Advanced Journal of Microbiology Research Volume 2013
Available online at http://advancedresearchjournals.org/AJMR

Full Length Research Paper

Isolation of plasmid-DNA from synthetic detergent degraders in wastewater from a tropical environment

Olusola Abayomi Ojo[1]* and Benjamin A. Oso[2]

[1]Department of Microbiology, Lagos State University, Badagry Expressway, Lagos-Nigeria.
[2]Department of Botany and Microbiology, University of Ibadan, Ibadan, Nigeria.

Accepted 18 February, 2012

The 'Tens Mini–prep' (Alkali – lysis) and the large – scale plasmid–DNA isolation methods were used to extract bacterial plasmids from the synthetic detergent–degraders from a tropical wastewater environment. The plasmid-profile of selected bacterial detergent–degraders was successfully detected with the large-scale (Maxi. Prep.) plasmid preparation method and the curing of the plasmids was successfully conducted which showed that the isolates harbor single plasmids (14 – 15 kbp) within the size range of the – PST marker (0.2 – 12 kbp) used. It was evident that the genetic information for detergent–hydrocarbon utilization was not plasmid mediated since the cured isolates grew on detergent supplemented medium after plasmid was removed. The bacterial and fungal isolates were characterized and identified by standard and conventional methods. Future studies with PCR and DNA sequence analysis would reveal the DNA fingerprint of each species of the detergent – degraders; this would enhance the processes of surveillance for these organisms in similar ecosystems and the detection of new serotypes as well as assist in environmental impact assessment (EIA) study for sustainable development.

Key words: Biodegradation, detergents, linear alkylbenzene sulphonate, plasmid-DNA, sustainable development.

INTRODUCTION

Detergents are cleaning products derived from synthetic organic chemicals. The cheapness of detergent production from petrochemical sources with its ability to foam when used in acid or hard water gives it an advantage over soaps (Okpokwasili and Nwabuzor, 1988). Surfactants are the components mainly responsible for the cleaning action of detergents. In commercial detergents, the surfactant component is between 10 and 20%. The other components include bleach, filler, foam stabilizer, builders, perfume, soil- suspending agents, enzymes, dyes, optical brighteners and other materials designed to enhance the cleaning action of the surfactant (Swisher, 1975; Okpokwasili and Nwabuzor, 1988).

Linear alkylbenzene sulphonates (LAS) is a commonly used anionic surfactant in detergents and it is easily biodegraded than non-linear alkylbenzene sulphonate (ABS) even though, total biodegradation still requires several days (Gledhill, 1975; Nomura et al., 1998).

Surfactants constitute a major ingredient of detergent components. Usually surfactants are disposed after use to sewage treatment plants (STPs). Here, biodegradation processes and adsorption on sludge particles remove these chemicals from wastewaters to a greater or lesser extent, depending on the chemical structure of the surfactant molecule and on the operating conditions of the STP. After treatment, residual surfactants, refractory co-products, and biodegradation products dissolved in STPs effluents or adsorbed on sludges are discharged into the environment. These chemicals through several transport mechanisms enter the hydro-geological cycle. Assessment of the environmental contamination levels of surfactants and related compounds is achieved through a wide range of laboratory biodegradation tests and eco-toxicological studies (Di Corcia, et al., 1999b). Since microorganisms are ubiquitous, it is important that effective detergent-degrader must elicit effective metabolic system whose degree of efficiency is determined by the quality of its genome.

*Corresponding author. E-mail: solayom@yahoo.com.

Plasmids are widely distributed throughout the prokaryotes; they vary in size from less than 1 x 106 D to greater than 200 x 106 D, and are generally dispensable. Plasmids are autonomous genetic elements that can replicate independently of the mainchromosome and can be separated physically from it in the laboratory. Plasmids often encode additional properties for the cell, such as antibiotic-resistance and metabolic activities. Bacterial plasmids are generally circular dsDNA molecules that are covalently closed in each strand (Towner and Cockayne, 1995). The only exceptions to this rule are the so-called 'linear' plasmids found in *Borrelia* sp. (Barbour and Garon, 1987) and *Sreptomyces* sp. The plasmid content of a cell normally comprises less than 5% of the total DNA (Towner and Cockayne, 1995).

Currently, there are many methods for isolating pure plasmid DNA but the method described by Birnboim and Doly, (1979), is often used. However, isolation of plasmids from Gram-negative and Gram-positive bacteria requires slightly different approaches, and different laboratories have their own preferences from amongst the various isolation methods.

MATERIALS AND METHODS

Sources of wastewater samples

Wastewater samples were obtained from sewage treatment plant (STP), detergent manufacturers and industries that utilize detergents as cleaning agent after production.

Sample collection

The effluent samples from Agbara cosmopolitan sewage treatment plant (CSTP) were taken from both domestic and industrial manhole systems in the morning and evening. The effluent samples from industrial concerns were taken at the depth of 0 – 30 cm from the effluent tanks. The only exceptions were Henkel Chemicals Ltd., Nigeria

Synthetic Fabric Co. and Swantex Nig. Ltd., where effluent collection was allowed to be taken at the outlet to the public drainage system. All the effluent generated was untreated according to the personnel of the companies. The experimental design was a randomized complete block design. Sampling was done with sterile plastic container (2- litre) and collection of effluent was randomly done at all points of discharge of effluent along the production line and stored in the refrigerator at 4°C.

Detergents used

Domestic detergents used included powdered 'Omo' which was purchased from Unilever Nigeria Plc., 'Elephant Extra' from PZ, Ariel from PT. Sayap Mas Utama, Jakarta Timur 13910 Indonesia. 'Persil' from Lever Brothers Ltd., Ireland. Teepol' was obtained from National Oil and Chemical Marketing Plc., (NOLCHEM) Lagos. Sodium Dodecyl Sulfate (SDS) was obtained from Fischer Scientific coy, New Jersey, U SA.

Chemicals

All Chemicals used in this study were of analytical grade and were purchased from JANSEEN CHIMICAL (Belgium), Fischer. Scientific Coy, BDH Chemicals, U K., Sigma Chemicals, St. Louis Missouri, USA.

Media composition

The medium used for isolation of detergent-utilizing organisms was the minimal salts medium supplemented with the test detergent at 0.01%(w/v) and agar 1.8%(w/v), Nutrient agar (BDH, U.K). All media used were sterilized at 121°C for 15 min using the autoclave (Thysse and Wanders, 1972; Okpokwasili and Olisa, (1991) Nwabuzor (1988).

Plasmid profile analysis

Isolation of plasmid DNA by 'mini - prep' is a regular but time-consuming technique in modern molecular biology, it usually takes 2 – 3 h to produce 12 – 24 samples of DNA from saturated bacterial cultures (Lech and Brent, 1987; Maniatis et al*.,* 1982). A modified 'alkaline lysis' procedure which is extremely quick and

reliable was used, that is, 'TENS MINI – PREP' in ten minutes (Kado and Liu, 1981; Zhou et al., 1990*).* TENS solution (TE buffer containing O.1N NaOH and 0.5% Sodium dodecyl sulfate, EDTA, Tris) and sodium acetate was used instead of potassium acetate. Neither lysozymes nor phenol – chloroform extraction is needed with this method. With the above modifications, the procedure can be completed in 10 min. This procedure can produce up to 18 samples in no more than 30 min. The yield of plasmid is high (2 – 3ng from 1.5 ml of a culture of cells) and the quality is good enough for further manipulations in processes such as
restriction enzyme digestion.

Protocols

Overnight bacterial culture of 1.5 ml was obtained and then spun for 10 s in a micro-centrifuge (5413 Eppendorf Geratebua, Nether + hinz Embh, 2000 Hamburg 63) (1000 rpm) to pellet cells. The supernatant was gently decanted, leaving 50 – 100 l together with cell pellet, this was vortexed (STUART Auto vortex mixer, Great Britain) 5 times (10 s) at high speed to re-suspend cells completely. Then 300 l of TENS was added. Then, the solution was mixed for 2 – 3 s until the mixture became sticky.

The samples were then incubated in ice for 10 minutes to prevent the degradation of chromosomal DNA. Thereafter, 150 l of 3.0 M sodium acetate (pH 5.2) was added to stop lysing process, then, it was vortexed for 1 min to mix completely. The micro-centrifuge (Eppendorf Centrifuge, 5413 Eppendorf Geratebua, Nether
+ Hinz Gmbh 2000 Hamburg 63) was used for spinning at 1000 rpm for 2min to pellet cell debris and *chromosomal DNA* .Then, the supernatant was transferred to a fresh eppendorf tube, where it was mixed with 900 l of 100 % ethanol which has been pre-cooled to -20^{o}C. Then, spun for 3 s to precipitate the plasmid DNA and RNA (white pellet is observed) from the supernatant. The supernatant was discarded, the pellet was rinsed twice with 1 ml of 70% ethanol and the pellet was dried under vacuum for 2 – 3 min. Pellet can be re-suspended in 20 – 40 l of TE buffer or distilled water for further use (Tris 25mM: EDTA 10mM) (Kado and Liu, 1981; Zhou et al., 1990).

Large-scale (Maxi-Prep.) plasmid DNA extraction

The detergent-degraders were previously stored on nutrient –detergent agar slant. Thereafter, pure cultures of each of the isolates were grown independently overnight on 100 ml LB (Luria broth) at 200 - 250 rpm. Then, 60 ml broth culture of each of the detergent-degrader isolates were transferred into microfuge tubes using sterile pipettes and were spun at 10,000 rpm for 1 min. The super-

natant was then removed before the pellets were re-suspended in 7.5 ml TE (Tris, EDTA). Meanwhile, 15 ml of the solution containing 1% SDS and 0.2 M NaOH (which must be less than 3 weeks old) was added and left for 5-10 min at room temperature. On careful observation at this point the solution became viscous. Then, 12.5 ml of 3M Sodium acetate (pH 4.8) was added to the solution in the tubes; this was mixed vigorously and thereafter kept on ice for 10 - 20 min. Thereafter, the samples were spun at 14,000 rpm for 10 min. Then, 30 ml of the supernatant from each sample was transferred to a fresh sterile microfuge tubes with care in order to avoid transfer of cell debris with the supernatant. Then, to each of the sample 30 ml of isopropanol was added and kept on ice for 30mins. Thereafter, the samples were spun at 14,000 rpm for 15 min. Then, the supernatant was discarded with care to avoid loss of the pellets with the supernatant. These pellets were the plasmid DNA. Then, 1000 l of 70% ethanol was added to the pellets and mixed by tapping the eppendorf tubes by the sides. Thereafter, the samples were spun at 14,000 rpm for 5 min. Thereafter, the supernatant was carefully removed without disturbing the pellets.

The pellets were air-dried at room temperature for 5 - 15 min. The pellets were later re-dissolved in 200 l of distilled water and stored at -20^{o} C .Gel for separation of plasmid was run at100 volts/hr. (Kado and Liu, 1981; Bhalakia, 2006).

Curing of plasmid

Curing of plasmid was done to trace plasmid encoded features among the selected bacterial 'detergent–degraders'. The modified methods of Ahrne et al. (1989) and Bhalakia (2006) were used. A stock solution of 10% SDS was prepared by the addition of 10g SDS to 100 ml of nutrient broth. The pH was adjusted to7.8 - 8.0. The mixture was steamed for 1 hour and kept aside as stock.
The overnight cultures of selected isolates in LB were diluted 100-fold and 0.5 ml volume of each of the isolates was transferred aseptically into fresh 30 ml volume of nutrient broth. The cultures were incubated with shaking for 3 h and SDS stock solution was added to give the required final concentration 1% (w/v). The cultures were incubated at room temperature with mild agitation for 72 h. The cells were then tested for detergent degradation: after transferring 0.5 ml sample of each of the isolates into nutrient broth supplemented with detergent 0.01% (w/v), the plasmid content was determined by carrying out the plasmid isolation procedure described above.

Protocol for running agarose gel electrophoresis

Powdered agarose (0.8%) was boiled in TBE (Tris, Boric Acid, EDTA) buffer intermittently until solution becomes clear gel. The Agarose solution was allowed to cool to 45^{o}C, then 7 l of ethidium bromide was added (which will act as intercalating agent) for 3 min. Intercalating agent penetrates into the genetic bases so that they fluorescence with it under U-V light. The clear gel solution was poured with comb in place and allowed to solidify. Thereafter, the gel tray was removed and the comb. The gel was placed into the tank containing the gel buffer. It is important that the buffer covers the gel completely.

Then, 2 l of the tracking dye (Bromophenol blue) was mixed with 1
l of the marker, thereafter, load into the first well. Bromophenol blue (BPB) helps to track the distance moved by the sample on the gel. Thereafter, 20 l of the Bromophenol blue with 20 l of the sample was mixed, and with this load into the remaining wells. The cover of the tank was carefully placed on it, and then plugged to the power source to run from the negative to positive direction making sure it does not run distance far more than ¾ of the gel for appro-ximately 1 h. Then, the gel was viewed via the U-V transilluminator using protective goggles (Maniatis et al., 1982).

RESULTS

Plasmid profile analysis

The gel electrophoresis result of the isolated plasmid–DNA from 8 bacterial detergent-degraders (Figure 1) showed no plasmid–DNA band when the 'Tens Mini-prep' method was used. This suggested that 'Tens Mini-prep' method has some limitations despite its widespread use because small quantities of plasmid DNA of this sort was undetectable by this method.

Large – scale (Maxi. Prep.) plasmid preparation

However, on applying the Large – scale plasmid–DNA isolation method, the plasmids were visible via the gel electrophoresis, this suggested that the detergent catabolic trait may have some relationship with the single plasmids (14 – 15 kbp) detected for each of the selected isolates (Figure 2).

Curing of plasmids

Further investigation via curing of plasmids confirmed that the plasmid–DNA was not the ultimate source of the detergent catabolic trait. The genomic DNA thus have to be analyzed and this would require the use of the PCR technique as well as the DNA sequence analysis to get to the source of the DNA sequence encoding for detergent / LAS utilization by the isolates. Moreover, the plasmid profile of these selected isolates which are single plasmids is adequate for typing of these isolates as 'deterge nt–degraders' from
wastewater.

Future studies would seek the use of high through –put technologies such as PCR, metagenomics and DNA microarray to uncover the genetic details of detergent–degraders.

Protocols for gel electrophoresis

The result of the gel electrophoresis was observed under the U-V transilluminator and the photograph taken with the Polaroid 677 camera. This is displayed in Figures 1 and 2.

The standard marker used −PST (Figure 3) serves as the basis of comparison and extrapolation.

DISCUSSION

The ability to utilize detergent components as carbon and energy sources for growth and biomass accumulation suggested some peculiarity in the genome of microoga-

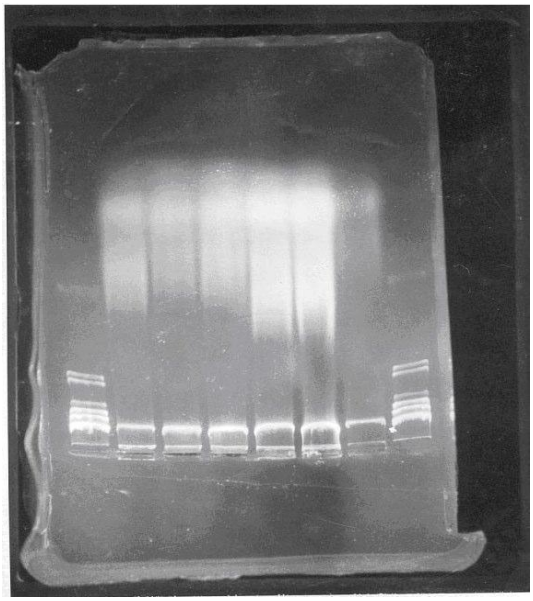

Figure 1. Plasmid – Profile of bacterial deter-gent – degraders (Tens mini-prep.). 1 2 3 4 5 6 7.

Lane 1 = - PST marker Lane 5 = *K. aerogenes*
Lane 2 = *P. aeruginosa* Lane 6 = *E. coli*
Lane 3 = *K. oxytoca* Lane 7 = –PST marker
Lane 4 = *Brevibacterium* sp.

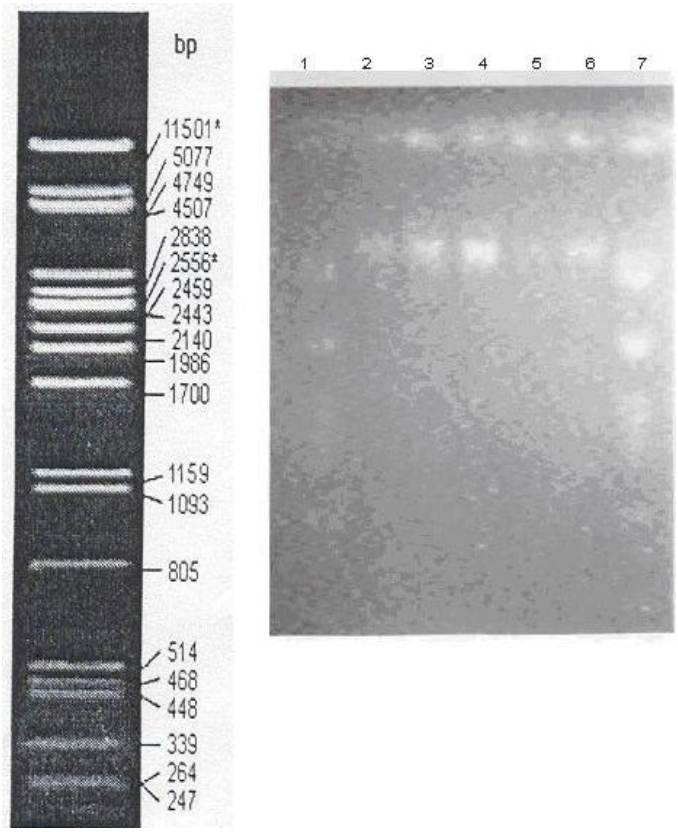

Figure 2. Large-scale (Maxi-Prep.) plasmid extraction.

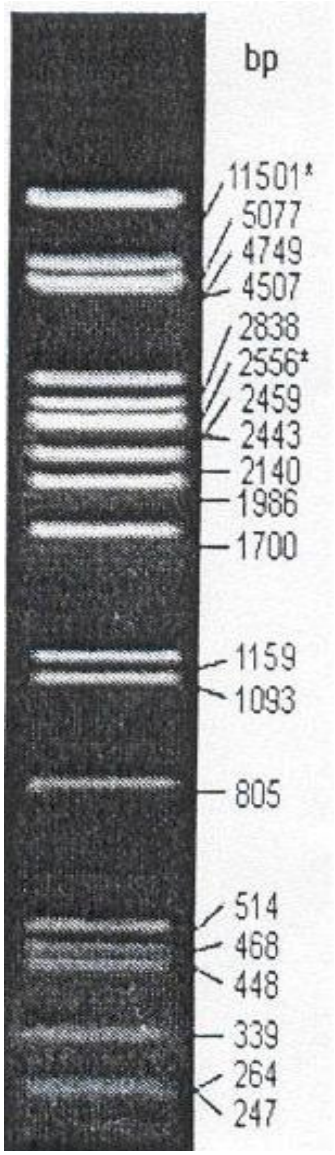

Figure 3. –PST marker.

nisms with this trait. Since not all microorganisms have the ability, particularly acclimation to xenobiotics compounds. Plasmid DNA- coded character often plays significant role in bacterial adaptation to xenobiotics in the environment (Kado and Liu, 1981). Current study revealed that bacterial chemo-taxis and ability to metabolize detergent components were not absolutely plasmid DNA–mediated because after curing of plasmid the isolates were tested on medium supplemented with detergent which they colonized with less vigor. Although, *E. coli* was unable to utilize the detergent after curing of plasmid. However, the convincing evidence on the genetic linkage of the catabolic trait would be found using high through-put techniques such as the PCR and DNA sequencing to trace the nucleotide sequence encoding for detergent – catabolism which may be resident in the

plasmid – DNA or the genomic DNA. The plasmid profile discovered in this study is adequate for typing of these selected isolates as the 'detergent–degraders' having single plasmid with band size range of 14 – 15 kbp. However, it noteworthy that the molecular marker (– PST Marker) used has the band size range of 0.2 – 12 kbp which can be extrapolated to read band size 14-15 kbp. The plasmid-DNA isolated from these selected detergent-degraders (*Escherichia coli, Enterobacter liquefasciens, Klebsiella liquefasciens* and *Klebsiella aerogenes*) corroborated the fact that there is a form of genetic similarity among these species since their plas-mid profile showed single band of size range of 14 – 15 kbp. These 3 isolates (*E. coli*, *Brevibacterium sp*. and *K. aerogenes)* grew profusely over others as at Day 10 and maintained relatively high population as at Day 30 (Ojo and Oso, 2008). This informed the observation made by Sigoillot and Nguyen (1992) that only a few microbial strains could carry out the first 2 steps in detergent mineralization, that is, terminal oxidation of alkyl chain and desulphonation while aromatic ring cleavage is achi-eved by fewer strains that possess very specific enzyme-tic activities.

Conclusively, Plasmid profile analysis also revealed the presence of single plasmid in all the bacterial isolates but plasmid – DNA may not be absolutely responsible for the detergent – hydrocarbon utilization trait. Future studies with PCR and DNA sequence analysis would reveal the DNA' fingerprint' of each species of the detergent – de-graders, this would enhance the processes of surveil-lance for these organisms in similar ecosystems and the detection of new serotypes as well as assist in environ-mental impact assessment (EIA) study for sustainable development.

REFERENCES

Ahrne S, Molin G, Stahl S (1989). Plasmids in *Lactobacillus* strains isolated from meat and meat products. Syst. Appl. Microbiol. 11: 320 – 325.

Barbour AG, Garon CF (1987). Linear plasmids of the bacterium *Borrelia burgdorferi* have covalently closed ends. Science 237: 409 – 411.

Bhalakia N (2006). Isolation and plasmid analysis of vancomycin-resistant *Staphylococcus aureus*. J. Young Investig. 15(4): 15–24.

Birnboim HC, Doly J (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7: 1513 – 1523.

Di Corcia A, Capuani L, Casassa F, Marcomini A, Samperi R (1999b). Surfactant structure and biodegradability. Environ. Sci. Technol. 33: 4119 – 4125.

Gledhill WE (1975). Screening test for assessment of ultimate biodegradability: linear alkylbenzene sulfonates. Appl. Environ. Microbiol. 30: 922 – 929.

Kado CI, Liu ST (1981). Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. 145: 1365 – 1373.

Maniatis T, Fritsch EJ, Sambrook EJ (1982). Molecular Cloning, A laboratory manual. Cold Spring Harbor Lab. N.Y.

Nomura Y, Ikebukuro K, Yokoyama K, Takeuchi T, Arikawa Y, Ohno S, Karube I (1998). Application of a linear alkylbenzene sulfonate biosensor to river water monitoring. Biosens Bioelectron. 13: 1047 – 1053.

Ojo OA, Oso BA (2008). Isolation and characterization of synthetic detergentdegraders from wastewater. Afr. J. Biotech. 7(20): 3753-3760.

Okpokwasili GO, Nwabuzor CN (1988). Primary biodegradation of anionic surfactants in laundry detergents. Chemosphere 17: 2175 – 2182.

Okpokwasili GO, Olisa AO (1991). River water biodegradability of surfactants in liquid detergent and shampoos. Water Res. 25: 1425 – 1429.

Sigoillot JC, Nguyen MH (1992). Complete oxidation of linear alkylbenzene sulfonate by bacterial communities selected from coastal seawater. Appl. Environ. Microbiol. 58(4): 1308–1312.

Swisher RD (1975). Surfactants: from recalcitrant to docile. In: *Proc.* Interational Biodegradation Symposium (Eds.) J. M. Sharpely and A. M. Kaplan. pp. 853 –865.

Thysse GJE, Wanders TH (1972). Degradation of n-alkane-1-sulphonates by Pseudomonas. Antonnie van Leeuwenhoek 38: 53 – 63.

Towner KJ, Cockayne A (1995). Analysis of nucleic acid profiles. In: Molecular methods for microbial identification and typing. Publ. Chapman & Hall 2-6 Boundary Row, London. pp. 29 – 57.

Zhou C, Yang Y, Yong AY (1990). 'Mini-prep' in ten minutes Biotechniques 8(2): 172–173.