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Microbial Spotting in Fresh and Cured Sago Processing Wastewater

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Abstract: ***In order to establish an aerobic granular sludge method for the effective treatment of highly synthetic sago industry wastewater and comprehend the granulation process in a multi-ion matrix, the structure and development of several granule types were examined in this work. Treatment of synthetic wastewater including varying percentages of actual sago industrial wastewater (0% to 100%) is done using a Hybrid Aerobic Bio-Reactor (HABR). The outcomes show that up to 100% of sago wastewater can be used to successfully generate aerobic granular sludge. Analysis using High Resolution Scanning Electron Microscopy (HRSEM) Furthermore, DNA Genomic Isolation indicated the presence of microorganisms in both the granule core and the outside layers, which served as additional bridging factors to hasten granule formation and preserve the structure. It is envisaged that this research will examine an alternative method for treating wastewater from the sago industry and provide some answers to the granulation mechanism's enigma.***

Keywords: Aerobic treatment, Industrial wastewater, Microbial identification, Scanning Electron Microscopy

1. Introduction

The treatment of industrial wastewater is a unique issue since it comprises a variety of pollutants. The hazardous impacts of industrial effluents, such as their influence on aquatic life and humans that can cause mutation and cancer, as well as their nearly non-biodegradable components have sparked severe concerns throughout the world. Today, a number of physicochemical and biological techniques have been created to lessen the dangers and pollution caused by chemicals. Because of its similar cheap costs and simplicity of operation, biological treatment is seen as one of these treatments that is appealing. Unfortunately, due to the uneven treatment efficacy and space requirements, various treatment methods can now be used to treat chemical industrial effluent. However, because to operational issues, increased energy demand, and expensive capital and maintenance costs, attempts to improve biological treatment systems or intensify the separation process have not been widely utilized.

In-depth study of biological wastewater treatment over the past two decades has successfully led to the engineering of microbial structures in activated sludge that enable microorganisms to produce stable granular sludge with spherical shape and distinct boundaries instead of traditional flocculent sludge. Gravity-based separation can be integrated inside the treatment unit due to the compact nature of the process and significant cost and land area reductions that result from using this type of sludge.

Typically, the process is accomplished in an Aerobic Hybrid Bio-Reactor (AHBR), which has both attached growth and suspended growth process in a single reactor with a cycle structure that selects for quickly settling microbial aggregates and reiterations repast and starvation situations frequently. Such a highly dynamic feed regime causes the development of solid and dense granules as well as the stimulation of particular metabolic pathways necessary for the biodegradation of substrates that are not readily biodegradable. For the purpose of treating synthetic wastewater in a laboratory-scale AHBR, we tried to create and use aerobic granules in the current work. Synthetic sago industry wastewater was prepared with a Chemical Oxygen Demand (COD) concentration of 4000 mg/L.

2. Methodology followed

Isolation of bacteria from Treated water of sago processing

About 1 ml of treated water from sago processing sample was serially diluted and 6th and 7th dilutions were spread plated in the sterile nutrient agar plate. After 24-48 h incubation at bacteriological incubator sample was observed for the bacterial growth. The bacterial colonies were counted and predominant culture was streaked in newer sterile nutrient agar plate. The predominant culture was subjected to molecular identification using 16S rRNA gene sequencing.

Genomic DNA isolation and PCR analysis

Genomic DNA was extracted from overnight grown cultures of the selected bacterial isolates using QIAGEN DNA isolation kit (Qiagen), suspended in 100 µl of elution buffer (10 mM/L Tris-HCl, pH 8.5) and quantified by measuring OD at 260 nm. PCR amplification was performed using a 50 µL reaction mixture containing 100 ng of template DNA, 20 µmol of 16S rRNA primers, 200 µM of dNTPs, 1.5 mM of MgCl2, 1U of Taq DNA polymerase (MBI Fermentas) and 10 µL of 10x Taq polymerase buffer. The sequences of 16S rRNA primers used were as follows.
27f: (5'-AGAGTTTGATCCTGGCTCAG-3')
1522r: (5′-AAGGAGGTGATCCANCCRCA-3')

Amplification was carried out with an initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 56°C for 45 sec, extension at 72°C for 1 min and final extension at 72°C for 5 min using a

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thermocycler (iCycler; Bio-Rad Laboratories, CA). PCR products were analyzed on 1% agarose gel for 16S rRNA amplicons in 1x TBE buffer at 100 V. The amplified product was sequenced using ABI PRISM 3730 Genetic Analyzer (Applied Biosystems).

Phylogenetic analysis

The sequences of these 16S rRNA genes were compared against the sequences available from GenBank using the BLASTN program (Altschul et al., 1990) and were aligned using CLUSTAL W software (Thompson et al., 1994). Distances were calculated according to Kimura's two-parameter correction (Kimura, 1980). Phylogenetic trees were constructed using the neighbour-joining method (Saitou and Nei, 1987). Bootstrap analysis was done based on 1000 replications. The MEGA4 package (Kumar et al., 2007) was used for all analyses.

Scanning electron microscopy of raw and treated sludge samples

The raw and treated sludge samples were analyzed using scanning electron microscopy analysis the determination of sludge degradation. Briefly, 10 µl of the sample was applied to copper grid assembly and air dried. The air-dried sludge sample samples were sputter coated with gold/platinum plasma sputter coater. The sputter-coated grid was analyzed with Hitachi FESEM with various magnifications.

3. Results and Discussion

Total plate count

The treated water sample from sago processing was estimated for the bacterial count. It was confirmed that, 8.32×1010 colony forming unit/mL (Figure 1a and 1b). The predominant isolate was streaked in nutrient agar plate for the colony morphology and purity (Figure 2) as *Lysinibacillus pakistanensis.* It is an aerobe, spore-forming, mesophilic bacterium that was isolated from rhizospheric soils, soybean, etc.

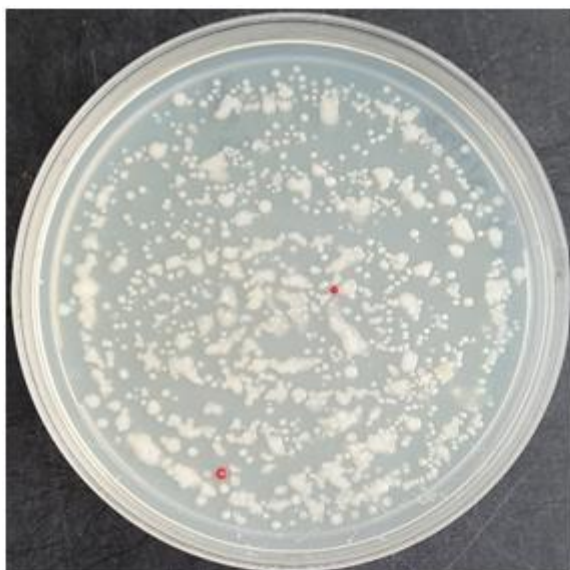

Figure 1 (a): Isolation of bacteria from treated water from sago processing (10^{-6} Dilution)

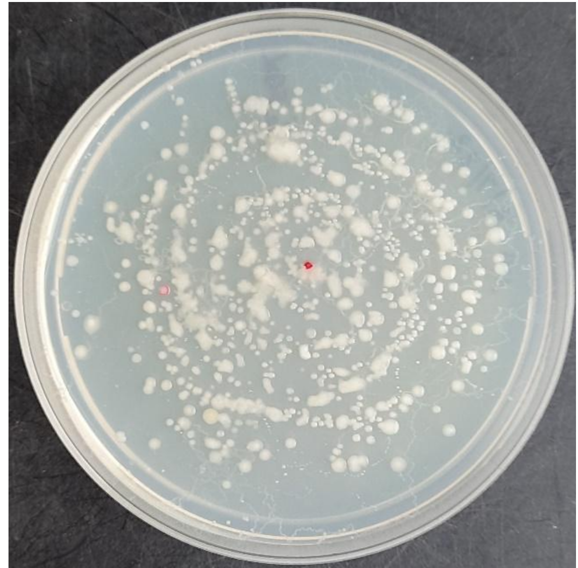

Figure 1 (b): Isolation of bacteria from treated water from sago processing (10^{-7} Dilution)

Figure 2: Selected bacteria from treated water from sago processing

Molecular identification of selected bacteria from sago processing treated water

The selected bacterium was used for the genomic DNA isolation and it was isolated at high pure conditions with single band in the agarose gel electrophoresis (Figure 3). The resultant DNA was amplified using 16S rRNA gene primers and it was greatly amplified with the signature product size of 1.5 kb (Figure 4). The amplified product was sequenced in a Sanger sequencing with both primers yielded good quality of sequences (Figure 5). The sequences thus obtained were BLAST in the NCBI website showed a great match against various Bacillus species. After running with phylogeny analysis using neighbour joining method showed closest clade with Lysinibacillus pakistanensis (Figure 6). Based on the BLAST analysis in the NCBI, RDB taxonomy analysis and phylogeny tree clearly revealed that that the given sample was belong to the taxa is Lysinibacillus pakistanensis.

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Figure 3: Genomic DNA of sago processing treated water

bacteria

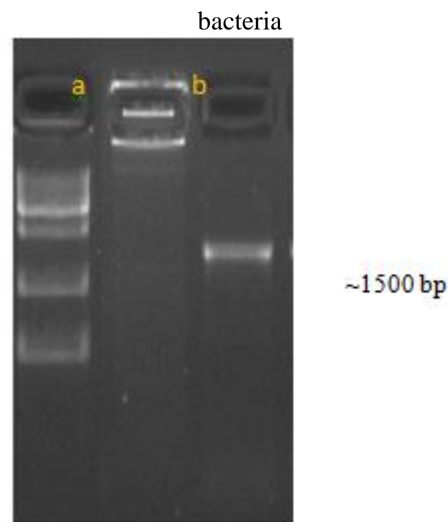

Figure 4: PCR amplification of 16S rRNA gene from sago processing treated water bacteria

>Seq_isolate of treated water Sago processing

**GTCGAACAGAAGATAGGAGCTTGCTCCTTTGACGTTAGCGGCGGACGGGTGAGTAACACGTG
GGCAACCTACCCTATAGTTTGGGATAACTCCGGGAAACCGGGGCTAATTACCGAATAATCTATGTCA
CCTCATGGTGACATACTGAAAGACGGCTTCTCGCTGTCGTTATAGGATGGGCCCGCGGCGCATTAGC
CCTAGTTGGTGATGGTAATGGCTCACCAAGGCGACAATGCGTACCCAACCTGAGAGGGTGATCGGCC
ACACTGGGACTGAGACACGGCCCAAATCCTACGGGAGGCAGCATTAGGGAATCTTCCACAAGGGGCA
AAAGCCTGATGGACCACCCCTCGCGTGAGTGAAAAAGGTTTTCGGATCTTAAAACTCTGTTGTAAGG
GAAAAACAAGTACAGTAGTAACTGGCTGTACCTTGACGGTACCTTATTAAAAAGCCACGGCTAACTAC
GTGCCACCAGCCGCGGTATACGGGTAGGTGGCAAGCTTTGTCCGGAATTATTGGACGTAAAGCGCGC
GCAGGCGGTCCTTTAAGTCTGATGTGAAACCCCACGGCTCACCCGTGGAGGGTCATTGAAAACTGGG
GAACTTGATTGCAGAAGAGGAAAGTGCAATGTCCAAGTGTACCGGTGANGTGCCTAAAGAATTGGAA
GGAACCCCATTGGCGAAGGCGACTTCTGGTCTGTAACTGACGCTGAGGCGCGAGTGAAGGAGCAAA
CAGGATTAGATACCTGGTAGTCCCCCCGAAAACAATGCATAGTAAAGTGTTGGGGGGTTTTCCCCC
CTATTGTTGAAGTAAAGGCATTAAGCATTCCCCCGGGGGGGTAGGGTGGCAGGACGGAAATTAAAAG
AAATTAACGGGGGCCCGCCCAACCGGGGGGGCAGGTGGTTTAATTTGAGGAAACGGGAGGAACCTT
ACCAGGTTTTGACATCCCGTTGACCACCGTAGGAAAATAGTTTTCCCTTCGGGGGCAACGGTGACAG
GGGGGGCATAAAGGTTTTTTTCAGTTGGTTTATTGAGATGTTGGGTTAATTCCCCCAACGAGGCAACC
CCCGTTTTTATTTGCCTTCATTTATTTGGGCATTTTAAGGGGACCATGCCGGGGAAAAACCGGGGGAA
GGGGGGGGGGAGGTAAAATAATCATGCCCCTTTTAACCTGGGGTACACACGTGTTCCAATGGAGGAT
ACAAACGTTGCCAACTCGCGGGGGGGGGGTATTCCGATAAATTTTCATTTTTGGTTTTTGGGGGGCA
ATTCCCTTCCATGAAGCCGGAATGGTTGGTAATTGGGGATCACCATGCCGCGGGGAAAAATTTCCCG
GCCCTACACACCCCCCGTCCCCCCCCGGGAGTTTGTAACACCCGAAGTGGGTGGGGTAACCTTTTGG
AGCCACCCCCCGAAGGGGGATAAATGATTTGTGG**

Figure 5: 16S rRNA gene sequence from sago processing treated water bacteria

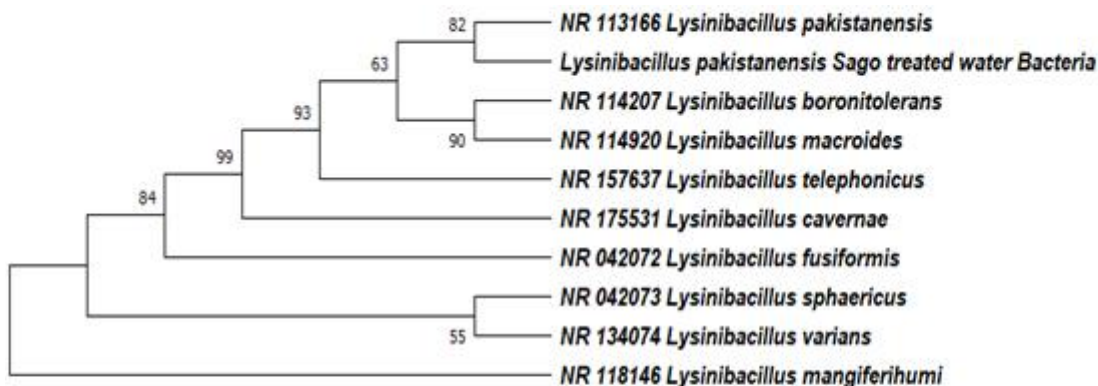

Figure 6: Phylogeny tree analysis of sago processing treated water bacteria

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The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). There were a total of 1384 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007).

Scanning electron microscopy of raw and treated sludge samples

The scanning electron microscopical images of the raw and treated sludge samples showed clear degradation of the sludge, and the macro particles were seen in the raw sludge samples and micronized particles were seen in the treated sludge sample (Figure 7a and 7b). In the samples, the bacterial and other microbial structures were also seen. The microbial digestion of the raw sludge was visualized after treatment with bacterial populations, due to the bacteriological enzyme action on the macro particles to become micronized were observed under 5000X magnifications.

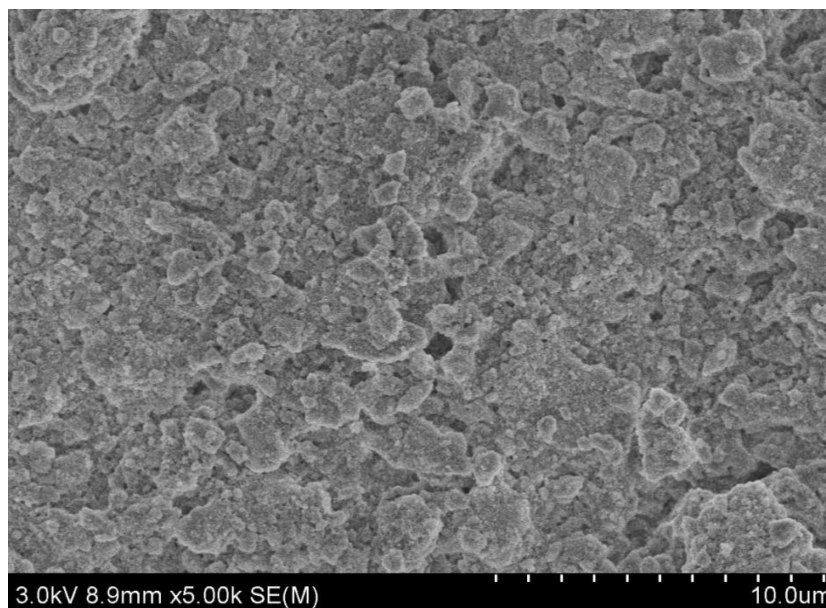

Figure 7 (a): SEM Analysis of raw sludge from sago processing

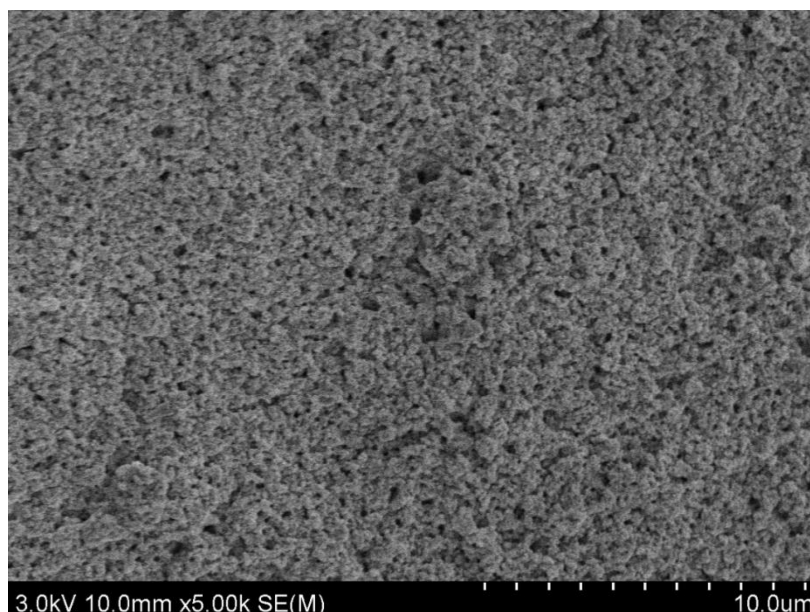

Figure 7(b): SEM Analysis of treated sludge from sago processing

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4. Conclusion

The structural and primary microbiological makeup of the aerobic activated sludge granules generated from three separate AHBR operations were characterised in the current study. A potent tool for examining the structure of microbial granules is provided by the combination of SEM and DNA isolation, which can also be used to other biofilm forms. Due to the ability to concurrently identify bacteria, ciliates, and fungi, this recently developed procedure applied to micro sections of microbial granules can now be utilised to track the development of such granules. The kind of wastewater and the AHBR's operational configuration affected the granules' compactness, size, and microbiological makeup. The investigated biofilms contained huge numbers of numerous bacterial morphological kinds. With synthetic wastewater serving as the only source of carbon and energy, aerobic granules with a strong microbial structure and great settling ability were created. Granules that had been grown displayed a high and constant rate of metabolism as well as a lengthy biomass residence period. The fundamental premise that aerobic granulation has the advantage of good biomass separation from treated effluent and, as a result, quicker settling time, is somewhat supported by the combination of these studies.

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