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Research Article

Emulsification Properties of Bacteria Isolates from Dairy Waste Water: Potential for Biosurfactant Production

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Abstract

Biosurfactants are amphipathic biomolecules produced by biological entities with vast applications. Waste water from a dairy processing industry showed maximum microbial heterotrophic activity; physicochemical analysis of the waste water showed the presence of the following: Cl, Ca, Mg, PO^{2-}_{3}, K as dissolved mineral contents. Heavy metals analysis showed the presence of Pb in trace concentration while Cu and Fe were richly in abundance. Physical properties of the waste water showed a relatively acidic pH of 6.0, conductivity of $893\Omega^{-1}cm^{-1}$ while solid contents such as TS, TDS and TSS were found as 1341, 580.45 and 760.55 g/l. initial oxygen concentration (mg/l) of the waste water was 6.23 mg/l while after five days the BOD quotient was 4.32 mg/l. Strains of *E.coli*, *Salmonella* sp., *Yesinia* sp., *Vibrio* sp., *Bacillus* sp., *Pseudomonas* sp., *Klebsiella* sp., were found after isolation using standard microbiology and biochemical techniques. Microbial load and standardization of the isolates were done using the Marcfarland solution, total heterotrophic number of the organisms (CFU/ml) showed the following: *E. coli* 3.2×10^5, *Salmonella* sp 3.0×10^5, *Yesinia* sp 2.2×10^4, *Bacillus* sp 3.8×10^6, *Pseudomonas* sp 3.7×10^6 and *Klebsiella* sp. 3.2×10^6. Emulsification assessment (emulsification index) of the organisms in the presence of crude oil only showed the following: *E. coli* 54.3%, *Salmonella* sp 43.51%, *Yesinia* sp 38.72%, *Bacillus* sp 56.21%, *Pseudomonas* sp 64.32% and *Klebsiella* sp. 62.31%. The same trend was seen in the presence of olive oil and coconut oil with *Pseudomonas* sp 67.3% and *Klebsiella* sp 71.09% showing the highest index of emulsification. Further investigation of emulsification potentials using oil drop collapse and spread plate techniques still showed optimized peak emulsification of strains of *Pseudomonas* sp. and *Klebsiella* sp in the presence of different oil used: kerosene, crude oil (bonny light), coconut oil and olive oil. The two consortiums were used for biosurfactant production in a submerged fermentation system.

Keywords: *Biosurfactants; Emulsification; Microbial Load; Physicochemical; Oil*

Research Background

Industrial waste water is one of the crucial sources of pollution in water body pollution [8]. The chemical industry including those of inorganic and organic bias in utility is of economic importance in terms of their impact on the environment. The wastewaters from this industry are generally polarize and may contain toxic pollutants. Chemical industrial wastes usually contain organic and inorganic matter in varying degrees of concentration. It contains acids, bases, toxic materials, and matter high in biological oxygen demand, color, and low in

suspended solids. Many materials in the chemical industries are said to be toxic, mutagenic, carcinogenic or simply hardly biodegradable upon impact and eco-toxicological assessments. Surfactants, emulsifiers and petroleum hydrocarbons that are being used in chemical industry reduce performance efficiency of many treatment unit operations [8].

Dairy whey a bye product in dairy processing industry is a contaminant with a high organic chemistry implication and after hydrolysis it may be used as cattle food resources and in the food industry for development of new products with no lactose content [9]. Cheese whey is a highly polluting product, consisting of 0.7% (w/v) protein, 5% (w/v) lactose, 93% (w/v) water and salts. This organic waste from dairy processing industries constitute to high organic oxygen quotient exertion in aquatic body in form of biochemical oxygen demand (BOD).

The continual increase in the number of industries in Nigeria has predisposed the surrounding biosystem with various noxious contaminants. The enroute path of this danger potential contaminants include the: soil, air, water and the surrounding biotic flora and fauna [13].

Dairy products production is a very common process around the world (Nigeria) and it brings a considerable amount of dairy whey, which represents a serious environmental problem for its disposal. Dairy industries whose major raw materials include cheese, probiotic starter cultures (living organisms) with lactose as major constituent sugar amount to very high quantity of organic matter (whey) in the environment [21]. Usually the treatments consist in biological and chemical processes for the removal of organic matter in the whey, in function of the chemical oxygen demand (COD), biochemical oxygen demand (BOD) and dissolved organic carbon removals [11]. Their main toxicological effect upon impact assessments is on the availability of oxygen current in the aquatic body needed for various biochemical activities by the biotic consortium inhabiting in thereof. Emulsifying agents such as bio surfactants are surface active bio molecules produced extracellularly or as part of a cell membrane by a variety of microorganisms ranging from yeast (non-filamentous fungi) bacteria and filamentous fungi with wide range of applications [20]. Recently, interests in microbial emulsifiers (surfactants) have increased because of their advantages when compared to the synthetic or chemical surfactants. Advantages which include their ability to display a high level of biocompatibility, biodegradation, biodigestibility, ease of preparation and ability to serve in a wide range of physicochemical parameter such as: temperature, pH and salt concentration [7].

Due to their unique functional properties, biosurfactants are used in several industries including organic chemicals, petroleum/ petrochemicals, mining, metallurgy, agrochemicals, foods/beverages and the pharmaceuticals [11]. The interfacial surface tension reducing ability of surfactant makes them able to play important role in oil recovery and remediation of spilled crude oil. During these exercise, biosurfactants aid the bioavailability of hydrophobic substrates through solubilization/ desorption. They also regulate the removal and attachment of microorganisms from surface interface.

Aim of the Study

This present study is aimed at assessing the emulsification potentials of bacterial isolates and their biodiversities in a diary effluent discharge using microbial load measurement and corresponding breakpoints of standard procedures.

Materials and Methods

Materials

All chemicals, reagents and equipments used in the present study were all of analytical grade, standardized and are products from designated renowned companies.

Methods

The study adopted the experimental design.

Dairy waste water sample collection

Dairy waste water were collected from two dairy processing sites located at Rumuoekeni (LONG.7° 03",25°.2" E; LAT. 4° 51",53°.03" N) and Rumuodumaya (LONG.7° 00",57°.3" E; LAT. 4° 53",70°.04" N), both in Obio/Akpor L.G.A of Rivers state, Nigeria. The samples were collected from the stated areas at 6:00 am in the morning using a hooked sample bottle.

The collections were done at the four perimeters of the marked drilling fronts with average of 9 m apart from each other. The collected waste water with the wheys were homogenously pooled together into a clean aseptic container and transferred to the laboratory.

Waste water analysis

Waste water from the dairy surge tank prior to microbial isolations were subjected to various physicochemical soil profiling test as described in journal of ATSDR [4].

The following tests were carried out:

- pH profiling test.
- Conductance test.
- Determination of macro and micro contents of the soil.
- Total organic carbon contents.

Heavy metals identification such as Fe, Hg, As, Cd, Cu and Pb using the atomic absorption spectra (AAS).

Isolation of the bacteria from the digested waste water

Organismal isolations from the waste water were carried out using standard microbiology and biochemical techniques as described by Ezeonu., *et al*. [10].

Inoculations of folds of diluted waste water on the prepared plate and subsequent sub culturing

From the 10^{-1} to 10^{-6} fold dilutions, inoculations were carried out on the prepared nutrient media and the differential media around bursen flame. Streaks were made from each side of the plate, marking an initial point, with sterilization of the wire loop after each side has been completed. After the inoculation, the inoculated plates were incubated for 3-4 days at 38°C using the incubator for colonies growth. All morphological contrasting colonies were purified by repeated streaking and sub-culturing on separate plates. This process was continued till pure bacteria colonies were obtained.

Microscopic features of the isolated bacteria

Three day old pure cultures were examined both under the microscope and by physical examinations. The colour, texture, spores and growth patterns were also observed.

Bacteria identification

After the gram staining experiment, a little bit of the culture suspensions was dropped on the slide and a drop of safranine red was added to it. A cover slip was placed over it and examination will be performed under the light microscope at X100 magnification. Identification will be carried out by relating features and the micrographs to "Atlas of Bacteriology" by Barnett and Hunters [5].

Heterotrophic counting

Total heterotrophic biomass from both the nutrient media and the mineral salt agar was counted from the grown media plate as described as follow:

$$\text{TCU/ml} = colony\ observed \times dilution\ factor \times volume\ of\ innoculum\ used$$

Screening of isolates for biosurfactant production

Prior to the screening for biosurfactants, the isolates identified was inoculated into 10 ml of broth medium each and the incubated at 37°C for 72h. The culture media was centrifuged at 3000 revolutions per minute (r.p.m.) for 30 minutes.

The supernatant was collected and the cells discarded. The supernatant was used for the various biosurfactant screening tests or assays as described by Mbachu., *et al.* [15].

Emulsifying assays

The various emulsifications screening assay were carried out with the culture suspensions:

- **Drop collapse assay:** The assay was carried out as described by Jain *et al*. [12].
- **Oil spreading assay:** This was carried out as described by Morikawa., *et al.* [16].

Emulsification capacity

The emulsification capacity of biosurfactant was developed by Cooper and Goldenberg [8].

$$E24 = Height\ of\ emulsion \div total\ height\ \times 100$$

Statistical analysis

Data obtained shall be expressed as mean ± SD and tests of statistical significance will be carried out using two-way analysis of variance (ANOVA). Mean values with $p < 0.05$ i.e 95% confidence interval were considered as significant.

Results and Discussion

Physiochemical Parameters	Control Water Sample	Waste Water Sample
pH	7.20	5.30
Water Conductivity ($\Omega^{-1}cm^{-1}$)	782	913
Chloride ion (Mg/L)	7123	1203.42
Dissolved oxygen (Mg/L)	7.02	5.76
Magnesium (Mg/L)	16.24	24.21
Potassium (Mg/L)	1.02	8.18
Calcium (Mg/L)	32.33	39.76
$B0D_5$	0.93	4.02
Iron (Mg/L)	1.25	22.29
Cadmium (Mg/L)	BDL	BDL
Mercury (Mg/L)	BDL	BDL
Arsenic (Mg/L)	BDL	BDL

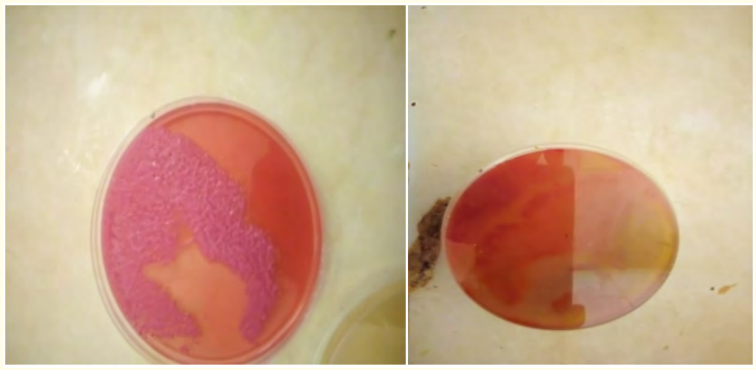
Lead (Mg/L)	BDL	10.32
Copper (Mg/L)	1.14	17.22
Total Organic Carbon (TOC) (mg/L)	38.40	184.41
Total Organic Matter (mg/L)	46.74	226.82

Table 1: *Physicochemical properties of the waste-water (N = 2).*

Physicochemical properties of the waste water analysed in the presence of the control experiment showed pH 5.30 and 7.2, water conductivity were seen at 913 and 782 ($\Omega^{-1}cm^{-1}$) respectively. This significant different between the two experimental treatments can be attributed to the nature of the contaminant in the waste water such as oil and other higher acidic contents (oleic, benzoic acids) as stated in the proceedings of the ASTDR, 2009. Dissolved mineral contents were found in the following order: Cl>Ca>Fe>SO_4>Mg>Cu>K>Pb>PO_3 while heavy metals of Hg, As and Cd were found at below detectable limit range (BDL) in the both treatments. Total organic matter (TOM) and total organic carbon (TOC) contents were found at 184.41, 38.40; 226.82, 46.74 respectively in the various treatments. Onugbolu., *et al.* [19] in their study on waste water from municipal dam reported a similar correlation of ions concentrations in the contaminated surge effluent. They revealed a higher concentration of the mineral ions in the following order 2.28, 1.84, 5.22 and 1789.22 mg/g respectively for K, nitrate, magnesium and chloride ions. Oxygen demand quotient for biochemical activities (BOD_5) quotients was found at 5.2mg/ml with initial dissolved oxygen concentrations at 6.2 mg/ml. Vallero [22] stated that dissolved oxygen concentration (DO) is said to have reciprocal relationship with the biochemical oxygen demand (BOD) in water, he went on to state that the presence of organic matter in water bodies increases biochemical activities of aquatic flora and fauna and such leads to their exponential multiplications (Bloom) and demand for oxygen for biochemical activities (oxidation, respiration etc). TDS, TSS and TS were recorded at 23036, 396.5 and 23433. This as reported in the proceedings of ASTDR, [4] that every exposed water body are characterized by the presence of solid particles which may be suspended within the costal water axis or dissolved in the olefiers of the water bed. The proceedings went further to state that these solid particles constituents of the water can be as a result of rock weathering, human activities such as querying, volcanic eruption in the water bed and water bodies eutrophications.

Heterotrophic Counts (CFU/g)	**Control Sample**	**Test Experiment**
Total Heterotrophic Counts (10^{-2})	2.7×10^8	3.8×10^9
Total Heterotrophic Counts (10^{-4})	1.9×10^5	2.8×10^7

Table 2: *Water microbial loading index and coliform unit (CFU/ml).*



Picture 1: *Soil microbial load from the irrigated soil with waste water.*

The results obtained during the microbial isolation and identification showed that dairy waste water has relatively high total heterotrophic and organic matter utilizing organisms. Water microbial proliferation recorded a bloom in the low dilution factors with total heterotrophic count of 3.8 x 10^7 CFU/g when compared with the control soil (2.7 x 10^5 CFU/g) for the 10^{-2} dilution factor.

Bacterial Isolate	Drop-Collapse	Oil Spreading Technique
E. coli sp	+	+
Pseudomonas sp.	++	++
Klebsiella sp.	++	+++
Pseudomonas sp.	+	+
Salmonella sp.	+	+
Bacillus sp.	++	++
Pseudomonas sp.	++	+++
Bacillus sp.	++	+
Bacillus sp.	++	++
Pseudomonas sp.	+	+

Table 3: *Screening of potential biosurfactant producing bacteria using several methods of emulsification assays.*

Key: Drop-collapse: -, No collapse, + slow drop, ++ vigorous drop.

Oil spreading technique: -, no clear zone diameter, +, clear zone diameter >1<3 (mm), ++, clear zone diameter >3<6 (mm), +++, clear zone diameter>6 and < 9 (mm).

Bacterial Isolate	Petroleum H-C	Unsaturated Oil
Vibrio sp.	32 ± 0.5	40 ± 0.54
E. coli sp	43 ± 0.7	46 ± 0.62
Pseudomonas sp.	40 ± 0.56	43 ± 0.56
Klebsiella sp.	43.3 ± 0.6	51.2 ± 0.74
Pseudomonas sp.	40 ± 0.62	42 ± 0.54
Salmonella sp.	43.8 ± 0.5	40 ± 0.48
Bacillus sp.	30 ± 0.42	31 ± 0.34
Pseudomonas sp.	48.0 ± 0.40	47 ± 0.57
Bacillus sp.	46 ± 0.75	45 ± 0.54
Bacillus sp.	51 ± 0.54	39 ± 0.32
Pseudomonas sp.	53 ± 0.54	42 ± 0.48

Table 4: *Emulsification index (%E_{24}) of bacterial isolates on different hydrocarbons as the sole carbon source.*

N = 3. Control with normal 1% saline 15 ± 0.14.

Upon emulsification assays carried out on the isolated bacteria, only three isolates out of the five organisms showed higher emulsification potentials during the study. Three emulsification assay carried out which include drop collapse, oil spread plate and emulsification index test on the isolates using crude oil, kerosene and coconut oil as the sole carbon source showed the following results: Using drop collapse assay as described by Jain., *et al.* [12] two out of the three isolates from the contaminated soil scored very positive (++) in drop collapse assay and also in oil displacement assay using kerosene and coconut oil as sole carbon sources.

Culture suspensions on crude oil showed positive correlation but much weaker when compared to assay outcome with kerosene and coconut oil but significantly showed a high emulsification than the control experiments without the culture cells. Drops of cell free culture from *Streptomyces* sp. remained intact on glass slide coated with kerosene and crude oil after one hour but showed the weakest collapse with slide coated with coconut oil. This isolate is considered non biosurfactant producer.

They may have utilized hydrocarbon for the production of other metabolites such as bioactive compounds, enzymes etc [1,2]. The same trend was also seen during the oil spread plate assay on the isolated bacteria. Strains of *Bacillus*, *Klebsiella* and *Pseudomonas* sp. showed the highest in oil displacement. Also as stated afore oil such as kerosene and coconut oil showed much promising emulsifying feature with the identified organisms than that done by crude oil. Okpokwasili and Amanchukwu [18] in their impact assessment of biodegradation of crude oil with strains of *Candida* sp. stated that crude oil especially that of bonny light are complex mixtures of hydrocarbons comprising of PAHs, aliphatic hydrocarbons and centrally total petroleum hydrocarbons (TPH) which will take on a hydrocarbonolistic organism(s) to act on and utilize. Also *Streptomyces* sp. showed no positive reaction upon immersion on the oil water interface in all the used oil. This according to Mbachu., *et al.* [15], [17] in their research reported that strains of imperfecti bacteria such as the *Actinomycetes* have much compromised ability to produce surface active compounds. Upon assessment on isolates from contaminated water; similar trend of emulsification capacity were obtained as that of the isolates from contaminated soil.

Out of the eight bacteria isolates, only three showed greater emulsification potentials on all the three emulsification assays carried out which include drop collapse, oil spread plate and emulsification index assays. Strains of *Pseudomonas* and *Vibrio* sp. showed the highest emulsification in drop collapse and oil spread plate assay. Strains of *Salmonella* showed the least among all the isolates in the-afore tested emulsification assays. Emulsification studies by crude cultures of the isolates from the contaminated soil showed that *Klebsiella* sp. showed the best emulsification activity with 50.2%, 55.8% and 47.9% emulsification respectively using H-C and Unsaturated oil respectively. It was followed by *Pseudomonas* sp. with 48%, 54% and 45.6% emulsification activity for H-C and Unsaturated oil as sole carbon source. Strains of *Streptomyces* showed the lowest emulsification index in all the three tested oil. This when compared with the control experiment showed a sharp significant variation with preparations without the cell suspensions that showed emulsification activity of 13%, 19% and 5% indexes with H-C and Unsaturated oil respectively.

Conclusion

Emulsifying organisms (surface active producing species) are much efficient in eco-monitoring and pollution control exercises as they can help to effect environmental bioremediation. Strains of *Pseudomonas* sp., *Klebsiella* sp. and *Bacillus* sp. among all the isolates from diary waste water from Rumuekini showed greater positive indices in all the emulsification tests carried out. This portrays a much cost effective and efficient way of hydrocarbon cleansing and recovery during field drilling and explorations.

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Ethics

Authors declared no ethical issues that may arise after the publication of this manuscript.

Author's Contributions

Nwanjoku Helen Chioma: Conceived and designed the experiments, performed the experiment and processed the data, analyzed the data and wrote the manuscript.

Ebede Solomon O: Co-supervised the research and revised the manuscript.

Nwanjoku Kingsley O: Carried out the experiment and provided the logistics.

Nwokoye KU: Provided the experimental logistics and revised the manuscript.

Anikwe Uchenna F: Carried out the experiment and revised the manuscript.

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