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Microbial community changes induced by thiocyanate released from sago factory effluent

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ABSTRACT

The realm of microbial diversity encompasses numerous organisms belonging to the kingdom of Bacteria, Eukarya and Archae. The toxic compounds may cause a shift in microbial community. Sago factory effluent collected from three sites was found to contain maximal Cyanide and Thiocyanate concentration of 1.7 mg/l and 12.2 mg/l respectively. After 10 days of incubation, maximal microbial growth was observed in site 3 while the microbial growth decreased in presence of 20 mM Thiocyanate. Shannon-Weiner and Simpsons' diversity index revealed the negative impacts imposed by Thiocyanate on bacterial community.

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KEYWORDS

Microbial diversity;
Shannon-Weiner diversity;
Simpsons' index;
Thiocyanate;
Sago factory effluent.

INTRODUCTION

Microbes are an integral part of our ecosystem. It wields its influence through the processes like decomposition, nutrient cycling, soil aggregation and pathogenicity. The recorded inventory of microbes present all over the world accounts for only a smaller fraction (0.1 – 1.0 %) of the total community. A conservative estimate predicts that the task of mapping the entire microbial community would take about 800 – 900 years. Knowledge on the soil microbial ecology is paramount for the restoration and sustainability of ecosystems. Contamination of soil with toxic compounds imposes detrimental effects on microbial activity thereby causing a shift in microbial community structure.

Thiocyanate is a stable, non-hydrolysable, non-

volatile inorganic compound that occurs from a diverse range of natural and industrial source. Sago industry employs Cassava (*Manihot esculenta cranz.*) as a substrate which results in the release of Thiocyanate and Cyanide upon the crushing process. Thiocyanate induced toxic effects include inhibition of a variety of enzymes involved in halide transport to the thyroid gland, stomach, cornea and gills. Many hemolithotrophic and chemoheterotrophic bacteria such as *Arthrobacter*, *Escherichia*, *Methylobacterium* and *Pseudomonas* species can utilise Thiocyanate as a source of energy and nutrients. Thiocyanate has been found to be utilised by *Thiobacillus thioeparus* and *Thiobacillus denitrificans*^[1], studied the degradation of Thiocyanate by *T.thiocyanoxidans*. *Pseudomonas* and *Arthrobacter* species are known to degrade Thiocy-

anate but they don't utilise them as an energy source. Bacterial strains of *Klebsiella sp.* are found to degrade both the cyanide and thiocyanide compounds^[2]. *P.Fluorescens*, *P.thiocyanatus*, *Klebsiella*, *Ralstonia*, *Burkholderia sp.*, fungal cultures like *Fusarium solani*, *Trichoderma* species have also been involved in Thiocyanate degradation.

Though few studies regarding the ability of bacteria to utilise Thiocyanate compounds have been carried out, the impact of Thiocyanate compounds on the microbial community needs extensive analysis. This study focuses on the microbial diversity changes occurring due to the presence of Thiocyanate in the sago factory effluent.

EXPERIMENTAL

Collection of samples

Soil samples from the nearby sago industry were collected from contaminated sites located near Attur, Salem District, Tamil Nadu. Triplicate samples were collected from three different sago factories located at Manjini (S1), Thalaiwasal (S2) and Kattukottai (S3). Soil from the top 3-10 cm layer of effluent contaminated sites was collected. The soil samples were then kept in sterile bags and transported to the laboratory and kept at 4 °C until further analysis. Chemicals used for the purpose of physico-chemical estimation and Sodium Thiocyanate were obtained from Merck Ltd, Germany. All microbiological media were procured from HiMedia Laboratory, Mumbai. Other chemicals used were of Analytical Grade from Qualigens Ltd., Mumbai.

Estimation of cyanide

Concentration of Cyanide present in the samples was determined by a modification of the Picric acid method of Fisher and Brown^[3]. A linear calibration curve was obtained with the standard cyanide solution as follows: an aliquot (0.05 ml) of cyanide-containing solutions (after centrifugation at 15,000 g for 10 min at 4°C) were added to 0.1 ml aliquots of a solution containing 0.5% (w/v) picric acid and 0.25 M Na₂CO₃. The resulting solution were placed in a water bath for 5 min, diluted to 1ml with 0.85 ml distilled water and cooled in tap water for 30 min. The absorbance was read at 520 nm against a blank of distilled water and Picric acid

reagent.

Estimation of thiocyanate

Thiocyanate assay was carried out by Ferric Cyanide method as reported in Standard methods^[4]. Thiocyanate was determined by measuring the absorbance at 420 nm after adding 0.2 ml of 10% (w/v) Fe(NO₃)₃, 0.2 ml of 5 M HNO₃ and 3.9 ml of deionised water to 0.1 ml of the sample^[5].

Microbial plating

The enumeration of the total microbes present in the sample can be assessed by the microbial plating technique. Ten grams of soil were diluted in 90 ml of sterile saline solution (0.9% NaCl, w/v), mixed thoroughly on a magnetic stirrer at 120 rpm for 120 min and then allowed to rest for 60 min to allow settling of the soil. Standard serial dilutions were followed and aliquots of each dilution were spread on Nutrient agar plates. The total bacteria and fungi grown were estimated for enumeration of diversity of the population.

Microbial count

The plate count method was used to monitor the number of culturable heterotrophic bacteria present in the sample. The bacterial count was done by the use of Neubers' counting chamber. In order to group colonies according to time of appearance, visible colonies grown on non-selective plates were enumerated daily during the incubation period. The number of bacteria in each class was expressed as a proportion (%) of the total colony number found after the experimental period.

Influence of time on microbial count

The impact of time on microbial count can be assessed by observing the colony counts over a period of time. The concentration of Thiocyanate, Temperature and pH was kept as constant. Periodic observation of colony forming units (CFUs) was undertaken.

Influence of thiocyanate concentration on microbial count

The impact of Thiocyanate concentration on microbial count can be assessed by observing the colony counts over a period of time. The concentration of Thiocyanate was varied from 5 – 20 mM while the factors like Temperature and pH was kept as constant. Peri-

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odic observations of colony forming units (CFUs) were undertaken.

Estimation of diversity index - shannon diversity index

The Shannon Weiner Diversity index^[6] is the negative sum of each OTU's proportional abundance multiplied by the log of its proportional abundance. The Shannon-Weiner diversity index^[7] was calculated as follows: $H' = - \sum (P_i \ln P_i) - [(S-1) / 2N]$, where, p represents the proportion of a phylotype relative to the sum of all phylotypes. Evenness (E) was calculated as: $E = H/H_{max}$ where $H_{max} = \log^2(S)$; Richness (S): Total number of species in the community.

Simpsons diversity index

Similarly estimation of microbial species by Simpsons Diversity index is carried out using the following formulae:

$D = \sum n(n-1) / N(N-1)$ Where, n = total number of organisms of a particular species;

N = total number of organisms of all species.

RESULTS

Estimation of cyanide and thiocyanate

The content of Cyanide and Thiocyanate in the samples is as in TABLE 1. The increased content of Cyanide and Thiocyanate compounds are a cause for concern. Although the maximum permissible limit of cyanide has been only 0.01 mg/l, the maximum con-

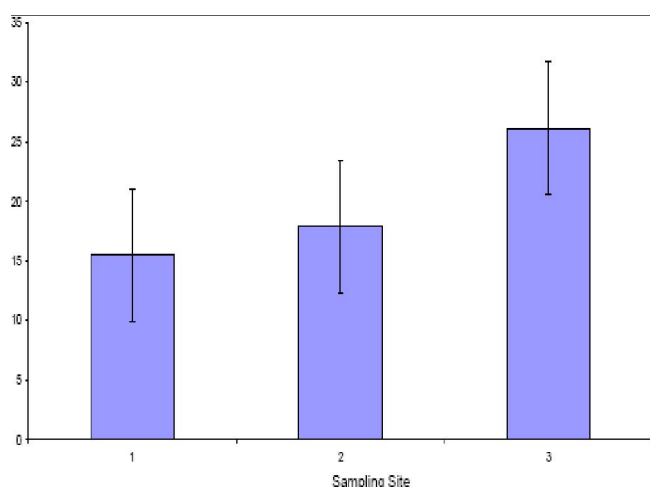


Figure 1 : Thiocyanate concentration at different sampling sites

TABLE 1 : Concentration of Thiocyanate and Cyanide

Parameter	Sample 1	Sample 2	Sample 3	Permissible Limit (CPCB)
Thiocyanate (mg/l)	15.47	17.85	26.12	1.00
Cyanide (mg/l)	2.33	1.82	2.45	0.01

centration of 2.45 mg/l has been observed in Site 3. The calculated value for Site 1 and Site 2 is 2.33 and 1.82 mg/l respectively. Similarly concentration of Thiocyanate has been observed to be of the range from 15.47 – 26.12 mg/l. Site 1 recorded Thiocyanate concentration of about 15.47 mg/l while Site 2 had Thiocyanate concentration of about 17.85 mg/l. The maximum Thiocyanate concentration was found at Site S3 with a concentration of about 26.12 mg/l exhibited an increased concentration of Cyanide and Thiocyanate. The Figure 1 represents the Thiocyanate and Cyanide concentration present in different sampling sites.

Total Microbial count

Total Number of species observed

Based on the isolation and plate count technique, the total number of microbial species present in the sampling area was recorded. The TABLE 2 depicts the scenario of total isolated species of bacteria and fungi from all three sites. The population represents the growth on plates when observed under the counting chamber. The bacterial population was found to be more predominant than fungal species. The number of species found in the sites also varied reasonably. Most number of species was observed in Site 1 than the other two sites. Site 1 logged 11 bacterial and 4 fungal species while site 2 recorded 6 bacterial and a lone fungal species while it was 7 bacterial and 5 fungal species in site 3. A total of 15, 7 and 12 microbial species have been identified in the three sites respectively.

The Figure 2 depicts the varied distribution groups of the microbial species reported earlier in the study. Bacterial groups included species belonging to the

TABLE 2 : Microbial Diversity – Total Number of species observed

Type	Site 1	Site 2	Site 3	Total
Bacteria	11	6	7	24
Fungi	4	1	5	10
Total	15	7	12	34

groups of *Bacilli*, *Alcaligenes*, *Staphylococcus*, *Klebsiella*, *Ochrobacterium* and *Aeromonas*. The fungal group comprised of *Basidiomycetes*, *Zygomycetes*, *Ascomycetes* and *Actinomycetes*. *Bacilli* and *Staphylococcus* (12% each of total population observed). *Zygomycetes* (14% of total population) formed the largest group of fungal species. *Klebsiella* and *Basidiomycetes* were part of a very small population.

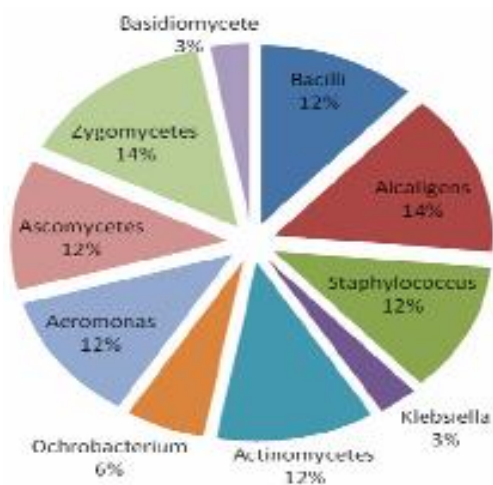


Figure 2 : Distribution of Microbial species

Influence of Time and Concentration factor on Microbial count

The response of various isolated microbial groups to the different time and concentration of Thiocyanate were observed. To determine colony diversity the colonies were grouped in classes (morphotypes) on the basis of visual differences using parameters such as colony colour, roughness or shininess of surface, diameter and edge. The number of different colony morphotypes and the number of the colonies belonging to each morphotype were considered in order to assess the colony diversity by the Shannon–Wiener diversity index^[8]. This approach was selected because of the ability of the Shannon–Wiener index to reveal changes using colony morphotypes. The following Figures 3 and 4 shows the response of various bacterial communities towards the increase in time and thiocyanate concentration. Assessment of heterotrophic bacterial count over different periods showed an increase in numbers with prolonged incubation period. S3 showed the maximal colony growth, with the visible colonies ranging from 5.1×10^{-3} in 3 days to about 5.8×10^{-3} in 10 days. S1 showed lower counts than the other sites with a 10 day

incubation period eliciting a response of only 4.4×10^{-3} colonies. Site 2 showed an increase of colony count from 4.4×10^{-3} to 5.1×10^{-3} within 10 days.

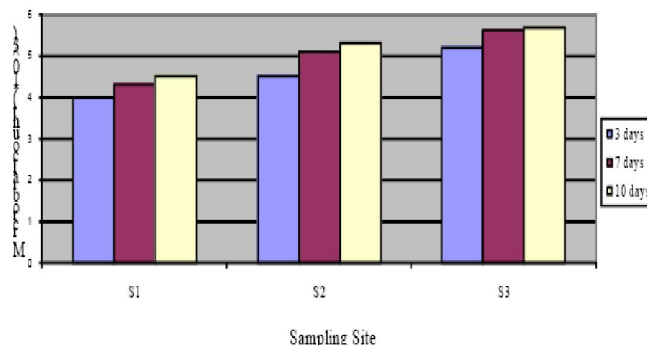


Figure 3 : Influence of Time factor on Microbial count

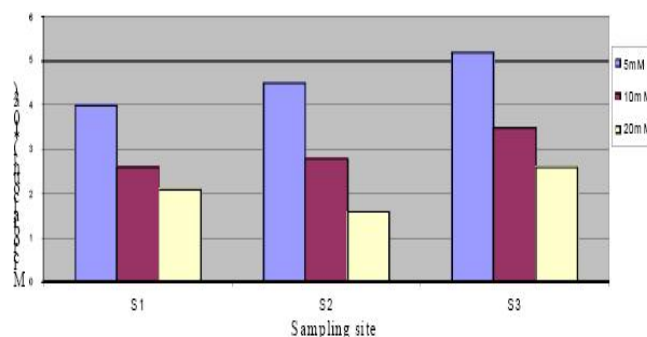


Figure 4 : Influence of Thiocyanate Concentration on Microbial count

Microbial diversity analysis

Assessment of shannon wiener diversity index

The Shannon wiener diversity index was calculated based on the colony units formed during different growth conditions. On observation, bacterial growth was observed over a period of 14 days. Regular sampling and determination of colony counts were taken. Shannon index was calculated by applying the formulae reported earlier. Indices were reported for the colony growth found at 3, 7 and 14 days. The concentration of Thiocyanate was maintained at 10, 20, 30 mM throughout the experimental period and the respective growth of organisms was observed.

The Figures from 5 to 7 shows the Shannon wiener diversity index values for the microbial groups able to grow in presence of 10, 20 and 30 mM Thiocyanate concentration. As such, the diversity indices were found to decrease with the progress of experimental period. Considerable decrease in H' was observed at 14 day sampling period. For sample 1, diversity value de-

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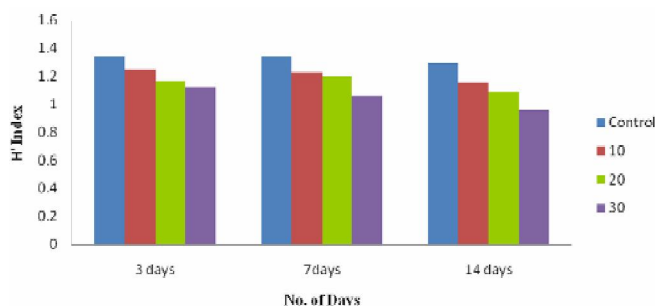


Figure 5 : Shannon Wiener Index - Sample 1

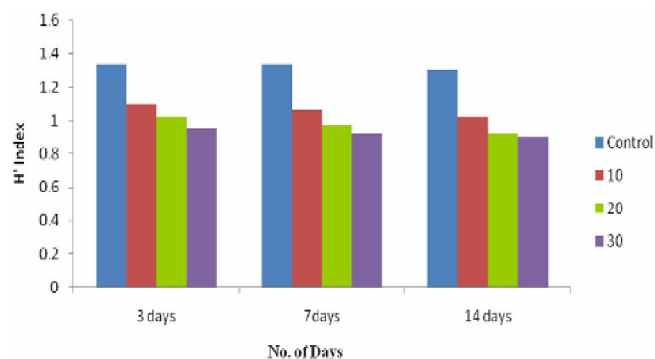


Figure 6 : Shannon - Wiener Index - Sample 2

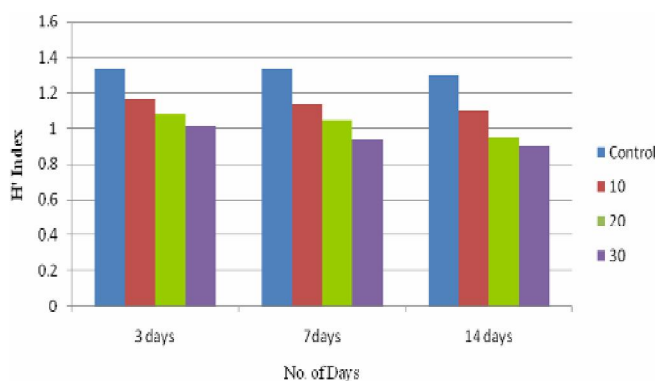


Figure 7 : Shannon-Wiener Index Sample 3

creased from 1.12 to 0.96 in presence of 30 mM Thiocyanate while it decreased from 1.17 to 1.09 at 20 mM concentration, but notable change has not been observed at 10 mM. For sample 2, diversity value decreased from 0.95 to 0.90 in presence of 30 mM Thiocyanate while it decreased from 1.02 to 0.92 at 20 mM concentration, and from 1.10 to 1.02 in presence of 10 mM Thiocyanate. Sample 3 showed a change in diversity value from 1.02 to 0.90 in presence of 30 mM Thiocyanate and from 1.08 to 0.90 at 20 mM. Growth index at 10 mM remained more or less similar throughout the period.

Simpson's diversity index

As observed from the experimental data presented

in the TABLE 3, the Simpson's diversity index at 30 mM Thiocyanate concentration was found to show significant difference in sample 2, where it decreased from 0.755 to 0.680. Unlike Shannon diversity index, these values are inversely proportional to the diversity status. Hence more the value, a lesser diversity status could be assumed.

TABLE 3 : Simpson's Diversity Status at 30mM Thiocyanate concentration

Parameter	Sample 1	Sample 2	Sample 3
Control	0.637	0.847	0.769
3 Days	0.592	0.755	0.531
7 Days	0.556	0.755	0.510
14 Days	0.520	0.680	0.500

Sample from site 1 showed a change in diversity index from 0.592 to 0.520 in 14 days, site 2 showed a change in diversity from 0.755 to 0.680 in 14 days while in site 3 the diversity showed a decrease from 0.531 to 0.500 after 14 days, thereby signifying a higher microbial diversity. During this study the control showed a change in index value from 0.637 to 0.76.

DISCUSSION

In this study about 1.82 – 2.45 mg/l of Cyanide have been observed which is around 10 – 25 fold higher than the permissible limits of Cyanides in effluents by the Indian standards. Cyanide concentration of about 2.33 mg/l has been observed at Site SF1 and 1.82 mg/l in site 2 while site SF3 effluent is found to contain the highest concentration of 2.45 mg/l Cyanide. This change in cyanide concentration at various sampling sites may be due to the varieties of Cassava being used for processing. According to previous reports, the concentration of Cyanide is found to decrease over a period of time. This is because Cyanide being an unstable compound gets degraded by the enzyme Linamarinase to Linamarin^[9]. The cyanogenic potential of known cassava cultivars ranges from less than 10 mg kg⁻¹ as HCN fresh weight basis to more than 500 mg kg⁻¹ as HCN fresh weight basis^[10]. Cassava production containing less than 50 mg/l cyanoglucosides are considered as harmless but its long term consumption might lead to chronic toxicity.

The Thiocyanate concentration observed in all sites

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is found to be higher than the tolerable limit for organisms. Site SF3 recorded the maximum of 26.12 mg/l Thiocyanate concentration and is found to exceed the permissible limit of CPCB by 25 fold. All cassava varieties contain varying concentrations of the cyanogenic glucosides, linamarin and lotaustralin, which are hydrolyzed to HCN by endogenous linamarase when the tissue is damaged. Cyanide concentrations of nearly 1000 mg/kg have been reported in tapioca foliage^[11, 12]. A concentration of 2 to 4 mg HCN/kg body weight can be lethal to the cattle. There are reports indicating that feeding large amounts of tapioca products without treatment could result in death of the animals, particularly non-ruminants^[13].

The analysis of count plate's data showed that both SCN⁻ concentration and incubation time affected the number of CFU deeply, and there was highly significant interaction between incubation time and SCN⁻. Significant changes have been observed in CFU numbers for all microcosms during the entire experiment. The differences between the CFU numbers of control and 10 were not significant after 14 days. Microcosm at 30 mM thiocyanate showed a significant lower number of culturable bacteria than the control after 7 days from the beginning of the experiment, but the number of CFUs increased over time and was higher than the control by the end of the experiment. The control had the greatest diversity of CFUs, and this did not change during the experiment.

The results of Shannon–Wiener diversity index (H') on the colony morphotypes showed that both SCN⁻ and incubation time affected the diversity and there was highly significant interaction between time and thiocyanate concentration. The Shannon index (H') seems a useful general diversity index that is influenced by both richness and evenness and is more sensitive to changes in abundance of the rare groups. The Simpson's index (D) is heavily weighted by the dominants. It is easily understood and is much less affected by coverage. Presence of higher sulphur content in the effluent might favour the growth of chemosynthetic bacteria.

The Simpson's Index (D) values were affected by the level of thiocyanate contamination and decreased over time. The index values tended to decrease with increasing thiocyanate concentration and time, confirm-

ing a higher diversity and the presence of some dominant morphotypes in concentration at any sampling time. The change in the diversity was paralleled by the heterotrophic culturable bacterial population density, as the concentration showed the lowest value of H' and the highest number of CFUs at the end of the experiment.

The plate count still appears to be a useful approach for assessing the effects of pollution and it has been amply supported by the works of Trevors^[14, 15]. The use of culture independent method approaches will remove the bias imposed by the isolation of bacteria on laboratory media but conversely will fail to take into account the differences in cellular activity. As many bacteria can exist in dormant forms, it is important to measure activity so that ecologically relevant bacteria are assessed and not inactive cells that do not contribute to ecosystem function.

CONCLUSION

The results of the study showed an increase in the growth of microbial community with time while a decrease in diversity of microbial population was observed with increased thiocyanate concentration. The impact of thiocyanate on microbial growth has been validated further by Shannon–Wiener diversity index and Simpsons' index. To overcome problems associated with non-culturable bacteria and fungi, various methods including fatty acid analysis and numerous DNA- and RNA-based methods could be employed.

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