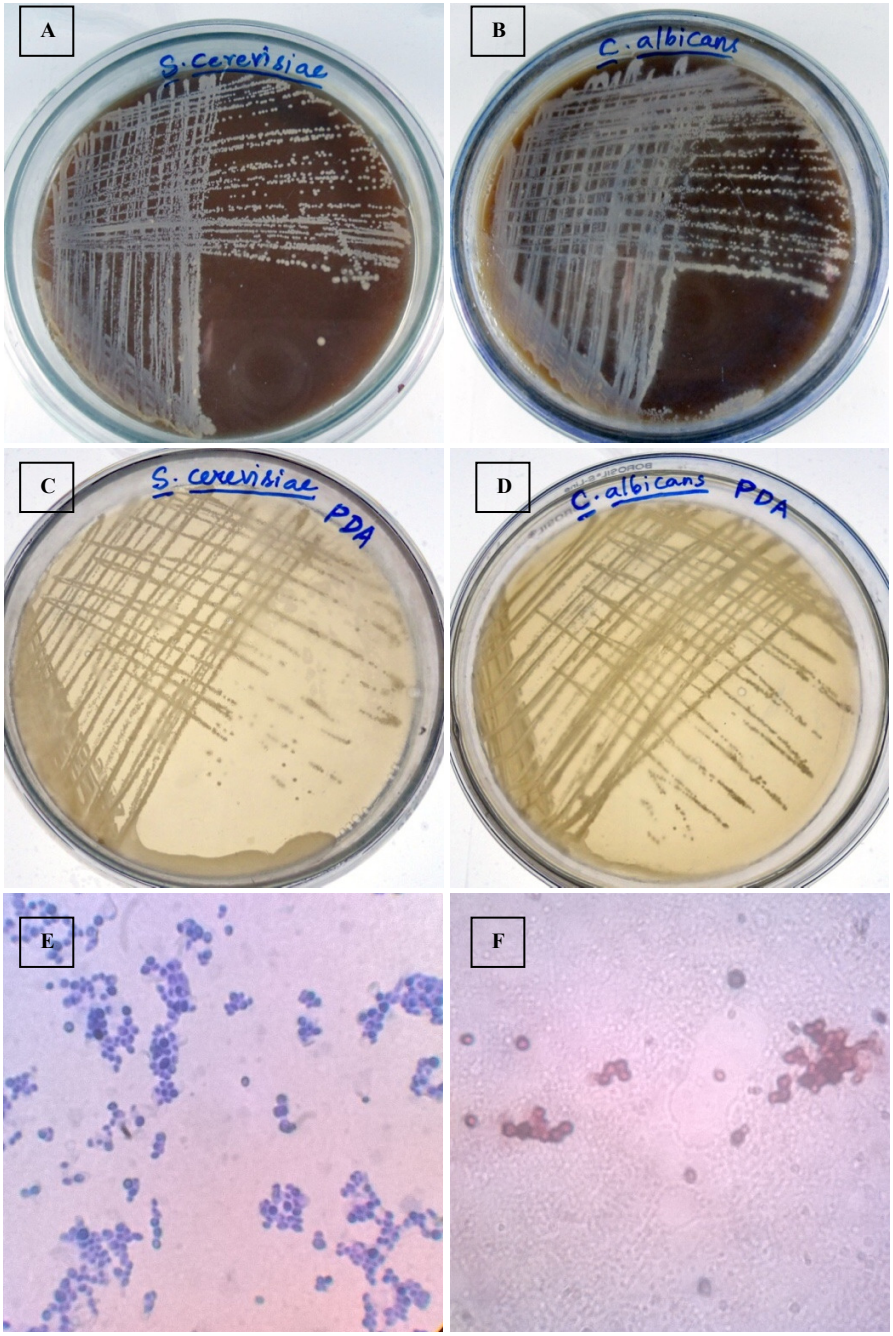

Figure 1. Morphological and microscopic characteristics of *S. cerevisiae* and *C. albicans* strains grown in SGTEA and PDA medium at 37 ˚C. Colony morphology of (A) *S. cerevisae* (B) *C. albicans* in SGTEA and colony morphology of (C) *S. cerevisiae* and (D) *C. albicans* in PDA.

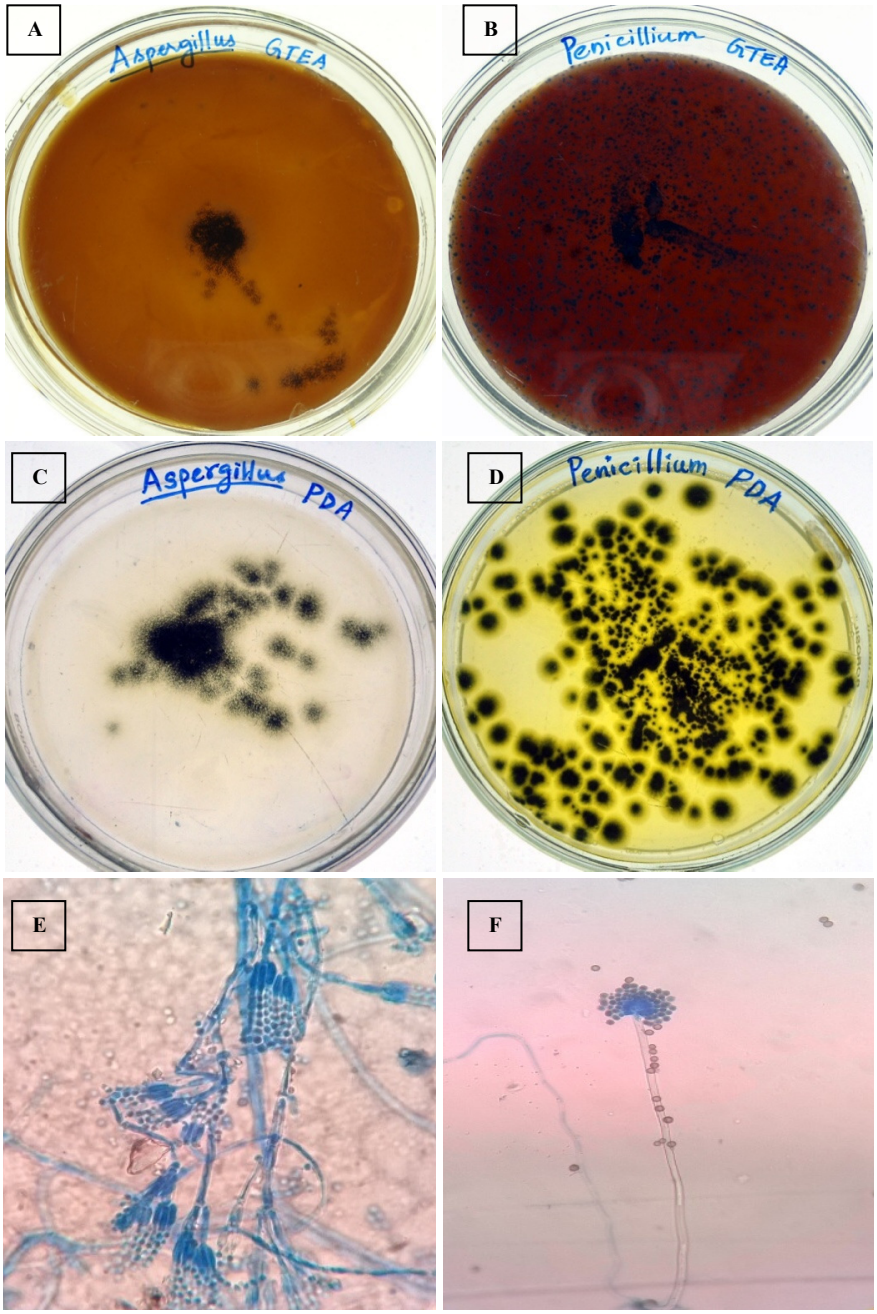

Figure 2. Morphological and microscopic characteristics of *A. brasilensis* and *P. chrysogenum* strains grown in SGTEA and PDA medium at 25 ºC. Colony morphology of (A) *A. brasilensis* (B) *P. chrysogenum* in SGTEA and colony morphology of (C) *A. brasilensis* and (D) *P. chrysogenum* in PDA.

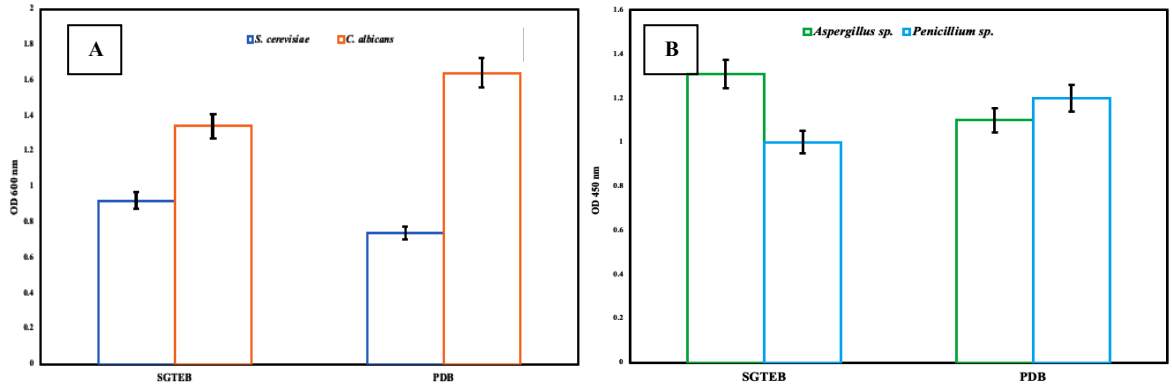

Figure 3. Graph showing Optical Density (O.D.) values of yeast and filamentous fungi read at 600 nm and 450 nm respectively in SGTEB and PDB media. O.D. values represent the amount of cell densities of yeast and filamentous fungi in each medium after a time period of 18 h. Values are mean of nine replications, bar values followed by different letters differed significantly at $P \geq 0.05$ according to *t* test.

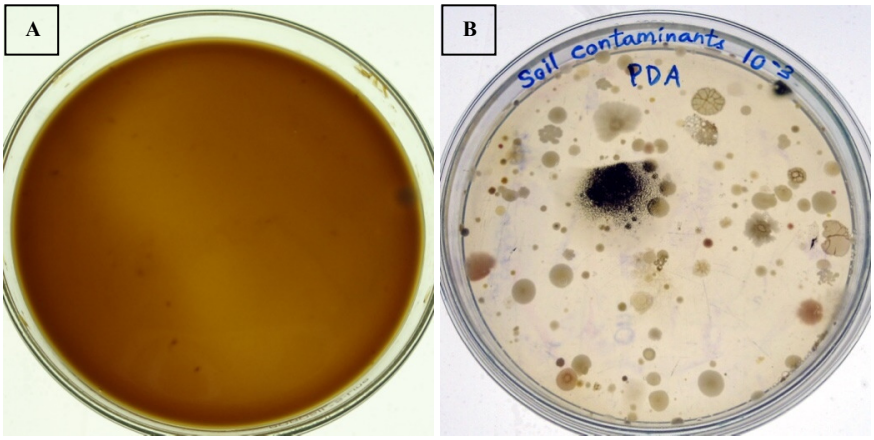

Figure 4. Bacteriostatic effect of A) SGTEA in comparison with B) PDA.

DISSCUSSION

SGTEA used in our study supported the growth of yeast and filamentous fungi due to possible presence of macronutrients and micronutrients in it. Harbowy et al. (1997) [13] showed that green tea contained simple sugars (11%) and other polysaccharides such as cellulose, pectin and lignin (4%), amino acids (3%), peptides/proteins (6%), caffeine (3%), lipids (3%), potassium (5%), other minerals (such as magnesium, manganese, ferrous and phosphorous in high amount (5%), whereas copper, nickel, sodium, molybdenum, zinc in trace amounts). Ramdani et al. (2013) [22] reported that the green tea leaves have crude protein (240/kg sample), ash (61.8/kg sample), total phenols (231/kg sample), total tannins (204/kg sample), and total saponins (276/kg sample). In addition to these these authors also reported the presence of Mn, Na and Cu in green tea leaves. Wang et al. (2014) [30] reported the presence of water soluble polysaccharides TPS1-2a and TPS1-2b by gel permeation based purification from green tea. For nitrogen (N) source, we assume that first the fungal strains would have assimilated the easily available amino acids and peptides from the medium and then after their reduced availability in the medium, the fungi would have degraded caffeine for their N energy requirements for growth and cell division. Das [5] confirmed that caffeine was assimilated as sole nitrogen source by most of the fungi. In our present study also, we assume that caffeine in SGTEA is degraded and utilized by the fungi as their sole source of nitrogen at the time of lack of nitrogenous substances in the medium. Numerous studies [8,11,26] have been performed with other substrates as an alternative to the PDA such as the use of cornmeal broth + 5% milk [12], Candida Bromocresol Green Medium (BCG) [6] and Peanut Sucrose Modifications Medium [26] over PDA and SDA for *Candida* spp. Trigueros et al. (2016) [29] reported the growth of *Saccharomyces cerevisiae* var. *boulardii* in cheese whey permeate (CWP), and these authors also carried out media optimization studies and also did fermentation kinetics modeling. Sandoval-Contreras et al. (2017) [25] reported slow growth of *A. niger* grows on lime fruit than the culture medium. Ilgin et al. (2020) [14] reported carob extract as a novel/renewable carbon source in *A. niger* growth and inulinase production. Anbu et al. (2017) [1] reported the growth of *P. chrysogenum* in fruit peel medium containing either pine apple, mango, sweet lime or pomegranate. Dar et al. (2015) [4] reported the growth and α-amylase in *Penicillium chrysogenum* using linseed oil cake as substratum, at pH 6. Microscopic observation reveals that *A. brasilensis* hyphae are septate and hyaline [15] and *Penicillium* conidia are observed to be round, forming brush-like clusters at the tip of phialides [19]

SGTE exhibited antibacterial activity against *Bacillus* sp. PVMX4 and *E. coli* ATCC 8739 strains and also against soil bacteria as evident by the microtiter based MIC assay and growth of fungal colonies alone in the SGTEA plates. Parvez et al. (2019) [21] observed MIC value of green tea extract was found at 125 µg/mL in case of MDR *E. coli*, MDR *S. aureus* strains. Friedmen et al. (2006) [9] reported that catechin concentration of 800 ± 141 nmol killed *B. cereus*. Namita et al. (2012) [18] reported that the tea catechins may cause cellular membrane disruption and prevention of supercoiling in DNA, and these finally leading to the destruction of bacterial cells. Norizon et al. (2013) [20] reported anti-quorum sensing activity of caffeine which in turn after the cell to cell communication and motility in *Pseudomonas aeruginosa* PA01. Zhou et al. (2002) [35] reported that the polysaccharide conjugates from Green Tea exhibited the growth of *E. coli* through the mechanism of outer membrane disturption.Therefore, crude SGT extract that contained caffeine, epigallocatechin and polysaccharide conjugates inhibited the gram positive and gram-negative test organisms at MIC values lower than the values considered as standard for MIC.

Green tea is a health drink made from the young leaves of *Camellia sinensis* that had undergone minimal oxidation during processing. After consumption of green tea, tea bag along with the spent green tea thrown in the garbage was found to support the growth of fungi. On this basis, we formulated a medium using spent green tea leaves and named it as

Spent Green Tea Extract Agar (SGTEA), and compared it with Potato Dextrose Agar (PDA), which lacked selectiveness because it supported the growth of wide range bacteria present in the environment. There was a need to add antibiotics in PDA in order to eliminate bacterial growth, but in case of SGTEA, the medium itself contained antibacterial compounds. Experiments conducted on SGTEA assayed to contain caffeine, catechin and other compounds, which showed bactericidal effect on wide range of bacteria. Therefore, SGTEA considered to be used as a selective medium for growth, identification and characterization of fungi and also for preserving fungal stocks.

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