A Comparative study on plasmid isolation from strains of *Enterobacter aerogenes* using different optimized procedures

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Abstract— *Enterobacter aerogenes* are gram negative, facultative anaerobic motile bacteria which possess plasmids. Plasmids are extra chromosomal DNA. Plasmid DNA has been used as the most powerful tool in several molecular and biotechnological applications. In this current study three modified protocols were examined including mini preparation method, hot and cold alkaline lysis method and compared their simplicity, rapidity, costs and plasmid quality for extracting plasmid DNA from bacterial strains of *Enterobacter aerogenes* from different sources. According to the results obtained all three methods are good and yielded plasmid DNA but each methods has its advantages and disadvantages. Among all three methods, the spectrophotometric highest yield of plasmid DNA (83 ug/uL) was obtained in mini preparation method and lowest yield (20 ng/uL) in cold alkaline lysis method. The best spectrophotometric 260/280 ratio (near to 1.8) also obtained in mini preparation method which indicates the purity of extracted plasmid DNA. Among all three methods tested in this study, Mini preparation method is more efficient, time saving, non-hazardous and less cost method as it doesn't require any expensive enzymes and hazardous chemicals.

Keywords— plasmids, DNA extraction, *Enterobacter aerogenes*, alkaline lysis, Mini preparation

I. INTRODUCTION

Enterobacter aerogenes is belonging to the family Enterobacteriaceae and the genus *Enterobacter* which is the member of the coliform group of bacteria. This bacterium is widely distributed in soil, water, sewage, hygienic chemicals and also found in plants (Farmer *et al*., 1985). *Enterobacter aerogenes* is a nosocomial and pathogenic bacterium that causes opportunistic infections including eye and skin infections, meningitis, bacteraemia (bacterial blood infection), pneumonia and urinary tract infections. *Enterobacter aerogenes* are resistance to several antibiotics as they contain resistance gene in plasmids (Mohammad R Alavi et al., 2011). Commercial significance of *Enterobacter aerogenes* is it produces Hydrogen gas during dark fermentation (Ibdal Satar *et al.,* 2017).

Plasmids are extra chromosomal circular, double stranded DNA about 1-5% of the size bacterial chromosome, present in cell cytoplasm which is capable of replicating independently from the chromosomal DNA. Plasmid DNA mostly found in bacteria but also in some eukaryotic microorganisms such as *Saccharomyces cerevisiae*. *Enterobacter aerogenes* also possess plasmids. Extraction of plasmid DNA from bacterial cells is an essential technique in molecular biology as plasmid DNA should totally isolated from bacterial genomic DNA, RNA and other molecules.

The isolation of plasmid DNA from bacteria is a critical technique. It is a vital step in many procedures for example cloning, PCR, transfection, in vitro translation, blotting, DNA sequencing, transfection and gene therapy. Plasmids provide genetic tools for differentiation among bacterial strains. Plasmids profiling and RAPD fingerprinting of plasmids DNA are commonly used methods for genetic differentiation of bacterial strains (De La Plaza et al., 2006 and Isabelle Mainville et al., 2006). Therefore the extracted plasmid DNA from bacteria can be used for several applications in molecular biology process. There are several protocols have been developed to purify plasmid DNA from bacteria. Growth of bacterial culture, Harvesting and lysis of cultured bacteria and purification of plasmid DNA (Kav et al., 2013) are the three major steps in all the protocols established for plasmid DNA extraction.

High-quality plasmid DNA extraction protocols are time consuming, expensive and laborious due to the complex multiple steps involved in culturing of bacterial cells and isolation of plasmid DNA.

The objective of this study was to compare three different plasmid DNA isolation methods from strains of *Enterobacter aerogenes* and find out the best working one for downstream applications.

II. MATERIALS AND METHODS

A. Samples collection

Enterobacter aerogenes bacterial pure cultures were obtained from water, air, milk, vegetables and fruits separately. Morphological (simple and gram staining), Biochemical (Catalase test, Citrate utilization test, Fermentation of sugar test, Hydrolysis of gelatine etc…) and Physiological studies (effect of temperature, pH and effect of growth in NaCl) were carried out to conform *Enterobacter aerogenes* strains.

B. Bacterial culture preparation

Eosin Methylene Blue (EMB) agar plates were prepared (Nacl, tryptone, yeast extract and H_2O) and the culture was streaked on the plate and kept incubation for overnight at 37º C. Then a single bacterial colony was picked from the plates and inoculated in 1-3 ml of LB broth and incubated at 37º C for overnight in the shaking incubator at 250 rpm separately.

C. Plasmid extraction

Plasmid DNA was extracted from bacterial cultures (*Enterobacter aerogenes*) from four different sources (air, water, milk and vegetables) mainly by three different methods with some modifications. Here are three methods that differ foremost in the type of bacterial lysis.

D. Method 1 (Mini preparation method)

The cultures were centrifuged at 5000 rpm for 5 min to pellet the cells. Culture medium was decanted and bacterial pellet was completely resuspended in 300 µL of ice cold P1 buffer (1M Tris pH 7.9, 0.5 M EDTA, RNase 10 mg/ml) by vigorous vortexing. Then 300 µL of P2 buffer (5M NaOH, 10 % SDS in distilled water) was added to each bacterial suspension and mixed the content by inverting the tubes rapidly five times. Tubes were kept on ice and 300 µL of ice cold P3 buffer (5M KAc pH 4.8) was added and tubes were inverted five times to disperse the solution through the viscous bacterial lysate. These tubes were centrifuged at 12 000 rpm for 15 min. Supernatant was carefully transferred to fresh tubes and 0.8 volume of isopropanol was added to each supernatant and pre-equilibrated to room temperature and then centrifuged at 12 000 rpm for 15 min. Supernatant was removed and DNA pellet was washed with adding 70% ethanol and centrifuged at 12 000 rpm for 5 min. Supernatant was decanted then DNA pellet was air dried. Each pellet was dissolved in 20 µL of sterile water.

E. Method 2 (Modified Hot alkaline lysis method established by Kado and Liu,1981)

The cultures were centrifuged at 5000 rpm for 5 min to pellet the cells. Pellet was resuspended in 1mL of solution containing 0.04 M Tris acetate, pH 8.0 (Adjusted with glacial acetic acid) and 2mM EDTA. Then 2ml of lysis buffer (0.05 M Tris, 3% SDS ,pH adjusted with 2N NaOH) was added and mixed. Sample was incubated at 68ºC for 40 min. Then 6ml of phenol/chloroform (1:1) was added to hot samples and mixed gently to complete emulsification. Phases were separated by centrifugation at 10.000 rpm for 15 min at room temperature and upper aqueous phase was transferred to new tube which contained 0.8 volume of isopropanol and pre-equilibrated to room temperature and then centrifuged at 12 000 rpm for 15 min. Supernatant was removed and DNA pellet was washed with adding 70% ethanol and centrifuged at 12 000 rpm for 5 min. Supernatant was decanted and DNA pellet allowed for air dry. Each pellet was dissolved in 20 µL of sterile water.

F. Method 3 (modified cold alkaline lysis method)

The cultures were centrifuged at 5000 rpm for 5 min to pellet the cells. Pellet was washed in 2ml of the TE buffer (0.05 M Tris pH 8.0, 0.01 M EDTA.) then resuspend in 40 uL of the same TE buffer. Then 0.6 mL of freshly prepared lysis buffer (TE buffer with 4% SDS, pH adjusted to 12) was filled into Eppendorf tube and the cell suspension was added to the lysis buffer and mixed gently. Lysis was completed by incubating at 37 ºC for 20-30 min. Then 30ul of 2M Tris, pH 7.0 was added to the sample for neutralization and mixed gently. Then 0.24 ml of 5M NaCl was added to the sample for precipitation of chromosomal DNA and protein and incubated on ice for 4 hours. Sample was centrifuged for 10 min at 12,000 rpm and the supernatant was removed and 500 µL of 70% ethanol was added to the pellet and centrifuged at 12,000 rpm for 5 min at 25°C. The pellet was resuspended in 20 µL sterile water.

Contaminated RNA was removed by adding 2 µL of 10 mg/mL RNAase and incubating at 37oC for 20 min in all three methods.

G. Visualization and quantification of Plasmid DNA

Finally, 3 µL of plasmid DNA extracted from above three methods were analyzed on 1.0% agarose/EtBr gel run in 1X TAE running buffer at constant voltage (50 volts) for 60 min separately. Then the gel was visualized under UV transilluminator. Plasmid DNA bands were assessed using Bio Rad Gel Doc 2000 Imaging System.
Concentration of plasmid DNA was measured using spectrophotometer. The absorbance of a diluted DNA sample was measured at 260 and 280 nm in order to assess the purity and the concentration of plasmid DNA.

III. RESULTS AND DISCUSSION

In this current study three modified protocols were examined including mini preparation method, hot and cold alkaline lysis method and compared their simplicity, rapidity, costs and plasmid quality for extracting plasmid DNA from bacterial strains of *Enterobacter aerogenes*. Extraction of plasmid DNA from bacterial cells is vital and crucial technique in molecular biology as plasmid DNA should entirely separate from highly abundant bacterial genomic DNA (gDNA) or chromosomal DNA, RNA and other molecules (carbohydrate and protein)

It is necessary to optimize the culture conditions, the culture medium and incubation times to get good yield of plasmid DNA. Quality and the quantity of the extracted plasmid DNA highly depend on the type of culture media used. In this study Eosin Methylene Blue (EMB) agar medium is used to get the pure culture of the bacterial strains of gram negative *Enterobacter aerogenes* from different sources (air, water, soil and food samples). Because EMB agar medium is a differential microbiological medium, which inhibits the growth of gram-positive bacteria and also to differentiate *Enterobacter aerogenes* from Escherichia coli. Therefore it is not necessary to add specific antibiotics in the culture media. *Enterobacter aerogenes* was observed in dark pink colour mucoid colonies while Escherichia coli was observed in green colour tiny colonies in the EMB agar medium.

Table 1: Quality comparison of plasmid DNA isolated by different methods

Name of the culture	Plasmid DNA extraction methods		
	Method 1	Method 2	Method 3
S1 (water sample)	69 µg/ul	30 µg/ul	28 µg/ul
260/280	1.89	2.07	2.24
S2 (Vegetable sample)	83 µg/ul	40 µg/ul	20 µg/ul
260/280	1.84	2.1	2.01
S3 (air sample)	74 µg/ul	55 µg/ul	30 µg/ul
260/280	1.87	2.26	1.94
S4 (Milk sample)	72 µg/ul	58 µg/ul	40 µg/ul
260/280	1.74	2.4	1.72

In this study, The *Enterobacter aerogenes* pure cultures were isolated from EMB agar medium and transferred and grown in standard Luria Bertani (LB) medium for plasmid DNA extraction.

Culture conditions influence the plasmid yield. It is necessary to understand the bacterial growth rate within a sample when use bacterial culture for any research. The bacterial cell density and the yield of plasmid can be increased by increasing the incubation time of bacterial culture. But the bacterial culture is overgrown it might produce large amount of dead and starving cells as the results of this, extracted plasmid DNA might be degraded or contaminated with genomic DNA (Chromosomal DNA) hence produce poor quality plasmid DNA. In this study, density of the bacterial culture was estimated by measuring Optical Density (OD600) using standard spectrophotometer. Here measured OD600 values were found to be within the range from 0.2-0.3 for the culture which was aliquoted ten-fold. The late log phase is the optimal growth phase of bacterial culture, which for *Enterobacter aerogenes* was 12 h.

Alkaline lysis is the method (first developed by Birnboim and Doly 1979) for extracting plasmid DNA from harvested bacterial cells in a culture medium. Quantity and quality of extract plasmid DNA relies upon the right lysis method used. In this lysis method, cells are broken open under alkaline conditions. Under these conditions, high molecular weight chromosomal DNA is denatured to form an insoluble clot and plasmid DNA remains stable in the supernatant. Large and small plasmid DNAs with variable copy numbers can be extracted by this method.

In this current study three different modified alkaline lysis methods were tested and compared their efficiency and simplicity. According to the results obtained all three methods are good and yielded DNA. Among all three methods, the spectrophotometric highest yield of plasmid DNA was obtained in method 1 (Mini preparation method) from all different sources (air, water, milk and food) and lower yield in method 3. Among all three methods, the best spectrophotometric 260/280 ratio (near to 1.8) also obtained in method 1.

High-quality plasmid DNA extraction protocols are time consuming, expensive and laborious due to the complex multiple steps involved in culturing of bacterial cells and isolation of plasmid DNA.

The method 1 is faster than other two methods. The mini preparation method (method 1) took almost 1 hour whereas the hot alkaline lysis method (method 2) took nearly 2 hours as it required incubation period of 40 min for lysis and the method 3 is very time consuming when compare to other two methods as it acquired 6 hours to be completed because this protocol consists of incubation on ice for 4 hours to precipitate chromosomal DNA and proteins. Therefore among all three methods tested, method 1 is more efficient and time saving method.

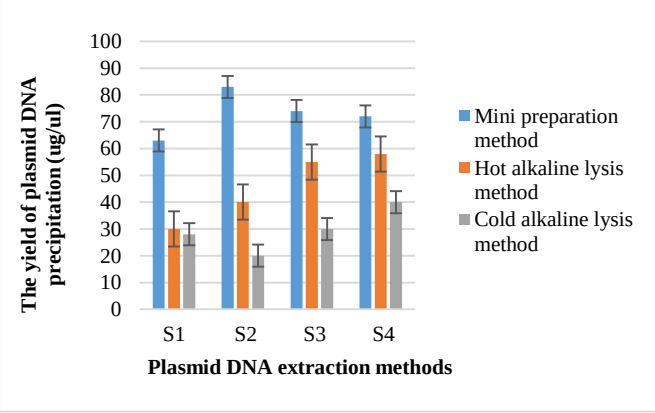
Most of the recommended protocols used for plasmid DNA extraction use hazardous chemicals (Chloroform, Phenol, ß-mercaptoethanol) and also require expensive enzymes (α-amylase, proteinase K, RNase, lysozyme).

In method 2, Phonol chloroform extraction is used to separate DNA from proteins and lipids. Exposure to phenol may cause irritation and severe exposure can cause liver and/or kidney damage. Chloroform can be poisonous if inhaled or swallowed (Committee on Acute Exposure Guideline Levels, 2012). Therefore method 1 and 3 are safe approaches as these methods do not required any hazardous chemicals.

I. CONCLUSION

From this study, we conclude that method 1 (Mini preparation method) is more efficient, less time consuming, safe and cost

Figure 1: The yield of Plasmid DNA from Enterobacter aerogens using three different extraction methods from four different sources.



effective method as it doesn't require expensive enzymes and hazardous chemicals. This procedure should be applicable to extraction of plasmid DNA from *Enterobacter aerogenes* with minimal facilities.

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