Biosurfactant Producing Metallo-Phenolic Tolerant Microbial Consortia for Nitrification

Y.P. Mpentshu, S.K.O. Ntwampe, and N. Mpongwana

***Abstract*— In this study, the use of metallo-phenolic tolerant microbial consortia was proposed for its biosurfactant supported COD reduction, under high metallo-phenolic conditions, with high concentrations of phenol and heavy metals (Zn^{2+} and Cu^{2+}) up to 850mg/L and 35mg/L, respectively.**

Results indicated that the toxicants had insignificant inhibition; albeit, comparative analysis indicated reduced emulsification efficiency of the biosurfactants under high toxicant loading, which will reduce the degreasing capabilities, thus reduced nitrification as the mass transfer will subsequently be reduced. The results showed potential for the consortia to be used in various types of wastewater including phenol and hydrocarbon contaminated water.

***Keywords*—** Biosurfactants, Heavy metals, Phenol, Wastewater

I. Introduction

Globally, water scarcity is both a governmental and environmental concern, with a current 1 billion persons (14.3% of the world population) having a limited access to drinking grade water [12]. In South Africa, a water stressed country, statistics show a decline in water levels in water reserves such as dams and rivers. The South African water stress level is because of rainfall shortages as indicated by recent statistics [1]. Water, as a basic unit for life is required for both industrial and human consumption purposes. In the South Africa available fresh water sources are largely being consumed by the agricultural sector, i.e. 63% of the surface water; thereby, necessitating the treatment and recycling water, even the wastewater largely from the agricultural sector, which is commonly contaminated by ammonium-nitrogen, a toxicant to both animals and humans [7]. Therefore, the recycling of treated water is vital to sustainability and the bulk use of water sources for human anthropogic activities. The forecasted demand for water in South Africa indicates that by year 2035, the consumption rate of water by various sectors and industries would've increased, as a result of the technologies currently being on an industrial scale [10, 9]. Generally, with the conventional water treatment methods currently in place, efficiency and environmental sustainability are compromised. This necessitates an improved method for the primary treatment of wastewater from industrial and agro-processing environments. The wastewater from such industries contains highly toxic phenolic compounds including heavy metals.

In this study, biosurfactant producing metallo-phenolic tolerant microbial consortia was assessed for their ability to nitrify.

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II. Materials And Methods

A. Inoculum Preparation

Isolation of microorganism was conducted in three sampling points, namely, wastewater from previous projects in the *BioERG* and the poultry slaughterhouse wastewater. Initially, nutrient yeast extract supplemented to nutrient broth was used an as an overnight media for inoculum preparation for the raw wastewater samples. These samples were then transferred to freshly prepared nutrient agar plates according to manufacturers' specifications to attain pure cultures. The isolated microorganisms were then inoculated into mineral salt media (pH 7) prepared to allow for nutrient supplementation during the incubation period of 120 hr at a temperature of 34˚C. The pH was measured using a Crison Basic 20 pH meter, which was calibrated daily. The microbial population was quantified using the Thoma counting chamber.

B. Microbial Growth and Identification

The microorganisms inoculated into Basal media were decanted into 1L reactors whereby an adequate supply of heat in the form of heated water from a water bath was used to ensure consistency in temperature conditions of the reactors. The reactors were constantly supplied with nutrients in the form of yeast extract infused Basal media for maximum microbial growth on a 3-week basis. In ensuring the microorganisms were firmly attached for increased biofilm formation efficiency, course sponge-like cubes were inserted after 48 hr incubation of the original inoculum. Dissolved oxygen supply was ensured through the use of air pumps (Resun air pump, AC-9906, Resun®, China) which were connected using silicone tubing to air diffusers (Mott element 6500, Mott Corporation, USA).

For microbial identification, genomic DNA was extracted

from the cultures received using the Quick-DNATM Fungal/Bacterial Miniprep Kit (Zymo Research, Catalogue No. D6005). The 16S target region was amplified using OneTaq® Quick-Load® 2X Master Mix (NEB, Catalogue No. M0486) with the primers presented. The PCR products were run on a gel and gel extracted with the ZymocleanTM Gel DNA Recovery Kit (Zymo Research, Catalogue No. D4001). The extracted fragments were sequenced in the forward and reverse direction (Nimagen, BrilliantDyeTM Terminator Cycle Sequencing Kit V3.1, BRD3- 100/1000) and purified (Zymo Research, ZR-96 DNA Sequencing Clean-up KitTM, Catalogue No. D4050). The purified fragments were analysed on the ABI 3500xl Genetic Analyzer (Applied Biosystems, ThermoFisher Scientific) for each reaction for every sample. CLC Bio Main Workbench v7.6 was used to analyse the .ab1 files generated by the ABI 3500XL Genetic Analyzer and results were obtained by a BLAST search (NCBI) https://blast.ncbi.nlm.nih.gov.

C. Toxicant Tolerance Tests for the Selected Biosurfactant Producers

Prior to the nitrification experiments and biofilm development, the isolated microorganisms were investigated for their potential environmental toxicant tolerance, using serial dilutions as a means of evaluation for toxicant tolerance. The isolated microorganisms were inoculated into fresh agar plates and incubated for 24 hr in a 37°C incubator. The 24 hr cultures were then inoculated into saline water containing some toxicants, namely, Zn^{2+}, Cu^{2+} and phenol with final concentrations being 600, 300 and 850mg/L, respectively. The dilutions were conducted up to the 10^{-4}. A volume (100µL) of the microorganism containing solution was inoculated into toxicant infused nutrient agar and incubated for 36 hr at 37°C.

D. Biofilm Engineering

The isolated microorganisms were inoculated into Basal media (pH 7) which was prepared as per instruction according to the specifications of Mpongwana [6] whereby yeast extract was used as a supplement for microbial growth. The microorganisms were incubated and grown in air supplied 1.5L glass reactors at 37°C, maintained using a 25L bench scale water bath. The aeration was supplied through silicone tubing (5mm diameter) connected to an air pump (Resun air pump, AC-9906, Resun®, China) supplying aeration through plastic air diffusers. The biofilm attachment of the microorganisms was on a porous sieve like membrane. The biofilm nutrient supply was conducted at weekly intervals whereby 0.3L of freshly prepared Basal media was supplied and microbial inoculation was constantly aliquoted into the reactors at 3-week intervals for the increment of microbial concentration.

E. Total Nitrogen Removal Experiments

In proving the nitrification and possible denitrification ability of the microorganisms in the engineered biofilm, initial concentrations of NH_4-N (48mg/L) and phenol (35mg/L) were introduced into the basal media infused biofilm. Samples were taken at 2 hr intervals and analysed for the residual concentrations of NH_4-N, NO_2-N and NO_3-N. Since the NO_x and gaseous nitrogen were not quantified, a decline in the concentration of NO_3-N was assumed as the initiation of denitrification.

F. Analytical Methods

Residual NH_4-N, NO_2-N and NO_3-N concentrations were quantified as per manufacturers' instruction using Merck ammonium (NH_4-N) (00683), nitrite (NO_2-N) (110057) and nitrate (NO_3-N) (14773) test kits. A Merck spectroquant® NOVA 60 was used to quantify the concentration of the analytes. The NH4-N test kit works on the Berthelot reaction method between ammonia, chlorine and phenolic compounds to form indophenol dyes. The nitrate test kit makes use of concentrated sulphuric acid in the presence of a benzoic acid derivative Nitrites were determined according to the method of Rider and Mellon [8] in which a reaction occurs between nitrite ions and 4 aminobenzenesulfonic acid and 1 aminoaphthalem, resulting in a reddish-pink colour which can be read at 520nm. The pH was measured using a Crison Basic 20 pH meter subjected to a routine daily calibration. The quantification of the population of microorganisms was conducted using a Jenway 7315 UV/visible spectrophotometer (Camlab, UK) at a wavelength of 600 nm.

III. Results And Discussion

A. Toxicant Tolerance Tests

In this study, the isolation of toxicant tolerant strains and their ability to biologically assimilate toxic nitrogenous compounds in the presence of common toxicants found in the wastewater, was undertaken to enhance the conventional methods currently utilised for the treatment of wastewater, in order to minimise challenges associated with water shortage and the rapid rate of wastewater generated. This is to have sustainable mitigation and/or treatment shortages strategies and improvements in recycling.

The proliferation of the microorganisms in the presence of toxicants in the growth media showed the microorganisms' tolerance to the toxicants studied. Heavy metals are known to be toxic to microorganisms; although; some maybe tolerant to higher concentrations of heavy metals, than those studied, some microorganisms have proven to sustain themselves in some metal recovery processes; therefore, the metal tolerance capabilities have been identified as being true. The selected microorganisms showed minimal growth after the normal 24 hr incubation period. However, the incubation was extended to 36 hr due to the presence of toxicants as microorganisms tend to have slower growth rates in the presence of potential growth limiting toxicants. The toxicant results indicated that the isolated microorganisms were capable of withstanding toxicity levels observed in wastewater treatment plants with minimal inhibition; albeit at increased lag phases.

B. Total Nitrogen Removal Efficiency

The relationship between biosurfactant producing biofilms and the nitrogen removal ability in the presence of phenol was studied. This was investigated since research has indicated that the presence of phenol could potentially be toxic to the nitrogen removal process, i.e. nitrification [11] which is the rate limiting primary step in nitrification and denitrification systems. The

initial concentration as determined by the test kits for the biofilm was 48mg/L and the incubation period was at 2 hr intervals. Fig. 1 shows the variations in concentration of the NH_4-N.

To prove the nitrifying ability of the microorganisms, NH_4-N was introduced to the Basal media, which was prepared according to the specifications in the Mpongwana [6], a study where nitrification was studied in the presence of cyanide. The microorganisms in the biofilm largely dominated by the *Chloroflex*i sp., showed an initial increase in concentration of NH_4-N from an initial concentration of 40mg/L to 55mg/L. This was hypothesised as being attributed to by the cell lysis initial shock experienced by the microorganisms a response to both phenol and nitrogen intoxication [14]. The chosen initial concentrations were a combination of the overall impact of the toxicants on microbial growth and to allow for microorganisms' metabolic activities to thrive since multiple toxicants affect microorganisms differently [14, 4].

The insignificant inhibition shown by the resistance of the microorganism to the toxic effects of phenol, could have been attributed by the percentage of *Chloroflexi* sp. In the consortia since they have been found to detoxify phenol [13, 5]. The results of this study have indicated that the constructed biofilms were able to eradicate the toxic nitrogenous compounds in a single reactor system in the presence of dissolved toxicant where a concentration of 48mg/L was used. This illustrated feasibility and considerable functionality of *Chloroflexi* sp. as they have been found to contribute to the success of the nitrogen removal experiments in traditional wastewater treatment experiments in the presence of various toxicants [2].

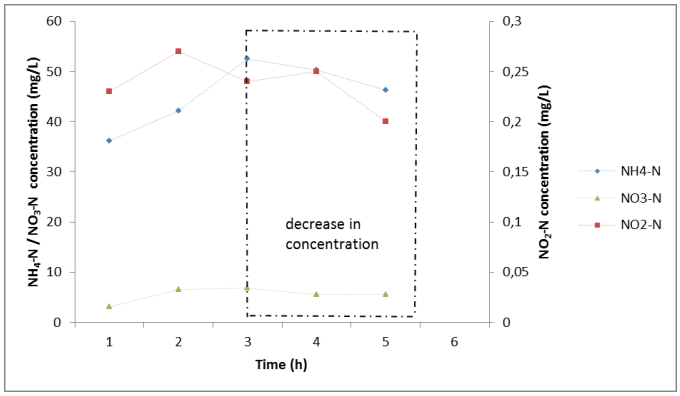

Fig. 1Nitrogen species removal results for biofilm in the presence of phenol and heavy metals

Furthermore, Yamada [13] and Kindaichi [3] studied different *Chloroflexi* sp. and found that they are capable of either nitrification or denitrification whereby both studies indicated the ability of these microorganisms to carry out nitrification and denitrification processes. However, no toxicants were reported, but for the purposes of the current study, no distinction was made as the microorganism identification was at species level since the identification was done for a biofilm consortium. The obtained results also indicated that the presence of heavy metals and hydrocarbons had insignificant impact on the removal of total nitrogen; however, due to variations of conditions in biofilm systems and microbial quality; the microorganisms displayed some colour changes due to variations in microbial concentrations as the experiment ensued; an indication of microbial adaptation even when toxicants are prevalent within the primary nutrient source.

IV. Conclusion

The microorganisms in the engineered biofilm were capable of nitrification; however, only partial denitrification was achieved. The heavy metals showed an insignificant inhibition as the microorganisms grew at high concentrations of toxicant during tolerance tests. Some of the microorganisms investigated were biosurfactant producers. Phenol, although the concentration was relatively low, the biofilms constituted by several microorganisms were able to nitrify even in the presence of phenol and heavy metals; proving sufficient tolerance against the continuous presence of these toxicants. This study has indicated a partial role played by metallo-phenolic resistant and biosurfactant producing engineered biofilms ability in reducing overall total nitrogen, in wastewater treatment.

References

[1] Boyer, L.R., Brain, P., Xu, X.M. and Jeffries, P., 2015. Inoculation of drought-stressed strawberry with a mixed inoculum of two arbuscular mycorrhizal fungi: effects on population dynamics of fungal species in roots and consequential plant tolerance to water deficiency. *Mycorrhiza*, 25, 215-227. https://doi.org/10.1007/s00572-014-0603-6

[2] Feng, L.J., Xu, J., Xu, X.Y., Zhu, L., Xu, J., Ding, W. and Luan, J., 2012. Enhanced biological nitrogen removal via dissolved oxygen partitioning and step feeding in a simulated river bioreactor for contaminated source water remediation. *International Biodeterioration & Biodegradation*, 71, 72-79. https://doi.org/10.1016/j.ibiod.2011.12.016

[3] Kindaichi, T., Ito, T. and Okabe, S., 2004. Ecophysiological interaction between nitrifying bacteria and heterotrophic bacteria in autotrophic nitrifying biofilms as determined by microautoradiography-fluorescence in situ hybridization. *Applied and Environmental Microbiology*, 70, 1641-1650. https://doi.org/10.1128/AEM.70.3.1641-1650.2004

[4] Kwak, W., McCarty, P.L., Bae, J., Huang, Y.T. and Lee, P.H., 2012. Efficient single-stage autotrophic nitrogen removal with dilute wastewater through oxygen supply control. *Bioresource Technology*, 123,.400-405. https://doi.org/10.1016/j.biortech.2012.07.076

[5] Ma, H., Ye, L., Hu, H., Zhang, L., Ding, L. and Ren, H., 2017. Determination and Variation of Core Bacterial Community in a Two-Stage Full-Scale Anaerobic Reactor Treating High-Strength Pharmaceutical *Wastewater. Journal of Microbiology and Biotechnology*, 27, pp.1808-1819. https://doi.org/10.4014/jmb.1707.07027

[6] Mpongwana, N., Ntwampe, S.K., Mekuto, L., Akinpelu, E.A., Dyantyi, S. and Mpentshu, Y., 2016. Isolation of high-salinity-tolerant bacterial strains, Enterobacter sp., Serratia sp., Yersinia sp., for nitrification and aerobic denitrification under cyanogenic conditions. *Water Science and Technology*, 73, 2168-2175. https://doi.org/10.2166/wst.2016.070

[7] Munro, S.A., Fraser, G.C., Snowball, J.D. and Pahlow, M., 2016. Water footprint assessment of citrus production in South Africa: A case study of the Lower Sundays River Valley. Journal of Cleaner Production, 135, 668-678. https://doi.org/10.1016/j.jclepro.2016.06.142

[8] Rider, B.F. and Mellon, M.G., 1946. Colorimetric determination of nitrites. *Idnustrial & Engineering Chemistry Analytical Edition*, 18, 96-99. https://doi.org/10.1021/i560150a003

[9] Schewe, J., Heinke, J., Gerten, D., Haddeland, I., Arnell, N.W., Clark, D.B., Dankers, R., Eisner, S., Fekete, B.M., Colón-González, F.J. and Gosling, S.N., 2014. Multimodel assessment of water scarcity under

climate change. Proceedings of the *National Academy of Sciences*, 111, 3245-3250.
https://doi.org/10.1073/pnas.1222460110

[10] Thopil, G.A. and Pouris, A., 2016. A 20 year forecast of water usage in electricity generation for South Africa amidst water scarce conditions. *Renewable and Sustainable Energy Reviews*, 62, 1106-1121.
https://doi.org/10.1016/j.rser.2016.05.003

[11] Urakawa, H., Rajan, S., Feeney, M.E., Sobecky, P.A. and Mortazavi, B., 2018. Ecological response of nitrification to oil spills and its impact on the nitrogen cycle. *Environmental Microbiology*. 23, 12-15
https://doi.org/10.1111/1462-2920.14391

[12] Xin, X.K., Li, K.F., Finlayson, B. and Yin, W., 2015. Evaluation, prediction, and protection of water quality in Danjiangkou Reservoir, China. *Water Science and Engineering*, 8, 30-39.
https://doi.org/10.1016/j.wse.2014.11.001

[13] Yamada, T., Sekiguchi, Y., Imachi, H., Kamagata, Y., Ohashi, A. and Harada, H., 2005. Diversity, localization, and physiological properties of filamentous microbes belonging to Chloroflexi subphylum I in mesophilic and thermophilic methanogenic sludge granules. *Applied and Environmental microbiology*, 71, 7493-7503.
https://doi.org/10.1128/AEM.71.11.7493-7503.2005

[14] Yao, S., NI, J., Chen, Q. & Borthwick, A. G. L. 2013. Enrichment and characterization of a bacteria consortium capable of heterotrophic nitrification and aerobic denitrification at low temperature. *Bioresource Technology*, 127, 151-157.
https://doi.org/10.1016/j.biortech.2012.09.098

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