Characterization of Bacteria Isolates from Surfaces of Corroded Zinc and Copper Coupons

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Abstract

This research characterized bacteria isolates from surfaces of corroded zinc and copper coupons. Corroded zinc and copper materials were collected carefully, from two different locations in Ken Saro-Wiwa Polytechnic Campus and were put in sterile McCartney bottles. Bacteriological analysis such as Gram staining, motility test, catalase test, indole test, oxidase test, methyl red test, Voges-Proskauer test, and citrate utilization test were carried out to ascertain the bacteria responsible for the corrosion. Two bacteria were isolated; Enterococcus which was seen to be responsible for the corrosion of zinc and Escherichia coli; which was responsible for the corrosion of copper.
It is recommended that materials that can inhibit biocorrosion should be used to control or treat metallic substances of corroded materials.

Keywords: Characterisation, bacteria, isolates, zinc coupon, copper coupon

1.0 INTRODUCTION

Metals deteriorate due to the many and different chemical attacks by various interactions within the immediate environment, and this is referred to as corrosion (Agwa *et al.*, 2017). Microorganisms like bacteria are known to be responsible for corroding metal surfaces (Maluckov, 2012).
Microbiologically induced corrosion (MIC) or biocorrosion is the process referring to the deterioration of metallic and non-metallic materials by the activity of microorganisms. Microorganisms adhere to nonliving and living tissue surfaces under submerged or moist natural conditions and in industrial environments exposed to moisture (Gu *et al.*, 2002). Microorganisms

adhesion to these surfaces changes the electrochemical characteristics of the material.
Complexity of the interaction which exists between microorganisms and metal surfaces as well as the different reaction kinetics between them has prevented a clear and sound understanding of the whole process. Basically, corrosion is an interfacial process. This is due to the settlement of microorganisms on metallic surfaces exposed to natural waters which can considerably modify the chemistry at the surface or solution interface compared with the composition of the bulk aqueous phase (Manga *et al.*, 2012). This interaction results to the forming of a layered structure often referred to as biofilm which can ultimately alter the corrosion resistance of the metallic substratum (Maluckov, 2012).
When metals such as zinc and copper are exposed to water, the water-borne microorganisms colonize its surface forming biofilm through a series of steps (Marangoni *et al.*, 2013). Many bacteria have long been shown to be associated with the process of biocorrosion since 1960's. According to Manga *et al.*, (2012) and Beech (2004), sulphate reducing bacteria (SRB), iron/manganese oxidising bacteria, iron reducing bacteria, sulphur oxidising bacteria and bacteria which produce organic acids and slimes are reported to be the dominant types of microorganisms microbiologically influencing corrosion in most terrestrial and aquatic habitats. These microbes are found in natural biofilm and they all contribute to the complex consortia of biofilm (Beech and Coutinho, 2003).
Although, the mechanism of the corrosion process has not been elucidated, bacterial biofilms have been implicated (Keevil *et al.*, 2001). Corrosion is a persistent challenge that is very hard to do away with completely, but easy to prevent and being achievable (Agwa *et al.*, 2017). Demonstration and characterization of this type of surface phenomenon under controlled conditions, however, have been difficult because of lack of analytical techniques (Lappin Scott and Costerton, 2006).
Carlos *et al.* (2019) investigated two hazardous bacteria isolated from a copper plumbing system using electrochemical impedance spectroscopy. Their conclusion was that the effect of Variovorax sp and Ralstronia pickettii on copper corrosion was inhibited in the tested conditions, increasing the protective properties of cuprites layer due to the development of biofilm and other copper oxides on the surface during the exposure time.
They further stated that there was a striking loss of variovorax sp biofilm formation capacity when interacting with Ralstonia pickettii, hence evidence to support the potential danger to tap water microbial contamination due to biofilm detachment of bacterial consortiums. For effective implementation there should be efficient monitoring and control strategies for the inhibition of biocorrosion. And it is very paramount to have knowledge of those microbial population which is responsible for the phenomenon, as well as the interplay of different microorganisms with metallic surfaces. Hence, this study is designed to characterized bacteria isolates from surfaces of corroded zinc and copper coupons.

2.0 MATERIALS AND METHODS

2.1 SAMPLE COLLECTION

Corroded zinc and copper samples were collected from back café and the water reservoir sites respectively at the Ken Saro-Wiwa Polytechnic, Bori, Rivers State, Nigeria and were put in separate sterile McCartney bottles. The samples were labelled and transported aseptically to the Department of Microbiology Laboratory, Rivers State University, Npolu-Oroworukwo, Port

Harcourt for bacteriological analysis.

2.2 SAMPLE PREPARATION

The stock analytical unit was prepared by weighing 1g of zinc and 1g of copper which were dispensed separately in 9ml of the diluents (normal saline solution) for easy examination to obtain pure cultures.

2.3 MICROBIOLOGICAL ANALYSIS

2.3.1 BACTERIA ENUMERATION

A serial six fold dilution was done on the weighed sample of corroded *zinc and copper separately* with dilution factor from10^{-1} to 10^{-6} dilution factor, then the third test tube 10^{-3} and fourth test tube 10^{-4} was used for the inoculation (Cheesbrough, 2005; Manga *et al.*, 2012)

2.3.2 INOCULATION AND INCUBATION

An aliquot (0.1ml) from two dilutions (10^{-3} and 10^{-4}) was plated in duplicates on Nutrient Agar, using the spread plate technique.

The plates were incubated at 37^{o}C for 16 to 24 hours. The colonies on the plates were counted and described morphologically. Colonies formed on Nutrient Agar was used to estimate the total heterotrophic bacterial count (THBC) (Cheesbrough, 2005; Manga *et al.*, 2012).

2.3.3 IDENTIFICATION OF TEST ORGANISM

Morphological and Biochemical test was conducted on the isolates for identification of the bacteria associated with corroded zinc and copper.

Biochemical tests such as Indole, Catalase test, Oxidase, Methyl red, Voges proskaure, Citrate Utilization test were carried out to confirm the isolates (Cheesbrough, 2005; Aditi *et al.*, 2017).

2.4 GRAM STAINING

This test was carried out and bacteria were grouped into Gram positive and Gram negative and also show the cellular morphologies and forms as described by Norris and Swain, (2007). Purple or violet colour showed Gram positive while pink or red colour showed Gram negative.

2.5 BIOCHEMICAL TESTS

2.5.1 OXIDASE TEST (FILTER PAPER METHOD)

This test identify whether an isolate contain the enzyme, Cytochrome oxidase (important in the electron transport chain). A little portion of the isolate (24 hours culture) was smeared on a filter paper impregnated with freshly prepared oxidase reagent (N, N-dimethyl-p-phenylenediamine). The reaction was observed within 10 seconds to see if there was any colour change. Deep purple colorations appeared within 5^{-10} seconds, which indicated a positive reaction, and a negative reaction was indicated by non-colour change (Shields and Cathcart , 2010).

2.5.2 MOTILITY TEST

The test was used in differentiating between motile and non-motile organisms. Semisolid strength nutrient agar was dispensed into test tubes, autoclaved and allowed to solidify. Using sterilized needle each isolate was inoculated by stabbing to half the depth of media and incubated at room temperature for about 48 hours. Growth that appeared away from the line of inoculation

was recorded as positive, while growth that confined to the line of stab was negative (Navena and Joy, 2014).

2.5.3 CATALASE TEST (SLIDE METHOD)

Catalase test was carried out to identify the isolates as they produce the enzyme, catalase. The enzyme that detoxified hydrogen peroxide broke it down into water and oxygen gas and bubbles were released. A sterile wire loop was used to transfer a loopful of the organism to a grease free slide emulsified with small distilled water. A drop of hydrogen peroxide (6%) was added and observed for effervescence within 3 seconds. The production of bubble indicates positive result and no bubble indicate negative (Elkins *et al.,* 2009).

2.5.4 METHYL RED TEST

This test was used to identify *E. coli*, by producing stable acid by mechanism of mixed acid fermentation of glucose.

Methyl red Voges-Proskauer (MRVP) broth (17 g) was suspended in 100ml distilled water. MRVP broth (5ml) was distributed into each test tube and autoclaved at 121oC for 15 minutes. A loopful of the test organism was inoculated into the broth and incubated for 48 hours. After incubation, 2-5 drops of methyl red indicator were added to the culture. Positive results indicated red colour as shown by E. coli and negative result indicated yellow colour (Nevena and Joy, 2014).

2.5.5 INDOLE TEST

This test was used to ascertain the ability of some isolates to hydrolyze the amino acid tryptophan to produce indole.

Tryptophan was made available by tryptone in the medium of 10ml of peptone water and dispensed in test tubes and sterilized by autoclaving. It was allowed to cool before inoculating isolates into the sterile broth. The broth culture was incubated at 37oC for 48 hours after which about 10 drops of Kovac's reagent was added into each of the culture test tubes .The test tubes were shaken and allowed to stand for 5 minutes. Positive result showed a red colour at the surface of the medium and negative result showed no red colour (Navena and Joy, 2014).

2.5.6 VOGES-PROSKAUER TEST

This test was used to detect acetone (an important physiological metabolite excreted by many microorganisms) in a bacteria broth culture. A loopful of the test organism was inoculated into MRVP broth and incubated for 24 hours. After incubation, about (10 drops) of a–naphthal and (10 drops) of potassium hydroxide were dropped into the broth culture and was shaken and allowed to stand for 15 minutes. Positive result indicated a pink or red colour at the surface of the medium while negative results indicated a copper colour at the surface of the medium (Navena and Joy, 2014).

2.5.7 CITRATE UTILIZATION TEST

Citrate utilization test was used in determining the ability of isolates to utilize sodium citrate as its only carbon source. Simmons citrate agar (Navena and Joy, 2014) was prepared according to manufacturer's instructions, transferred into test tubes and autoclaved. The tubes were slanted and allowed to cool and solidify. The slant was inoculated by touching the surface of the slant

from 18-24 hours .The tubes were incubated at 35oC for 18 to 24 hours.
The development of blue colour denoting alkalinisation was observed and recorded as positive, while negative result showed no blue colour (Navena and Joy, 2014).

2.6 MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF THE ISOLATES

The morphology and biochemical characteristics were used to describe and determine the identities of isolates. The bacteria isolates identified were based on colonial morphology such as size, colour, elevation, transparency, margin, texture and shape.
The biochemical characteristics include oxidase test, motility test, catalase test, methyl red test, indole test, Voges-Proskauer test and citrate utilization test as described earlier (Cheesbrough, 2005; Manga et al., 2012; Navena and Joy, 2014).

3.0 RESULTS AND DISCUSSION

3.1 RESULTS

After incubation, each bacterial isolates were identified by gram stain and biochemical tests. The results of microbial isolation and identification done through colonial morphology description, gram staining and biochemical tests are shown in Tables 1, 2 and 3 respectively.

Table 1: Morphological characteristics of isolates from corroded zinc and copper

Isolate Code	Shape	Elevation	Surface	Margin	Colour	Opacity	Size
Zn1	Spherical	Raised	Rough	Undulate	Whitish	Translucent	Small
Zn2	Circular	Raised	Rough	Entire	Milky	Opaque	Large
Cu1	Circular	Raised	Smooth	Entire	Milky	Opaque	Small

Table 2: Microbial isolates from corroded zinc and copper through Gram staining

Isolate Code	Gram Reaction	Shape	Suspected organism
Zn1	-	Rod	*Enterococcus*
Zn2	-	Rod	*Enterococcus*
Cu	-	Rod	*Escherichia coli*

Key: (-) = Negative

Table 3: Biochemical characteristics of isolates from corroded zinc and copper

Biochemical and Sugar Fermentation	Zn1	Zn2	Cu1
Catalase	-	-	+
Oxidase	-	-	-
Citrate test	-	-	-
Indole	-	-	+
Methyl Red	-	-	+
VP	+	+	-
Motility test	-	-	-
Coagulase	-	-	-
Glucose	AG	AG	AG
Lactose	AG	AG	AG
Mannitol	AG	AG	AG
Urease	-	-	-
Suspected Organism	*Enterococcus*	*Enterococcus*	*Escherichia coli*

KEY: A-Acid, G- Gas, (-) Negative, + Positive

3.2 DISCUSSION.

In the present study, the isolate with code Zn1 was spherical in shape, had a rough surface and a raised elevation. It was translucent and whitish in colour for its colony/morphology characteristics as shown in Table 1. The isolate with code Zn 2 was circular in shape, had a rough surface and raised elevation. This reconciled with report by Mahbubar et al., 2010 for shape of bacteria responsible for biocorrosion. It was opaque and milky in colour. Its colony/ morphology characteristics are shown in Table 2. The isolate Cu1 was circular in shape, had a smooth surface and raised elevation, with an entire margin.

It was opaque and milky in colour for its colony/morphology characteristics as shown in Table 2 (Bergey, 1984; APHA, 2010; Manga et al., 2012) The isolate of Zn1 exhibits rod shape, small size and showed pink colour which indicated gram negative in Gram staining reaction as shown in table 2. A pink colour was seen at the surface of the medium showing that the isolate reacted positively to the Voges-Proskauer Test in the biochemical test.

Table 4: Morphological and Biochemical characteristics of isolates from corroded zinc and copper

	Colony Characteristics						Gram Stain		Biochemical and Sugar Fermentation												
S/N & Isolate	Form/ Shape	Elevation	Surface	Margin	Colour	Opacity	Reaction	Shape	Catalase	Oxidase	Citrate	Indole	Methyl	VP	Motility	Coagulas	Glucose	Lactose	Mannitol	Urease	Suspected Organism
1.Zn 1	Spherical	Raised	Rough	Undulate	Whitish	Translucent	-	Rod	-	-	-	-	-	+	-	-	AG	AG	AG	-	*Enterococcus*
2.Zn 2	Circular	Raised	Rough	Entire	Milky	Opaque	-	Rod	-	-	-	-	-	+	-	-	AG	AG	AG	-	*Enterococcus*
3.Cu1	Circular	Raised	smooth	Entire	Milky	Opaque	-	Rod	+	-	-	+	+	-	-	-	AG	AG	AG	-	*Escherichia coli*

KEY: A-Acid, G- Gas, (-) Negative, + Positive

It was also able to ferment sugar in the glucose, lactose and Mannitol test by the liberation of acid and gas and hence Enterococcus was suspected (Bergey, 1984; APHA, 2010). Unlike the Zn2, Cu1 was circular, opaque and produced a milky colour. The isolate of Zn2 also possessed the same biochemical characteristics as Zn1 and hence, Enterococcus was also suspected (Bergey, 1984; APHA, 2010). The isolate with code Cu1 showed the same characteristics for the colony, biochemical and Gram staining as shown in tables 1, 2 and 3 respectively. The isolate was positive for the methyl red test by the exhibition of a characteristic red colour.

The production of bubbles for the catalase test also indicated that the isolate produced catalase. The isolate also proved to be positive to indole test by the exhibition of a characteristic red colour when tested with Kovac's reagent. The isolate from the tables shown above showed that the isolate was negative to the other biochemical tests carried out. The isolate with code Cu1 was also able to ferment glucose, lactose and Mannitol by the liberation of acid and gas. From the results of colony and biochemical tests, the suspected organism responsible for the corroded copper is Escherichia coli (Bergey, 1984).

Zn1 and Zn2 are the same in biochemical test but in morphological characteristics, they possess a different character which is seen in Table 1. The isolate code Zn1 was spherical in shape; undulate in margin, translucent in opacity, small in size and also whitish in colour. Zn2 was circular in shape, entire in margin, opaque in opacity, large in size and also milky in colour (Bergey, 1984). Zn 1 and Zn 2 are same because they reacted in the same manner to all the biochemical tests and Gram staining reaction that determine the identification of the suspected organisms which is Enterococcus (Bergey, 1984).

4.0 CONCLUSION

Biocorrosion is currently one of the most prevalent phenomenons in the environment and its effect can lead to complete deterioration of metallic substances. From the results of the analysis carried out, it can be understood and concluded that bacteria are associated with the corrosion of zinc and copper coupons. Bacteria such as Enterococcus was responsible for the corrosion of zinc from the two samples collected for zinc while Escherichia coli were responsible for the corrosion of copper.

It is therefore recommended that materials that can inhibit biocorrosion should be used to control or treat metallic substances of corroded materials.

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