Characterization and Antibiotics Sensitivity Pattern of Enterobacteriaceae in Obafemi Awolowo University Sewage Oxidation Pond

Sokan-Adeaga Micheal Ayodeji[1], Sokan-Adeaga Adewale Allen[2] and Sokan-Adeaga Eniola Deborah[3]

[1] Obafemi Awolowo University

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Abstract

Some members of the family Enterobacteriaceae were isolated from Obafemi Awolowo University sewage oxidation pond (target site) and characterize by various biochemical test (citrate test, Gram stain, catalase test, idole production, fermentation of sugar, starch hydrolysis, Nitrate reduction, oxidative-fermentative test, gelatin hydrolysis and Methyl Red Voges-Proskauer test); the antibiotic sensitivity pattern of the isolate were also carried out. All was done under an aseptic condition. The total bacterial count (TBC) obtained from the target site was higher than those obtained from the relative sites (1: before the sewage enter) and (2: after leaving) the oxidation pond. The biochemical tests identified the following: Citrobacter diversus, Salmonella arizonae, Typical Salmonella, Escherichia coli and Providencia alcalifaciens, all belonging to the family Enterobacteriaceae. Investigation of the antibiotic sensitivity test revealed that Cefuroxime and Ampicillin were not effective against all the bacteria isolates. However they each exhibit a varying degree of resistance and susceptibility to Ciprofloxacin, Tetracycline, Norfloxacin, Amoxycillin, Ofloxacin, Chloramphenicol, Gentamycin and Nitrofurantoin. Hence the study confirms that Obafemi Awolowo University sewage oxidation pond has a very high number of Enterobacteriaceae which show relative resistance to various antibiotics.

Index terms— enterobacteriaceae, sewage oxidation pond, total bacteria count, antibiotic sensitivity, biochemical characterization.

1 I. Introduction

he increased use of waters by humans especially as a receptacle for the disposal of human waste, the effects of added organic matters and pathogens are of public health concern (Prescott et al., 2005;Al-Bahry et al., 2011). Contamination of drinking water by faecal waste has led to a major epidemic of disease caused by water-borne pathogens (Nester et al., 2001;Felfoldi et al., 2010). Sewage has been recognized over the years as a major source of water contamination. In the urban environment, sewage discharges are major component of water pollution and contributes to oxygen demand and nutrient loading thereby promoting growth of toxic algae and the aquatic plants resulting in destabilization of aquatic ecosystem (Olajire and Impekperia, 2000;Morrison et al., 2001). Untreated domestic sewage contains large quantity of pathogenic organisms which are released into water bodies. These pathogens include: viruses, bacteria, protozoa and parasitic worms which are causative agents of many communicable diseases such as typhoid fever, diarrhea, amoebic dysentery, cholera and infectious hepatitis (Farmer and Kelly, 1991;Chiu, 2004;Gillespie et al., 2011). The heterogeneous composition of sewage

allows the development of diverse heterotrophic bacteria populations including the members of the "family Enterobacteriaceae" (Atlas and Bartha, 1997; Croxen and Finlay, 2010).

Enterobacteriaceae is a family of Gramnegative, facultatively anaerobic, non-spore-forming rods. Morphological and biochemical characteristics of this family include being motile, catalase positive, and oxidase negative; reduction of nitrate to nitrite; and acid production from glucose fermentation (Farmer and Kelly, 1991;Grimont and Grimont, 2006;Denton, 2007). However, there are also many exceptions. Currently, the family comprises 51 genera and 238 species. The number of species per genus ranges from 1 to 22. Twenty-two genera contain only one species, while seven genera have more than ten species (Brisse et al., 2006;Janda, 2006). Enterobacteriaceae is closest to Vibrionaceae and Pasteurellaceae as sister clades with all members except for the genera Arsenophonus and Thorsellia being clustered together in one clade (Borenshtein and Schauer, 2006). Of the 30 genera with two or more species, 21 are likely to be monophyletic based on clustering on 16 rDNA sequence and other data. However, seven genera are likely to be polyphyletic requiring further reclassification (Paradis et al., 2005;Pham et al., 2007, Auch, 2010). Enterobacteriaceae has been heavily sequenced from across the spectrum of the family diversity with 180 complete genomes covering 47 species and 21 genera. The genome size ranges from 422,434 bp, coding for just 362 ORFs, to 6,450,897 bp, coding for 5,909 ORFs T Characterization and Antibiotics Sensitivity Pattern of Enterobacteriaceae in Obafemi Awolowo University Sewage Oxidation Pond (Hedegaard et al., 1999; Konstantinidis and Tiedje, 2005). Enterobacteriaceae is ubiquitous in nature. Many species can exist as free living in diverse ecological niches, both terrestrial and aquatic environments, and some are associated with animals, plants, or insects only. These groups of microbes are pathogens in human, animal and/or plant causing a range of infections (Gillespie et al., 2011). There are numerous applications using members of Enterobacteriaceae including biocontrol in agriculture, production of numerous recombinant proteins and nonprotein products, control of infection diseases, anticancer agents, biowaste recycling, and bioremediation **??**Dento, 2007). Genome-based phylogeny and genomics are expected to further delineate the members of Enterobacteriaceae and refine the classification of the genera and species within this family (Pham et al., 2007).

Due to its high sanitary efficiency, treatment of waste water by stabilization ponds is recommended for sensitive coastal areas; it is suitable for peri-urban settings and requires large surface area (Picot et al., 1992). Oxidation (stabilization) pond is a simple scientifically designed pond with 2-6 feet depth, in which algal-bacterial growth in situ helps in the reduction of biochemical oxygen demand of wastewater (Ghrabi et al., 1993). These ponds are effective, low-cost and simple technology for the treatment of wastewater before it is discharged to an aquatic ecosystem (Mahajan et al., 2010) and are commonly used in tropical countries to purify wastewater. The efficiency of the pond depends on climatological conditions like light, temperature, rain, wind and also the wastewater quality. Oxidation pond typically operate in an extended aeration mode with long detention and solids retention time (Sperling and Lemos, 2005) and is a widely adopted technique for the treatment of domestic and industrial wastes. It is one of the methods widely used in the tropical areas of the world for treating wastewater (Hosetti and Frost, 1995). Oxidation pond comprises different groups of organisms such as bacteria, algae, protozoa, fungi, viruses, rotifers, nematodes, insects and crustacean larvae etc. which coexist and compete with each other (Nair, 1997). The bacteria present in the pond respire aerobically and anaerobically by decomposing the biodegradable organic content of the waste and release carbon dioxide, ammonia and nitrates (Tharavathy and Hosetti, 2003). These compounds are utilized by the algae, which together with sunlight and photosynthetic process releases oxygen, enabling the bacteria to breakdown more waste and accomplish reduction in BOD levels (Tharavathy and Hosetti, 2003). Initial research on oxidation ponds (1946 to 1960) describes pond activity in terms of mutualistic behaviour of algae and protozoa through photosynthesis (Nair, 1997). According to the conditions of the oxidation pond aerobic, facultative and anaerobic bacteria grow and stabilize the organic substances present in the wastes through biological processes (Hosetti and Frost, 1995).

Contemporary populations of enteric bacteria, when compared with those from the pre-antibiotics era, display a higher tolerance in their nonspecific responses to several antibiotics (Houndt and Ochman, 2000). The increase in antimicrobial resistance, observed in a bacterial population, may result from the clonal selection of organisms that tolerate sublethal antimicrobial doses and that present greater fitness under conditions of selective pressure, or from the spreading of resistance genetic determinants through horizontal gene transfer. The most plausible hypothesis is that, in the natural environment, both mechanisms are responsible for the dynamics of the bacterial population. In different environments, bacteria are expected to experience distinct selective pressures for antibiotic resistance and, hence, distinct patterns of antibiotic resistance acquisition and evolution. Urban wastewater treatment plants represent important reservoirs of human and animal commensal bacteria in which antibiotic resistance determinants and/or organisms persist in the final effluent and are released to the environment (Reinthaler et al., 2003;Tennstedt et al., 2003).

Previous works done on community sewage oxidation pond in tropical countries had centered only on the physicochemical characterization and microbiological examination at the family level. There is hardly any detailed documentation in available literature on the characterization and antibiotics sensitivity of the family Enterobacteriaceae isolated from a sewage oxidation pond in Nigeria. Thus the main objective of this study was to isolate and characterize the five genera of Enterobacteriaceae isolated from Obafemi Awolowo University sewage oxidation pond; and also assess their antibiotic sensitivity pattern.

2 II. Experimental Procedures a) Study Design

The study was experimental and laboratory based, involving morphological characteristics, biochemical characterization; and antibiotics sensitivity test of the bacterial isolates found in sewage samples.

3 b) Study Location

The study was carried out at Obafemi Awolowo University (O.A.U) Oxidation pond located at the outskirt of the O.A.U campus, Ile-Ife, Osun State, Nigeria.

4 c) Sample Collection

The sewage samples were collected with the aid of sterilized sampling bottles from three (3) sampling sites designated as follows: Point A: Where the sewage enter into the oxidation pond.

Point B: The Obafemi Awolowo University Oxidation Pond. Point C: Meeting point (confluent) between the effluent of the oxidation pond and the stream that flows along Ede road.

A long rope was tied around the neck of each bottle, covered and sterilized by autoclaving at 121 o C for 15 minutes. The bottles were allowed to gradually sinked into each sample collection point to collect each sewage sample. Each bottle were allowed to filled to the brim, bought out of the sewage sample collection point and immediately covered to prevent contamination from gaining entrance into the sewage sample. The sample bottles were labeled accordingly, transported to the laboratory and used immediately.

5 d) Isolation and Enumeration Procedure i. Serial Dilution of Sewage Sample and Plate Count

Pour plate dilution technique described by Seeley and Van Demark (1981) was used to determine the total bacterial count (TBC). The stock samples to be examined were thoroughly mixed to ensure the uniform distribution of the microbes in the sample. Point A: 1 in 100 dilution was used for sample from point A. Point B: 1 in 1000 dilution was used for sample obtained from point B. Point C:1 in 10 dilution was used for sample from point C.

After the samples have been serially diluted, 0.5ml of the different dilutions was aseptically transferred into each of the sterile -petridishes containing set, solidified Eosin Methylene Blue (EMB) Agar. The culture plates were inverted to avoid moisture droplets falling on the growth which could prevent formation of discrete colonies. The plate was the incubated at 37 o C for 24 hours. Before preparing pure isolates, viable counts were carried out on each of the three different plates (i.e. plate A, B and C), where the number of bacteria per ml of the sample were obtained by multiplying the number of colonies on each of the plate by each plate dilution factor.

ii

6 . Media for Isolation of Bacteria Isolates

The following media were used for the bacteria isolation viz:-Eosin Methylene Blue (EMB) Agar -Nutrient Agar -Normal Saline iii.

7 Isolation and Preparation of Stock Culture

The bacterial colonies were noted and counted; and five different colonies were differently transferred into five different sterile Petri dishes each containing a sterile EMB Agar. The plates were then inverted and incubated at 37 o C for another 24 hours.

Isolate from each of the five different plates were each transferred into five different tube containing a set nutrient agar in a slanting position. After which they were kept in the refrigerator from where they were picked for subsequent Biochemical Tests performed.

8 e) Morphological Characteristics (Identification Procedures)

For the morphological characteristics of the isolate (i.e. Enterobacteriaceae) only Gram Staining was carried out to know the shape of each of the five isolates since they are all Gram-negative based on the fact that they are all isolated from EMB Agar.

9 f) Biochemical Characterization of Bacterial Isolates

The following biochemical tests were carried out to characterize and identify the FIVE BACTERIA ISOLATES (Enterobacteriaceae) obtained.

i

10 . Catalase Test

This test is carried out to find out the production of catalase by bacteria isolate. The hydrogen peroxide is usually toxic and is decomposed immediately by the enzyme catalase as soon as it is formed. A loopful of

hydrogen peroxide was emulsified into the culture from the plate on a clean grease free slide. The occurrence of effervescence caused by the liberation of oxygen bubbles indicated a positive test (i.e. the presence of catalase in the culture under test).

This test was carried out to detect the production of the enzyme, catalase by an organism. The enzyme converts Hydrogen peroxide to Water and Oxygen as shown in the equation below: **??**——————————2H 2 O 2 –2H 2 O + O 2 ii. Citrate Utilization Test

The coliform bacteria may be differentiated by their ability to utilize citrate as a sole source of carbon. The culture was incubated at 37 o C for 2 -5 days and examined for change in colour of the bromothymol blue indicator. A change in colour of bromothymol blue indicator from green to blue indicates utilization of the citrate i.e. positive or otherwise negative.

11 g) Fermentation of Sugars

In this test, the prime concern is to determine what sugars are fermentable by the unknown. If the organism does ferment a particular sugar, acid will be produced and gas may be produced. The presence of the acid is detectable with a pH indicator. Gas production is revealed by the formation of a void in the inverted vial of the Durham tube. The fermentable sugars used are: Glucose, Mannitol, Maltose, Sucrose and Fructose.

Each tube of each fermentable sugar was inoculated with a loopful of the test organism from the 24-hour old peptone water culture and incubated at 37 o C for 7 days respectively. Observation were made daily for the production of acid and gas in each Durham.

12 h) Indole Test

This test is important in the differentiation of Coliforms and depends on the production of indole from trytophane by the bacterium. Tryptone water is used rather than from peptone water, but peptone water was used to prepare a 24-hour old broth culture.

Each tube containing sterile tryptone water was inoculated with a loopful of a broth culture of the organism. The tubes were incubated at 37 o C for 5 days. After, incubation, 0.5ml Kovac's reagent was added to the content of each tube, shaken gently, and then allowed to stand. A deep red colour developed in the presence of indole, which separated out in the alcohol layer. This is a positive reaction, otherwise indicates negative result.

13 i. Methyl Red Voges Praskauer (MRVP) Test

This test help to help distinguished the Coli and Aerogenes bacteria from each other. The MRVP medium prepared and distributed into test tubes plugged with cotton wool. Each tube was aseptically inoculated with a loopful of 24 hour-old culture of the isolate and incubated at 37 o C for 5 days. Uninoculated controls were also incubated. After incubation period, the content of each tube was aseptically divided into two portions labeled M and V respectively. To the portion labeled M, 5 drops of methyl red solutions was added. To the other portion labeled V, 0.5ml of 6% ?-napthol added, followed by 0.5ml of KOH. The content of each test tube was mixed thoroughly. The test tubes were allowed to stand for 5 minutes and observation was made for the formation of colour.

A development of red colour in the M portion indicates a positive reaction while development of yellow colour in the M Portion indicates a negative reaction. To the V portion, the development of a red colouration constitutes a positive reaction otherwise negative reaction.

14 ii. Oxidative-Fermentative Test

Bacteria which attack carbohydrate either do so aerobically (i.e. OXIDATIVELY) or anaerobically (i.e. FERMENTATIVELY). The carbohydrate most frequently used is dextrose but lactose, sucrose or any other carbohydrate may also be used.

For each carbohydrates, two tubes of medium was stab inoculated with a 24 hours -old culture. The surface of the medium in one tube was covered with sterile paraffin and later covered with a sterile cotton wool, while the surface of the medium in the other tube was only covered with a sterile cotton wool, the tubes were then incubated at 37 o C for up to 14-days and examined. A change in the colour of medium from green to yellow indicated acid production. While fermentative organisms produced acid in both tubes, oxidative organisms produced acid in the tube covered with sterile cotton wool only.

15 i) Nitrate Reduction Test

Many microorganisms are capable of reducing nitrate to nitrite or even further to hydroxylamine, ammonia or nitrogen. Thus an intermediary in the reaction is NITRITE and the first test applied was for its presence.

Each tube of nitrate medium was inoculated with a loopful of a 24hour-old peptone culture of the bacteria. Incubated at 37 o C for 5-days and examined for the presence of gas in the inverted Durham tube. Tubes without gas were were test for the presence of nitrite using Gries -Ilosvay reagent. A red, pink or maroon colour indicated a positive reaction otherwise negative. The negative tubes were further treated for the presence of residual nitrate by the addition of zinc dust.

16 j) Hydrolysis of Gelatin

The medium was dissolved in distilled water and boiled to dissolved completely after which twenty millilitres (20ml) was poured into sterile petridishes and allowed to solidified. 24 hours old culture of isolate was streaked across the plate and incubated at 37 o C for 5 days. After incubation, the plates were flooded with the reagent (mercuric chloride solution) and observation.

Unhydrolysed gelatin forms a white opaque precipitate with the reagent while hydrolysed gelatin appears therefore as a clear zone when flooded with the reagent.

17 k) Hydrolysis of Starch

Twenty millimetres of molten starch were poured into sterile petridishes and allowed to cool and set. The test organisms were each streaked across the surface of each of the plate containing the set starch agar. The plates were incubated at 37 o C for 5 days. After incubation, gram's iodine was flooded on the plates.

i. Antibiotics Sensitivity Test Medium: Sensitivity Test Agar (S.T.A) Peptone water ii. Preparation 48 grammes of S.T.A were dissolved in 1litre of distilled water. The mixture was warmed on a hot plate to dissolved. The medium was sterilized by autoclaving at 121 o C for 15 minutes and allowed to cool. Twenty millilitres of S.T.A were dispensed into sterile petridishes and allowed to set.

iii. Procedure A loopful obtained from a 24 hour-old culture of the organism was placed at one edge of the surface of the S.T.A. A sterile swab was used to spread the organism uniformly on the surface of the medium.

An antibiotics disc was aseptically removed with the aid of a sterile forceps and placed in the centre of the petridish, such that no air bubbles are trapped between the disc and the plate. The plates were then incubated at 37 o C for 24 hours and the diameters of clear zones of inhibition around the discs were measured in millilitres with a transparent ruler. Zones with diameter of less than 2mm were recorded as resistant while, those with 2mm and greater were taken to be susceptible or sensitive.

18 III. Results

19 a) Features of Sewage Samples Examined

A total of three samples were taken from three different sampling sites designated as point: A, B and C. The features of each sample was noted and observed. The observed features are shown in Table **??**.

20 Table 1: Features of sewage samples from the sample collection sites b) Total Bacterial Counts in Sewage Samples

The result of the total bacteria counts in each sewage sample obtained/collected from the three different sampling sites is shown in Table **??**.

Table **??** The sewage sample obtained from point B has the highest number of Bacterial population, followed by that from point C while that from point A has the least total bacterial count (TBC). Total bacterial population in descending order: point B > point C > point A.

21 c) Observed Growth of Bacterial Colonies on Eosin Methylene Blue (EMB) Agar

The cultural/morphological characteristics of the bacteria isolates obtained from point B (O.A.U oxidation pond) is depicted below in Table 3. Table 3 above shown all the five (5) isolates which appear pink, hence they are all Gram-negative (-) and also appeared rod in shape and are all straight. Hence all five isolates: 1,2,3,4 and 5 are Gram-negative and straight rods.

22 d) Biochemical Characterization

Results obtained from the biochemical characterization of bacteria isolates from the sewage sample collected from Obafemi Awolowo University oxidation pond (point B).

i. Catalase Test Table 4 showed that all the five isolates were positive for catalase test. This implies that all isolates were able to synthesize the enzyme catalase.

ii. Citrate Utilization Test All the isolates except isolate 4 (which is citrate negative) had the ability to utilize citrate as the sole source of carbon as the citrate broth of all the isolates (except isolate 4) changed from green to blue colour at the end of the incubation period (i.e. 5 days) as shown on Table 4.

iii. Indole Production Isolates: 1, 4 and 5 were positive for indole production, while Isolates: 2 and 3 were negative for indole production. In these positive tubes, there were production of indole as there were changes in colour in these tubes from yellow to a deep red colour in the upper layer of these tubes from yellow to a deep red colour in the upper layer of these tubes, after the addition of Kovac's indole reagent after 5 days incubation period, as shown on Table 4.

23 iv. Methyl Red and Voges Proskauers Tests (MRVP Test)

Table 4 showed that all isolates were positive for methyl red test as the isolates changed the colour of their medium to red on addition of methyl red while all the isolates were negative for voges Proskauers Test as indicated by the cream colour obtained in all the tubes inoculated with the isolate each on addition of Barritt's reagent.

v. Nitrate Reduction Test Table 4 shown Isolates: 1, 3, 4 and 5 reduced Nitrate to Nitrite while Isolate: 2 produced gas in the inverted durham tube which implied the complete reduction of nitrate to nitrogen gas production.

vi.

24 Hydrolysis of Gelatin

Isolates: 1, 3, 4 and 5 were not able to hydrolyze gelatin on addition of mercuric chloride after the 5 days incubation period as white opaque precipitates were formed. While isolate: 2 was able to hydrolyzed gelatin on addition of mercuric chloride after 5 days incubation period as a clear zone was produced as depicted on Table 4.

25 vii. Hydrolysis of Starch

Isolates: 1 and 4 were able to hydrolyzed starch with the presence of a blue-black colouration which developed on flooding the plates with Gram's iodine while isolates: 2, 3 and 5 were not able to hydrolyze, as a clear zone were formed on flooding the plates with Gram's iodine after the 5 days incubation period Table 4. The results on Table 5 shown that all the isolate were facultatively anaerobic as they each produced acid (i.e. colour change from green to yellow) in both the oxidative and fermentative tubes at the end of 14 days incubationperiod.

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27 viii. Fermentation of Sugars

All the isolate fermented Glucose and Fructose with the production of acid and gas. Mannitol was fermented by all the isolates (except isolate 5) with the production of acid and gas. Maltose was fermented only by isolates: 1, 3 and 4 (but not by isolates: 2 and 5) with the production of acid and gas. Sucrose was only fermented with only the production of acid but without gas production by isolates: 1, 4 and 5 (but not by isolate: 2 and 3) as shown in Table 6. 7 showed the antibiotics sensitivity pattern of the bacterial isolates as obtained on the PD 002 Gram-negative Discs.

Isolate 1 were sensitive to Ciprofloxacin, Tetracycline, Norfloxacin, Amoxycillin, Of loxacin and Gentamycin but resistant to Chloramphenicol, Cefuroxime, Ampicillin and Nitrofurantoin.

Isolate 2 were sensitive to Ciprofloxacin, Norfloxacin, Ofloxacin and Gentamycin but resistant to Tetracycline, Amoxycillin, Chloramphenicol, Cefuroxime, Ampicillin and Nitrofurantoin. Isolate 3 were sensitive to Ciprofloxacin, Tetracycline, Norfloxacin and Ofloxacin but were resistant to Amoxycillin, Chloramphenicol, Cefuroxime, Ampicillin, Gentamycin and Nitrofurantoin.

Isolate 4 were sensitive to Ciprofloxacin, Tetracycline, Norfloxacin, Amoxycillin, Ofloxacin, Chloramphenicol, Gentamycin and Nitrofurantoin but were resistant to Cefuroxime and Ampicillin. The results of the total Enterobacteriaceae count in sewage samples showed that the number of Enterobacteriaceae in point A is very low, which is probably due to the inability of the bacteria to reproduce or proliferate and dispersity of the bacteria because of the speed by which the sewage is flowing into the oxidation pond caused by the high pumping pressure that is being use to conveyed the sewage from the O.A.U Community through the sewers to the oxidation pond. The population of Enterobacteriaceae in point B is very high, this is probably due to the favourable environmental conditions such as the alkalinity of the environment, optimal growth temperature (20 -38 o C), high concentration of organic matter, mutual association between the pond algae and the bacteria and the stagnancy of the pond. The population of Enterobacteriaceae in point C is intermediate between the numbers of Enterobacteriaceae in point A and point B which is due to the poor infrastructure facilities in the pond and the poor disinfection of the effluent from the oxidation pond.

The results of the biochemical characterization of the Enterobacteriaceae isolated from Obafemi Awolowo University sewage oxidation pond showed that: Citrobacter diversus, Salmonella arizonae, Typical salmonella, Escherichia coli and Providencia alcalifaciens were all catalase positive, vogespraskauer negative, citrate positive except E.coli that was citrate negative, methyl red positive, indole positive except Salmonella arizonae and Typical Salmonella that were indole negative, thus corroborating the results of the work carried out by Balows (1991) and Brooks et al., (2003). The results also showed that all the Enterobacteriaceae isolated from Obafemi Awolowo University sewage oxidation pond were capable of nitrate reduction. Citrobacter diversus, and Typical Salmonella, E. coli and P. alcalifaciens were only capable of reducing nitrate in nitrite, while S.arizonae was capable of complete reduction of nitrate to nitrogen gas production. The results also showed that the isolated Enterobacteriaceae were facultative anaerobes. Several workers (Farmer and Kelly, 1991;Gillespie et al., 2011) also reported similar results for all Enterobacteriaceae.

The results and this investigation also showed that all the isolated Enterobacteriaceae fermented sugars such as glucose and fructose producing acid and gas, while all the isolated Enterobacteriaceae except P. alcalifaciens, fermented mannitol to produced acid and gas. Maltose was fermented with the production of acid and gas by

all the isolated Enterobacteriaceae except S. arizonae and P. alcalifaciens. Sucrose was only fermented with the production of acid and no gas production by all the isolated Enterobacteriaceae except S. arizonae and Typical Salmonella, corroborating the results of the work carried out by Madigan et al., (1997); Brock and Madigan, (1998) and Madigan et al., (2008) on Enterobacteriaceae. The results of Gelatin hydrolysis showed that all the isolated Enterobacteriaceae except S. arizonae were Gelatin-negative, while the results of the starch hydrolysis showed that C. diversus and E.coli were capable of starch hydrolyses, while S. arizonae, Typical Salmonella and P. alcalifaciens were not able to hydrolyse starch.

Enterobacteriaceae form part of the normal flora of the intestinal tract of man and animals (Denton, 2007). The Enterobacteriaceae encountered in this study were tested against antibiotic disc to determine their relative susceptibility. The results of the antibiotic sensitivity test showed that Ciprofloxacin, Norfloxacin and Of loxacin were effective against all the Enterobacteriaceae isolates except Providencia alcalifaciens that proved resistant to the antibiotics. Gentamycin proved effective against all the Enterobacteriaceae isolates except Typical Salmonella that was resistant to the antibiotic. However, all the Enterobacteriaceae isolates were resistant to Cefuroxime and Ampicillin, while all the Enterobacteriaceae isolates except Escherichia coli proved resistant to Chloramphenicol, and Nitrofurantoin. Similar results were obtained for Enterobacteriaceae and some other bacteria by previous workers (Paterson, 2006;Pitout, 2008).

The relative resistance of C. diversus, S. arizonae, and Typical Salmonella, E. coli and P. alcalifaciens towards antibiotics treatment is of great public health concern. Previous reports have also indicated that some Coliforms bacteria isolated from raw sewage and sewage effluents exhibit resistance to a number of antibiotics and that the resistant strains were capable of transferring their resistance to susceptible C. diversus, S. arizonae, Typical Salmonella, E.coli and P. alcalifaciens (Houndt and Ochman, 2000). In a comparative study of three activated sludge treatment plants, Reinthaler et al. (2003) concluded that, although no significant increases in antibiotic resistance phenotypes were observed over the course of sewage treatment, this process may contribute to the dissemination of resistant bacteria to the environment. In addition, Tennstedt et al. (2003) reported the presence of antibiotic resistance determinants in self-transmissible genetic elements of bacteria residing in the activated sludge and final effluent released from a wastewater treatment plant.

The high rate of antibiotics resistant in isolates recovered from the sewage oxidation pond is of concern because it may suggest the ineffectiveness of these drugs in the treatment of infections caused by these organisms. The sewage entering the oxidation pond of Obafemi Awolowo University is contributed by some heterogeneous group of people including students, workers and farmers who live or work on the campus. Thus, one may suggest that the antibiotics pattern of the Enterobacteriaceae isolates obtained in this work is a reflection of the nature of the faecal materials from the population. There is high tendency that successive abuse of antibiotics by some, if not many of these peoples on many occasions must have contributed to the development of resistant features by the Enterobacteriaceae isolates obtained in this work. This suggests caution in the use of antibiotics in the treatment of infections caused by the isolated Enterobacteriaceae.

28 V. Conclusions

The aim of this research was to isolate, characterize and screen the antibiotics sensitivity pattern of the Enterobacteriaceae in the Obafemi Awolowo University (O.A.U) sewage oxidation pond. This study was able to identified five different species belonging to the family Enterobacteriaceae from the oxidation pond. The species were Citrobacter diversus, Salmonella arizonae, typical salmonella, Escherichia coli and Providencia alcalifaciens; and these organisms are usually associated with intestinal infections which may spread to other parts of the body. These species also showed relative resistance to antibiotics treatment, and thus pose serious public health challenge.

Since the effluents from this oxidation pond are discharge into a nearby stream which may be used by villagers living along the stream flow, the University authority should pay an immediate attention to the improvement of these ponds to safeguard the health of the villagers and other people who may have contact with the stream. Thus, the sewage oxidation pond should be properly manages and maintains for effective performance. The effluents from the oxidation pond should be disinfected properly by adequate chlorination before discharging it into the environment.

29 Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this research paper.

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Dilution of Sample	Temperature of Incubation	Point of sample Collection	Total Bacteria Count in (CFU/ml)
10 -2	37 o C	A: Point of entry of sewage.	1.40 × 10 1
10 -4	37 o C	B: O.A.U oxidation pond	8.29 × 10 2
10 -1	37 o C	C: Discharge of sewage effluent into stream along Ede road.	2.98 × 10 2

Figure 1: :

3

Characteristics	Test	1	2	3	Isolates
Cultural Agar Colonies	Medium	EMB	EMB	EMB	Distinct Circular Raised with convex
	Position	Distinct	Distinct		
	Shape	Circular	Circular		
	Elevation Size	Raised with convex level Small	Raised with convex level Small		
	Margin	Entire	Entire		
	Surface	Smooth and glistering	Smooth and glistering		
	Pigment	Dark green	Green		
	Medium	NA	NA	NA	
	Size	Small	Small	Small	
Cultural Agar Colonies	Margin	Entire	Entire	Entire	
	Surface	Smooth	Smooth	Smooth	
	Elevation	Low convex level	Low convex level	Low convex level	
	Pigment	Cream colour	Cream colour	Cream colour	
Morphological Test	Gram stain	-Rod (straight)	-Rod (straight)	-Rod (straight)	
	Cell form				

EMB: Eosin Methylene Blue Agar

NA: Nutrient Agar

Figure 2: Table 3 :

4

Biochemical Reaction	Isolates 1	2	3	4	5
Catalase Test	+ ve	+ ve	+ ve	+ ve	+ ve
Citrate Utilization Test	+ ve	+ ve	+ ve	-ve	+ ve
Indole Production	+ ve	-ve	-ve	+ ve	+ ve
Methyl Red	+ ve	+ ve	+ ve	+ ve	+ ve
Voges Proskauer	-ve	-ve	-ve	-ve	-ve
Hydrolysis of Gelatin	White opaque (-ve)	Clear zone (+ ve)	White opaque (-ve)	White opaque (-ve)	White opaque (-ve)
Nitrate Reduction Test	Nitrate to Nitrite	Complete reduction of nitrate (Gas produced in inverted durham tube).	Nitrate to Nitrite	Nitrate to Nitrite	Nitrate to Nitrite
Starch Hydrolysis	Blue-black (+ ve)	Clear zone (-ve)	Clear zone (-ve)	Blue-black (+ ve)	Clear zone (-ve)

Figure 3: Table 4 :

5

Isolates (Bacteria)	Oxidative medium	Fermentative medium	Conclusion
1	Changed from green to yellow with gas produced.	Changed from green to yellow with gas produced.	Facultative Anaerobes
2	Changed from green to yellow.	Changed from green to yellow with gas produced.	Facultative Anaerobes
3	Changed from green to yellow with gas produced.	Changed from green to yellow.	Facultative Anaerobes
4	Changed from green to yellow with gas produced.	Changed from green to yellow with gas produced.	Facultative Anaerobes
5	Changed from green to yellow with gas produced.	Changed from green to yellow.	Facultative Anaerobes

Figure 4: Table 5 :

6

Carbon Source	Reaction Given by Bacterial Isolates 1	2	3	4	5
Glucose	AG	AG	AG	AG	AG
Mannitol	AG	AG	AG	AG	AG
Maltose	AG	NIL	AG	AG	NIL
Sucrose	A	NIL	NIL	A	A
Fructose	AG	AG	AG	AG	AG

Note: A: Acid Production
G: Gas Production

[Note: NIL: No Production of Acid and Gas e) Antibiotics Sensitivity TestTable]

Figure 5: Table 6 :

7

Isolate 5 were sensitive to Gentamycin but resistant to Ciprofloxacin, Tetracycline, Norfloxacin, Amoxycillin, Ofloxacin, Chloramphenicol, Cefuroxime, Ampicillin and Nitrofurantoin.

Disc Code	Antibiotics	Concentration (µg)	Diameter of Zones of Inhibition of Isolates 1	2	3	4	5
	CIP	5	18	22	20	18	0
	TE	50	18	0	20	18	0
PD 002 Gram-	NB	10	15	12	6	18	0
negative URINE	AX	20	4	0	0	19	0
LEVEL TYPE 2	OF	5	25	23	23	19	0
	C	10	0	0	0	20	0
	CF	30	0	0	0	0	0
	AM	25	0	0	0	0	0
	GN	10	12	12	0	18	20
	N	100	0	0	0	17	0

Note: CIP -Ciprofloxacin
TE -Tetracycline
NB -Norfloxaci
AX -Amoxycillin
OF -Ofloxaci
C -Chloramphenico
CF -Cefuroxime
AM -Ampicillin
GN -Gentamycin
N -Nitrofurantoin

Figure 6: Table 7 :

[Microbiology] , *Microbiology* p. . (3rd edition)

[Nester et al. ()] , E W Nester , D G Anderson , C Roberts , Jr , N N Pearshall , M T Nester . 2001.

[Brooks et al. ()] , G F Brooks , J S Butel , S A Morse . 2003.

[Madigan et al. ()] , M T Madigan , J M Martinko , P V Dunlap , D P Clark . 2008.

[Pitout ()] , J D Pitout . 2008.

[Hosetti and Frost ()] 'A review of the sustainable value of effluents and sludges from wastewater stabilization ponds'. B B Hosetti , S Frost . *Ecol Eng* 1995. 28 p. .

[Olajire and Impekperia ()] 'A study of water quality of the Osun River; metal monitoring and geochemistry'. A A Olajire , T A Impekperia . *Chemical Society Ethiopia* 2000. 14 p. .

[Reinthaler et al. ()] 'Antibiotic resistance of E. coli in sewage and sludge'. F F Reinthaler , J Posch , G Feierl , G Wust , G Haas D Ruckenbauer , F Mascher , E Marth . *Water Res* 2003. 37 p. .

[Morrison et al. ()] *Assessment of the impact of point source pollution from the Kieskanmahoek sewage treatment plant on the Kieskanmahoek river -ph, electrical conductivity, oxygen demanding substance and nutrients*, G Morrison , O S Fatoki , Persson , A Ekberg . 2001. Water S.A. 27 p. .

[Tharavathy and Hosetti ()] 'Biodiversity of algae and protozoa in a natural waste stabilization pond: A field study'. N C Tharavathy , B B Hosetti . *J Environ Biol* 2003. 24 (2) p. .

[Sperling and Lemos ()] *Biological wastewater treatment in warm climate regions*, V M Sperling , D C Lemos . 2005. London: IWA Publishing.

[Madigan et al. ()] *Brock biology of microorganism*, M T Madigan , J M Martinko , J Parker . 1997. New Jersey: Prentice -Hall International. p. . (8th edition)

[Brock Biology of Microorganisms] *Brock Biology of Microorganisms*, New York, USA: Pearson Higher Education. (12th edn)

[Picot et al. ()] 'Comparison of the purifying efficiency of high rate algal pond with stabilization pond'. B Picot , A Bablaoui , B Moersidik , Baleux , J Bontoux . *Water Science Technology* 1992. 25 p. 12.

[Felfoldi et al. ()] 'Detection of potentially pathogenic bacteria in the drinking water distribution system of a hospital in Hungary'. T Felfoldi , Z Heeger , M Vargha , K Marialigeti . *Clin Microbiol Infect* 2010. 16 p. .

[Auch et al. ()] 'Digital DNA-DNA hybridization for microbial species delineation by means of genome-togenome sequence comparison'. A F Auch , Jan M Klenk , H P Goker , M . *Stand Genomic Sci* 2010. 2 p. .

[Farmer and Kelly ()] 'Enterobacteriaceae'. J J I Farmer , M T Kelly . *Manual of clinical microbiology*, A Balows, Wjj Hausler, K L Herrmann, H D Isenberg, H J Shadomy (ed.) (Washington, DC) 1991. American Society for Microbiology. p. . (5th edn)

[Denton ()] 'Enterobacteriaceae'. M Denton . *Int J Antimicrob Agents* 2007. 29 (3) p. . (Suppl)

[Konstantinidis and Tiedje ()] 'Genomic insights that advance the species definition for prokaryotes'. K T Konstantinidis , J M Tiedje . *Proc Natl Acad Sci* 2005. 102 p. .

[Gillespie et al. ()] J J Gillespie , A R Wattam , S A Cammer , J L Gabbard , M P Shukla , O Dalay , T Driscoll , D Hix , S P Mane , C Mao , E K Nordberg , M Scott , J R Schulman , E E Snyder , D E Sullivan , C Wang , A Warren , K P Williams , T Xue , H S Yoo , C Zhang , Y Zhang , R Will , R W Kenyon , B W Sobral . *PATRIC: the comprehensive bacterial bioinformatics resource with a focus on human pathogenic species*, 2011. 79 p. .

[Hedegaard et al. ()] 'Identification of Enterobacteriaceae by partial sequencing of the gene encoding translation initiation factor 2'. J Hedegaard , S A Steffensen , N Norskov-Lauritsen , K K Mortensen , H U Sperling-Petersen . *Int J Syst Bacteriol* 1999. 49 p. . (Pt 4)

[Houndt and Ochman ()] 'Long-term shifts in patterns of antibiotic resistance in enteric bacteria'. T Houndt , H Ochman . *Appl Environ Microbiol* 2000. 66 p. .

[Balows ()] *Manual of Clinical Microbiology*, G I Balows . 1991. p. . (5th edition)

[Jawetz] *Mel nick and Adeberg's Medical Microbiology*, Jawetz . McGraw-Hill Publishers. 198 p. . (23rd Ed)

[Atlas and Bartha ()] *Microbial ecology fundamentals and applications*, R M Atlas , R Bartha . 1997. Benjamin/ Cumming Publishing Company Incorporation. California. (4th edition)

[Prescott et al. ()] *Microbiology*, L M Prescott , J P Harley , D A Klein . 2005. New York: McGraw Hill. p. .

[Croxen and Finlay ()] 'Molecular mechanisms of Escherichia coli pathogenicity'. M A Croxen , B B Finlay . *Nat Rev Microbiol* 2010. 8 p. .

[Multiresistant Enterobacteriaceae: new threat of an old problem Expert Rev Anti Infect Ther] 'Multiresistant Enterobacteriaceae: new threat of an old problem'. *Expert Rev Anti Infect Ther* 6 p. .

[Janda et al. ()] 'New members of the family Enterobacteriaceae'. J M Janda , M Dworkin , S Falkow , E Rosenberg , K H Schleifer . *The prokaryotes: proteobacteria: gamma subclass*, E Stackebrandt (ed.) (New York) 2006. Springer. 6 p. . (3rd edn)

[Tennstedt et al. ()] 'Occurrence of integronassociated resistance gene cassettes located on antibiotic resistance plasmids isolated from a wastewater treatment plant'. T Tennstedt , R Szczepanowski , S Braun , A Pühler , A Schlüter . *FEMS Microbiol Ecol* 2003. 45 p. .

[Al-Bahry et al. ()] 'Opportunistic pathogens relative to physicochemical factors in water storage tanks'. S N Al-Bahry , A E Elshafie , R Victor , I Y Mahmoud , J A Hinai . *J Water Health* 2011. 9 p. .

[Pham et al. ()] 'Phylogeny and species identification of the family Enterobacteriaceae based on dnaJ sequences'. H N Pham , K Ohkusu , N Mishima , M Noda , M Shah , X Sun , M Hayashi , T Ezaki . *Diagn Microbiol Infect Dis* 2007. 58 p. .

[Paradis et al. ()] 'Phylogeny of the Enterobacteriaceae based on genes encoding elongation factor Tu and F-ATPase beta-subunit'. S Paradis , M Boissinot , N Paquette , S D Belanger , E A Martel , D K Boudreau , F J Picard , M Ouellette , P H Roy , M G Bergeron . *Int J Syst Evol Microbiol* 2005. 55 p. .

[Brock and Madigan ()] *Public prentice hall international editions London*, T D Brock , M T Madigan . 1998. p. . (Biology of micro organisms)

[Paterson ()] 'Resistance in gram-negative bacteria: Enterobacteriaceae'. D L Paterson . *Am J Infect Control* 2006. 34 p. .

[Nair ()] 'Role of organisms in sewage treatment I: Types of organisms'. G Nair . *Proc Acad Environ Biol* 1997. 6 (1) p. .

[Chiu et al. ()] 'Salmonella enterica serotype Choleraesuis: epidemiology, pathogenesis, clinical disease, and treatment'. C H Chiu , L H Su , C Chu . *Clin Microbiol Rev* 2004. 17 p. .

[Al-Hasan et al. ()] 'Temporal trends in Enterobacter species bloodstream infection: a population-based study from'. M N Al-Hasan , B D Lahr , J E Eckel-Passow , L M Baddour . *Clin Microbiol Infect* 2011. 1998-2007. 17 p. .

[Borenshtein and Schauer ()] 'The genus Citrobacter'. D Borenshtein , D B Schauer . *The prokaryotes: proteobacteria: gamma subclass*, M Dworkin, S Falkow, E Rosenberg, K H Schleifer, E Stackebrandt (ed.) (New York) 2006. Springer. 6 p. .

[Grimont and Grimont ()] 'The genus Enterobacter'. F Grimont , P A D Grimont . *The prokaryotes: proteobacteria: gamma subclass*, M Dworkin, S Falkow, E Rosenberg, K H Schleifer, E Stackebrandt (ed.) (New York) 2006. Springer. 6 p. .

[Brisse et al. ()] 'The genus Klebsiella'. S Brisse , F Grimont , P A D Grimont . *The prokaryotes: proteobacteria: gamma subclass*, M Dworkin, S Falkow, E Rosenberg, K H Schleifer, E Stackebrandt (ed.) (New York) 2006. Springer. 6 p. .

[Ghrabi et al. ()] 'Treatment of wastewater by stabilization ponds-Application to Tunisian conditions'. A Ghrabi , M Ferchichi , C Drakides . *Wat Sci Tech* 1993. 28 (10) p. .

[Mahajan et al. ()] 'Wastewater treatment at winery industry'. C S Mahajan , S D Narkhade , V A Khatik , R N Jadhav , S B Attarde . *Asian J Environ. Sci* 2010. 4 (2) p. .