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Research Article

The percentage distribution of bacteria, fungi and yeast of coffee leaves, cherries, and soil of coffee growing areas of Tamil Nadu

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Abstract

Isolation of Bacteria, Fungi and Yeasts from soil and leaves of coffee plants and coffee cherries collected from coffee-growing areas of Tamilnadu was attempted. The present study focus on the percentage distribution of fungi, bacteria and yeast. The microbial load varied from as less as 1 cfu/cherry to as high as 86 cfu x10^6 /g of coffee ground soil. The microbial load increased after heavy rainfall on cherries that were drying on the ground. At all stages, bacteria were usually the most abundant group, followed by filamentous fungi and finally yeast. Counts of bacteria, yeasts and fungi varied considerably with the stages of maturation. Of a total of 554 isolates, 426 were identified to at least genus level comprising 44 genera and 64 different species. The 143 isolates of Gram-negative bacteria included 17 genera and 26 species, the most common of which were members of the genera *Aeromonas, Pseudomonas, Enterobacter and Serratia*. Of 131 isolates of Gram-positive bacteria, 23 were spore-forming and included six *Bacillus* species, and 118 were non-spore-formers of which over half were *Cellulomonas* with lesser numbers of *Arthrobacter, Microbacterium, Brochothrix, Dermabacter and Lactobacillus*. Of the 87 yeast isolates, 60 were identified into 12 genera and 24 different species and almost all were fermentative. The most common genera, in decreasing frequency, were *Pichia, Candida, Arxula,* and *Saccharomycopsis*. Almost all 192 fungal isolates were identified to genus level and 52 were identified to species level. *Cladosporium, Fusarium and Penicillium* each comprised about one third of the isolates and were found on all farms. 20% of the isolates were *Aspergillus*. Beauvaria, Monilia, Rhizoctonia and Arthrobotrys species were also occasionally found. The genera and species identified include members known to have all types of pectinase and cellulase activities.

Keywords: coffee plants and coffee cherries, microbial load, bacteria, filamentous fungi, yeast.

Introduction

Caffeine (1, 3, 7-trimethylxanthine) is a commercially important purine alkaloid synthesized by plants. It is an active psychostimulant, which increases alertness and sustains concentration by overcoming fatigue. Environmentally, caffeine has been suggested as a chemical indicator of ecosystem since it is difficultly metabolized (Ogunseitan, 2002). Therefore, release of some

caffeine-containing wastes, such as coffee pulp/husk from coffee producing factory, infused tea leaves and sewage from the factory of tea polyphenols preparation, leads serious ecological pollutions and becomes a big disposal problem (Pandey et al.,2006)**.**Solid wastes, such as, coffee pulp and husk, are the major contributors of environmental pollution from the coffee estates (Bressani, 1979; Adams and Dougan, 1981). The presence of caffeine in soil can also affect soil fertility as it inhibits seed germination and growth of seedlings (Friedman and Waller, 1983). Coffee pulp containing waste water is often discharged to the surrounding water bodies resulting in contamination of freshwater (Buerge *et al*., 2003; Glassmeyer *et al*., 2005.) To date, four major approaches for reducing caffeine content from caffeine containing products are conventional breeding, physicochemical methods, Genetic engineering and microbial degradation whilst approach for wastes decaffeination is rarely concerned. In this aspect microbial degradation of caffeine (1,3,7-trimethylxanthine) and related methylxanthines has been the focus of research in the recent past owing to major advantages that it has over conventional techniques of decaffeination (Dash and Gummadi,2006).Exploring microbial diversity under these extreme environmental conditions (Caffeine tolerance) and understanding adaptation mechanisms of extremophiles to survive in such extreme conditions will lead to much new knowledge of biology and also contribute to the future development of biotechnology.

Materials and Methods

Sample Collection and Isolation of Microbes

Coffee pulp was collected from coffee cherry, leaves, Soil from coffee growing areas and processing site of the coffee estate in a sterilized container. Soil (100 g) and the other samples were soaked in 1 L distilled water for 30 mins. Solution containing microorganisms obtained by filtration and diluted 10^3-10^6 times, and spread over the surface in Petri dishes (0.5 ml solution for each dish).After incubation at appropriate temperature and time, the fast-growing colonies were picked up for further studies.

Isolation and identification of Bacteria

Solid screening medium (SSM) for isolating the caffeine-tolerant bacteria was prepared by mixing the mineral solution with caffeine (2.5 g/ L) and agar (1.5%) and autoclaved at 121°C for 10 min. Solid purifying medium (SPM) was also prepared as SSM except different concentrations of caffeine (1 to 10 g/L) was supplemented. Liquid amplifying medium (LAM) was obtained after addition of caffeine (0.5 g /L) and sucrose/glucose (5.0 g /L) in the mineral solution and disinfection.

Soil (100 g) and the other samples were soaked in 1 L distilled water for 30 mins. Solution containing microorganisms obtained by filtration and diluted 10^3-10^6 times, and spread over the SSM surface in Petri dishes (0.5 ml solution for each dish).After incubation at 30°C for 2-3 d, the fast-growing single colony was picked up with tooth tip and inoculated on SPM surface for further purification. Colonies, which could grow normally on the SPM,were separately picked up again and transferred to the tubes containing 1.0 ml LAM medium (each colony for one tube),then incubated at 150 r min-1 and 30°C in a shaking incubator for amplification.Three days later, 0.3 ml of bacterial solution was mixed with 0.1 ml dimethylsulfoxide and stored at -70°C and the remaining solution was used for further tests.

Pure cultures were maintained on nutrient agar medium at 4ºC and were sub-cultured at an interval of every 2 week. Various morphological, physiological and biochemical tests were performed to identify the bacteria.

Isolation and identification of Fungi

For isolation of fungi associated with the coffee samples, the direct plating technique was applied in DRBC medium - Dicloran Rose de Bengal Chloramphenicol (glucose 10.0 g; peptone 5.0 g; KH_2PO_4 1.0 g; $MgSO_4.7H_2H$ 0.5 g; Agar 15.0 g;

bengal rose 25.0 mg; dicloran 2.0 mg; chloramphenicol 100.0 mg; distilled water 1.0 L). A total of 100 coffee beans were plating directly without surface disinfection and 100 beans were plated with surface disinfection with 70% alcohol and 1% sodium hypochlorite according to Samson *et al.* (2000). The plates were incubated for 5–7 days at 25 °C. The overall percent contamination was expressed as the percentage of particles yielding visible growth of fungi.

The isolated fungi were purified and identified according to Klich (2002), Frisvad *et al.* (2004) and Samson *et al.* (2004).The isolates were incubated in CYA medium - Czapek yeast Agar (K_2HPO_4 1.0 g; concentrate Czapek $NaNO_3$ 30.0 g; KCl 5.0 g; $MgSO_4.7H_2O$ 5.0 g; $FeSO_4.7H_2O$ 0.1 g; $ZnSO_4.7H_2O$ 0.1 g $CuSO_4.5H_2O$ 0.05 g; distilled water 100mL) in MEA - Malt Extract Agar (malt extract 20.0 g; peptone 1.0 g; glucose 30.0 g; Agar 20 g; distilled water 1 L) at 25 °C and CYA at 25 °C and 37 °C. After incubation for 7 days, the microscopic and macroscopic characteristics described by Klich (2002b) were observed.

Isolation and identification of Yeasts

Ten g of each sample were added to 90 ml diluent saline peptone (SPO) [0.1% bactopeptone (Difco, Detroit, MI, USA), 0.85% (w/v) NaCl (Merck, Darmstadt, Germany), 0.03% $Na_2H_2PO_4$, $2H_2O$ (Merck), adjusted with 1 M NaOH (Merck) and 1 M HCl (Merck) to pH 5.6]. After mixing in a stomacher (Lab Blender 400, Seward, London, UK) at normal speed for 30 s, 10-fold dilutions were prepared and spread onto malt yeast glucose peptone (MYGP) agar [3 g yeast extract (Difco), 3 g malt extract (Difco), 5 g bactopeptone (Difco), 10 g glucose (Merck), 100 mg chloramphenicol (Oxoid) and 20 g Agar (Difco)] per litre of distilled water, adjusted with 1 M NaOH and 1 M HCl to pH 5.6] and incubated at 25 °C for 5 days. The number of colony forming units (CFU) was recorded. From a suitable dilution of each sample, 10 representative colonies were picked and recultivated in MYGP broth at 25 °C for 2 days, and further purified by streaking onto MYGP agar. A total of 110 yeast isolates were obtained.

Results and Discussion

The microbial load varied from as less as 1 cfu/cherry to as high as 86 cfu x10^6 /g of coffee ground soil (Table 1) . The microbial load increased after heavy rainfall on cherries that were drying on the ground. At all stages, bacteria were usually the most abundant group, followed by filamentous fungi and finally yeast. Counts of bacteria, yeasts and fungi varied considerably with the stages of maturation. Of a total of 554 isolates, 426 were identified to at least genus level comprising 44 genera and 64 different species. The 143 isolates of Gram-negative bacteria included 17 genera and 26 species, the most common of which were members of the genera *Aeromonas, Pseudomonas, Enterobacter and Serratia*. Of 131 isolates of Gram-positive bacteria, 23 were spore-forming and included six *Bacillus* species, and 118 were non-spore-formers of which over half were *Cellulomonas* with lesser numbers of *Arthrobacter, Microbacterium, Brochothrix, Dermabacter and Lactobacillus*. Of the 87 yeast isolates, 60 were identified into 12 genera and 24 different species and almost all were fermentative. The most common genera, in decreasing frequency, were *Pichia, Candida, Arxula,* and *Saccharomycopsis*. Almost all 192 fungal isolates were identified to genus level and 52 were identified to species level. *Cladosporium, Fusarium and Penicillium* each comprised about one third of the isolates and were found on all farms (Table 2 & 3). Most of the isolates were *Aspergillus*. Beauvaria, Monilia, Rhizoctonia and Arthrobotrys species were also occasionally found.

The understanding of these microbes and their surveillance under high caffeine concentration and genetic manipulation of the same for the benefit of safe environmental disposal of coffee spent and biodecaffeination lead to the growth of biotechnology towards human welfare.

Table 1. Represents the maximum microbial load found from 10^3 to 10^6

Bacteria	**Cfu/cherry**	**Cfu/leaf**	**Soil cfux10^6**
Aeromonas	$4X10^4$	$3X10^4$	54
Brevibacterium	~	~	32
Enterobacter	$3X10^3$	~	42
Serratia	$4X10^4$	$2X10^4$	36
Pseudomonas	$6X10^6$	$2X10^6$	86
Bacillus	$5X10^6$	$3X10^6$	78
Cellulomonas	$4X10^3$	$1X10^3$	34
Arthrobacter	$3X10^4$	$2X10^4$	23
Microbacterium	$2X10^3$	$4X10^3$	22
Brochothrix	$2X10^4$	~	12
Lactobacillus	$2X10^5$	~	42
Dermabacter	$1X10^4$	$1X10^2$	12

~ nil cfu-colony forming units

Table 2. Represents the maximum microbial load found from 10^3 to 10^6

Yeasts	**Cfu/cherry**	**Cfu/leaf**	**Soil cfux*10^6***
Pichia	$6X10^4$	$2X10^4$	14
Torulopsis	~	~	22
Candida	$3X10^3$	~	26
Arxula	$2X10^4$	~	~
Saccharomycopsis	$5X10^6$	~	27
Saccharomysis	$2X10^6$	$3X10^6$	28

~ nil cfu-colony forming units

Table 3. Represents the microbial load found from 10^3 to 10^4

Fungi	**Cfu/cherry**	**Cfu/leaf**	**Soil cfux*10^4***
Aspergillus	++	~	+++
Cladosporium	+	+	+++
Fusarium	+	+	+++
Penicillium	+	++	+++
Baeuveria	++	+	+
Monilia	+	~	+
Rhizoctonia	+	~	~
Arthrobotrys	~	~	+

~ nil cfu-colony forming units
+ propugules less ++ moderate +++ more

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