Enhancing the Yield of Therapeutic Plasmids in Laboratory-Scale Maxiprep Preparations through a Modified Alkaline Lysis Approach

ABSTRACT

Plasmid purification is an important step in molecular biology and good quality / quantity of plasmids are essential for cloning, production of therapeutic plasmid. This study was aimed to enhance the yield of therapeutic grade plasmid in maxiprep preparation by modifying culturing conditions and the traditional alkaline lysis method. To increase the yield of bacterial cell mass carrying the plasmid of interest, LB broth supplemented with 1.9% yeast extract (LBY) was used. The LBY broth with extra supplementation of yeast extract increased the yield of bacterial cell mass to 0.39g / 250 ml of culture, compared to plain LB broth. Furthermore, the traditional alkaline lysis method was improved by using calcium chloride, Sodium chloride and PEG6000 (40%) which boosted the yield of plasmid with increasing amount of supercoiled plasmid. The modified method yielded five times more plasmid (3.530 mg/ 250 ml of culture) than commercial purification kit (566 µg / 250 ml of culture). The plasmid purified using the modified method was pure, cost-effective and ideal for cloning and transfection. This modified method of purification enhanced the yield of supercoiled plasmid which is essential for therapeutic purpose.

Keywords: *Plasmid – purification – yield - Polyethylene glycol and calcium chloride.*

1. INTRODUCTION

Plasmids are extrachromosomal DNA molecules of 3 to 20 Kbp in size having a relative molecular mass ranging from 2 X 10^6 to 13 X 10^6 [1]. Plasmids as a eukaryotic expression vector was first identified in 1990, since then it became an important part in several biotechnological application [2]. Compared to conventional protein/peptide-based vaccines, DNA vaccines are more stable, cost-efficient, easy to manufacture, induce both cellular and humoral immune response [3]. These advantages of DNA vaccines make them more attractive approach for vaccine development.

The DNA vaccine technology is only 30 years old and numerous DNA vaccines were developed to prevent infectious diseases, allergy, and cancer. To date, five DNA vaccines have received commercial licensing [4] and more than 1700 DNA vaccines are in different phases of clinical trial including phase II and phase. The steps in DNA vaccine development includes cloning of gene of interest, bulk volume culture and purification of plasmid [5]. The yield of recombinant plasmid depends on several factors viz. gene inserted, bacterial strain, culturing medium, culturing condition and purification process [6]. The main goal in plasmid production is increasing the yield and decreasing the production cost [7,22,23].

For laboratory-scale preparations of plasmid, shake flask method is the commonly employed method. In commercial scale preparations fermenters are routinely used to achieve high yield of bacterial cells with recombinant plasmid. Initial stages (Phase I and phase II) of clinical trials require minimal quantity of plasmid for which the shake flask method is sufficient and commercial purification kits are routinely used in laboratory-scale testing. These commercial kits usually provide a plasmid yield of 500 µg to 1 mg depending upon the copy number of plasmids. However, most of these commercial kits use RNAse A derived from bovine pancreas which is not permissible in clinical grade plasma as per current regulations [8-9].

Several conventional methods are available for the purification of plasmid. The conventional methods that are used for the purification of plasmid are boiling method, alkaline lysis, and Cesium chloride (CsCl) ultracentrifugation. Each method has its own advantages and disadvantages. The boiling method is a very simple method. But the RNA contamination is very high in boiling method. Alkaline lysis is the widely adapted method for plasmid purification. But without RNAse A, the RNA contamination is higher. Both the boiling method and alkaline lysis requires further purification with phenol/ chloroform which affects the integrity of the plasmid. Cesium chloride (CsCl) ultracentrifugation method with ethidium bromide is used to isolate supercoiled plasmid DNA effectively and this method requires ultracentrifuge, time consuming, expensive, and hazardous.

The traditional alkaline lysis was modified with calcium chloride for plasmid purification in miniprep preparations to increase the quality of the plasmid [10]. However, when this method was

adopted in maxiprep preparation, the yield of plasmid was very low. Hence, this study aims to optimize a plasmid extraction method for maxiprep preparation that can yield high quality and quantity of plasmid, suitable for cloning and therapeutic purpose.

2. MATERIALS AND METHOD

2.1 Bacterial strain and plasmid

The DH5α cells harbouring pVAX1 plasmid (pVAX1-gB) carrying the glycoprotein B (*gB*) gene of ILT available in our laboratory were used in this study. The pVAX1-gB plasmid is 5.6 kbp in size.

2.2 Bulk culturing of plasmid

To optimize the yield of DH5α cells two broth compositions viz. Luria Bertani (LB) broth and Luria Bertani broth supplemented with 1.9 % added yeast extract (LBY) were used [11]. Glycerol stock of DH5α cells containing pVAX1-gB was streaked on to a LB agar plate containing kanamycin (50 µg/ ml) and incubated at 37ºC overnight. Single recombinant colony was picked up and inoculated into 5 ml of LB broth and in LBY broth with yeast extract (1. 9%) containing kanamycin (50 µg/ ml) and incubated at 37 °C overnight with 180 rpm in an orbital shaker. About 2.5 ml of starter culture was diluted in 250 ml of LB broth, both with and without added yeast extract, and incubated at 37 °C overnight with 180 rpm. Broth was chilled on ice for arresting cell growth and centrifuged at 3000 rpm, 4°C for 10 minutes in Beckman Coulter Avanti JXN26 centrifuge. The supernatant was discarded. The tubes containing bacterial cell pellet were air -dried by keeping the tubes inverted on a filter paper to completely remove the residual medium. The dry weight of bacterial pellet was weighed to measure the dry pellet yield.

2.3 Alkaline lysis

Alkaline lysis was carried out for the lysis of DH5α cells to release plasmids [12]. Briefly, the bacterial pellet was resuspended in 10 ml of solution I (50 mM glucose, 25 mM Tris-Cl, 10 mM glucose - pH 8.0), vortexed for 30s. Subsequently, 10 ml of solution II (0.2 N NaOH, 1% SDS) was added and the tube was inverted gently for 3-4 times and incubated at 10 minutes at room temperature. Finally, 15 ml of

chilled solution III (3M potassium acetate pH-5.5) was added, the tube was gently inverted few times for proper mixing and incubated at room temperature for 10 minutes.

2.4 Modified method of plasmid purification

The lysis procedure is followed by purification procedure that already has been described with modifications [8-9]. The entire lysate was filtered through a 100 µm nylon filter. Calcium chloride ($CaCl_2$-5M) was added to the filtrate at 3:1 ratio and centrifuged immediately at 9000 rpm, 10 minutes at 25 °C. The supernatant was transferred to another fresh tube and equal volume of isopropanol was added. The mixture was centrifuged at 13,000 rpm for 30 minutes at 25 °C in Beckman Coulter® Avanti JXN26 centrifuge. The supernatant was discarded and the plasmid pellet was resuspended with 5 ml of Nuclease free water. Then, 5 ml of 40 % PEG 6000 in 2.5 M NaCl solution was added and incubated at 4 °C for two hours. After incubation, the PEG-plasmid mixture was centrifuged for 20 minutes at 13,000 rpm , 25 °C. The plasmid pellet was washed twice with 70 % ethanol at 13,000 rpm, 4°C, air dried and reconstituted with 500 µl endotoxin free nuclease free water. The plasmid yield was compared using a commercial purification kit (Endofree plasmid maxiprep kit - Qiagen, # 12362) as per manufacturer's instructions. The quality and quantity of extracted plasmid was assessed in a nanodrop microvolume spectrophotometer (DeNovix – DS11) and by agarose gel electrophoresis on 1% agarose gel in Tris-acetate EDTA (TAE) buffer containing 0.5 µg/ ml of ethidium bromide.

2.5 Restriction digestion of plasmid extracted through modified method

The extracted plasmid was digested with *Bmt I* (NEB®) and *Afl II* (NEB®). Restriction digestion was carried out in 20 µl volume consisting of 10 µl of pVAX1-gB plasmid, 2 µl of cut smart buffer (10X), 1 µl of *Bmt I* enzyme, 1 µl of *Afl II* enzyme and 6 µl of nuclease free water. The restriction digestion mixture was incubated at 37 °C for 1 hour. The digested product was run on 1.0 % agarose gel.

2.6 Transfection of HEK 293 T cells with extracted plasmid

Transfection of pVAX1-gB plasmid was carried out in HEK 293T cells in 6-well cell culture plate. The cells were seeded at a concentration of 2×10^5 cells and transfection was carried out when the cells

attained 90-95 per cent confluency. Transfection was carried out using jetPRIME transfection agent (Polyplus®) as follows: Two µg of pVAx1-gB plasmid was mixed with 200 µl of jetPRIME buffer, vortexed for 30 seconds. Then 4 µl of jetPRIME reagent was added and incubated at 25 °C for 10 minutes. The DNA transfection agent complex was added dropwise in to the cells and incubated at 37° C. After 4 hours of transfection, medium was changed and incubated at 37 °C for 48 hours. Then the cells were harvested by trypsinization, washed thrice with PBS, and reconstituted with 50 µl of PBS at -80°C.

2.7 Western blotting

Western blotting was carried out after running the transfected cell culture lysate on SDS – PAGE [12]. Transfected cells were mixed with equal volume of 2X Lammeli's buffer and were denatured at 95 °C for 10 minutes. The denatured protein was loaded on to 5% stacking and separated in 10% resolving sodium dodecyl sulphate polyacrylamide gel. The sample was run on Medox vertical slab gel electrophoresis unit at 80 V, till the tracking dye reached the end of resolving gel. After electrophoresis, gel was blotted on a PVDF membrane using 1X Towbin buffer in Turbo Trans Blot SD cell (BioRad®) for 10 minutes at 25 V. The protein transferred membrane was then stained [13]. Initially the PVDF membrane was blocked overnight at 4°C with 3 % skimmed milk in PBST (0.05 % Tween-20 in PBS) and then washed thrice with PBST. Subsequently, ILTV polyclonal serum (1:100) was added to the membrane and incubated at 37°C for one hour. The membrane was washed thrice with PBST and anti-chicken IgY HRP conjugate (1:2000) was added to the membrane and incubated at room temperature for one hour. The membrane was washed thrice with PBST and 10 ml of substrate 3,3'-diamino benzidine hydrochloride (DAB) was added with 10 µl of 30 % hydrogen peroxide at room temperature till the color developed. The color development reaction was stopped by washing in water. The membrane was dried in room temperature and stored in dark.

3. RESULTS AND DISCUSSION

The plasmids for clinical use should provide a good yield and quality. In laboratory settings, the yield of plasmid using the shake flask method is always lower compared to fermenter-based methods. Shake flask cultures produce cell biomass ranging from 1-8 g/l [14]. The most commonly used broths for the bulk cultivation of *E. coli* cells are LB broth, terrific broth (TB) and 2X yeast extract tryptone (2xYT).

Terrific broth is known to produce higher cell biomass compared to LB broth. LB broth is composed of 10 g of tryptone, 5 g of yeast extract, and 10 g of sodium chloride per liter. In contrast, TB broth contains 24 g of yeast extract, 12 g of tryptone, 4 g of glycerol, 2.3 g of KH2PO4, and 16 g of K2HPO4 per litre [15]. It has been reported that the elevated amount of cell biomass in TB broth was due to the increased level of yeast extract and not due to the presence of glycerol [16]. Hence, in this study an additional amount of 1.9% yeast extract was added to LB broth to enhance the bacterial cell mass yield. The dry pellet yield (g/ 250 ml) of DH5α cells containing recombinant pVAX1 -gB plasmid in LB broth and LBY broth after 18 hours of overnight culture were 1.142 ± 0.016 and 1.535 ± 0.016. The addition of yeast extract enhanced the yield of bacterial cell mass to 0.39 g/ 250 ml of culture volume (Table.1). Hence, LB broth supplemented with yeast extract may be used to enhance the yield of *E. coli* cells carrying the recombinant plasmid.

The yield of plasmid purified using commercial kit ranged from 500 µg to 1 mg. Most of the studies on DNA vaccine for poultry used 100 -150 µg of plasmid per bird for Intramuscular route of administration [17-19]. A single preparation is only sufficient for approximately 5 Nos. of birds. The cost of commercial kits is higher and utilizes RNAse A which must be avoided in clinical preparation to prevent Bovine Spongiform Encephalopathy (BSE) [9]. Hence modified traditional alkaline lysis was explored as an alternative. A super solution containing solution III and $CaCl_2$ was used earlier to precipitate RNAs in miniprep [5]. However, this method did not provide good yield in maxiprep and this method was suitable for culture volume up to 50 ml. The $Cacl_2$ will precipitate high molecular weight RNA and subsequent addition of polyethylene glycol (10%) will precipitate only the DNA leaving small molecular weight RNA in solution. The combination of 10% PEG and $Cacl_2$ described earlier was also not successful in yielding high quality and quantity of plasmid in maxiprep preparation [10]. Hence, 40% PEG 6000 in 2.5M NaCl was used instead of 10% PEG with 500mM NaCl in maxiprep preparation which significantly enhanced the yield of plasmid in maxiprep preparation. The mean yield of plasmid purified using the commercial kit was 566 µg / 250 ml of culture volume. The mean yield of plasmid purified by modified alkaline lysis was 3.530 mg / 250 ml of culture volume. The modified alkaline lysis method yielded five times higher yield compared to purification by kit. The purity of plasmid in commercial kit and modified alkaline lysis ranged

from 1.84 and 1.99, respectively (Table. 2). The PEG -NaCl-plasmid mixture was incubated for 2 hours and the yield of plasmid did not increase even after overnight incubation. Our method yielded more plasmid than earlier reports (0.56 mg/ L and 1.6 – 4.3 mg /L) using the shake flask method [15, 20]. The higher yield in our experiment might be due to the combined effect of increased cell biomass from LB broth with additional yeast extract and improved plasmid yield by purification using the modified alkaline lysis method.

The quality of the plasmid was also checked using agarose gel electrophoresis. The plasmid (pVAX1-gB) purified using commercial kit and modified method was electrophorosed on 1% agarose gel to identify the various forms of plasmid. Three forms of plasmid viz. super coiled, linear, and covalently circular were observed in the plasmid purified using the commercial kit. Whereas highly supercoiled and covalently circular forms were observed in plasmid purified by modified method (fig.1). The modified alkaline lysis yielded higher amount of supercoiled plasmid than the commercial kit. The plasmids for therapeutic purpose should have more than 90% supercoiled form. The linear or nicked form plasmids are not preferrable for therapeutic purpose [21].

To assess the suitability of plasmid for downstream applications, the extracted plasmid was subjected to double digestion with restriction enzymes and transfection studies in cell culture. The extracted plasmid was double digested with *Bmt I* and *Afl* II enzymes to verify the insert release. Restriction digestion resulted in two fragments of 2.9 kb and 2.6 kb in size (fig.2). The transfection experiment revealed no cytotoxic effects from the purified plasmid in HEK-293 T cells. The transfection and expression of glycoprotein B was verified by Western blotting which showed a band size of approximately 58 kDa specific to ILTV glycoprotein B (fig. 3). This suggested that the extracted plasmid can be used for cloning and transfection studies.

The plasmid extracted using the modified method described in this paper is highly cost effective. The purification cost per dose (approx. 100 µg) using a commercial kit include Rs.250 to 500/-. Whereas, the purification cost per dose (100 µg) using the modified method in laboratory scale is not more than Rs. 2/-.

4. CONCLUSION

Plasmids are indispensable part in molecular biology and plasmid DNA vaccines are gaining importance nowadays. Commercial kits are costlier and the traditional methods are suitable for lesser culture volume. Hence, ways to increase the yield of plasmid were studied. Addition of yeast extract enhanced the bacterial cell mass yield and the traditional purification methods were modified. Plasmids purified using modified method described in this paper are cost effective and suitable for clinical use as it contains high amount of supercoiled plasmid DNA free of RNAse A. Apart from clinical use, the plasmid prepared in this method can also be used for cloning and transfection studies.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declares that no generative AI technologies such as large language models (ChatGPT, COPILOT, etc.,) and text-to-image generators have been used during the writing of manuscript.

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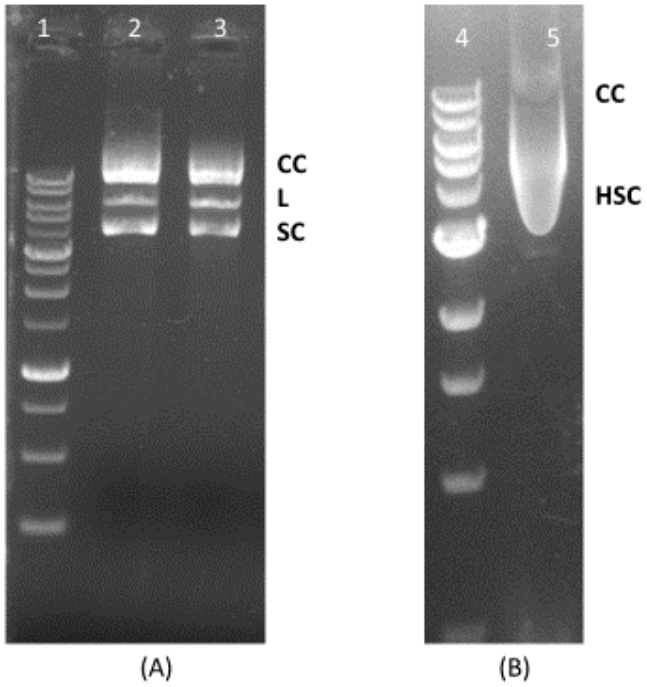

Fig.1. Agarose gel electrophoresis showing plasmid isoforms

A. plasmid pVAX1-gB extracted using commercial kit; B. Plasmid extracted by modified method; CC-covalently circular; L-linear; SC – supercoiled; HSC – highly supercoiled , Lane 1& 4 – 1 Kb ladder; Lane 2, 3, 5 – pVAX1-gB plasmid

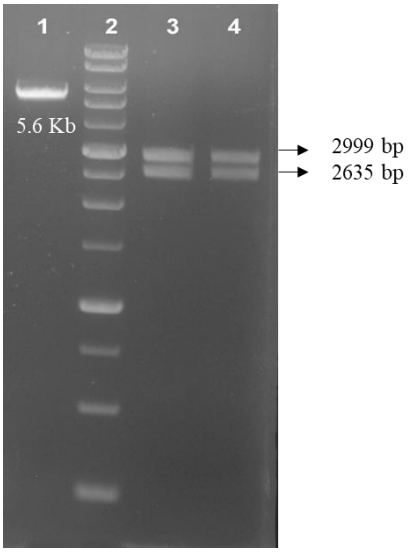

Fig.2. Agarose gel electrophoresis of pVAX1-gB plasmid digested with *Bmt I* and *Afl II* enzymes

Lane 1 – plasmid digested with *Bmt I* enzyme ; Lane 2 – 1Kb ladder; Lane 3& 4 – Double digested plasmid.

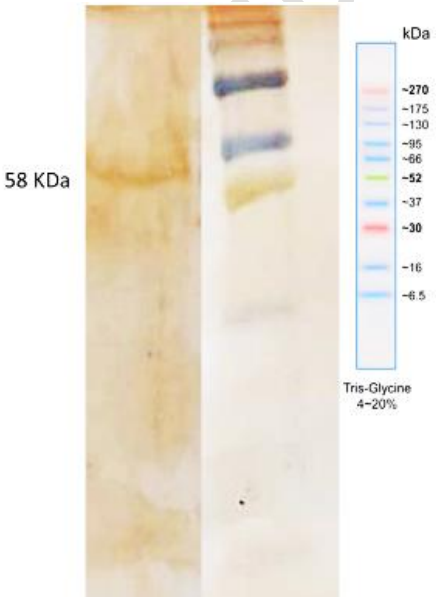

Fig.3. Western blot analysis showing 58 KDa glycoprotein B of ILTV expressed in HEK-293 T cells by transfection of PVAX1-gB plasmid

Table. 1. Yield of bacterial cell mass in LB and LBY medium

Medium	**LB broth**	**LBY broth**

Bacterial cell mass yield (g/ 250 ml)	1.20	1.52
	1.13	1.56
	1.13	1.48
	1.15	1.55
	1.10	1.57
Mean ± SE	1.142 **±** 0.0166	1.535 **±** 0.0169

Table. 2. Yield of plasmid in different purification method

Method	**Commercial purification kit**		**Modified alkaline lysis method**	
	Yield	**Purity (A260/ 280)**	**Yield**	**Purity (A260/ 280)**
	0.51	1.81	3.425	2.06
	0.525	1.83	3.555	1.98
	0.575	1.92	3.375	2.00
	0.725	1.86	3.568	1.99
	0.495	1.80	3.725	1.96
Mean ± SE	0.566 ± 0.061	1.84 ± 0.021	3.530 ± 0.061	1. 998 ± 0.017