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Evaluation of Transformation Ability of Caesium Chloride Density Gradient Centrifugation and Ion Exchange High Performance Liquid Chromatography Purified Plasmids

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Abstract: The aim of present study was to compare the biological activity of plasmid purified by $CsCl_2$ buoyant density gradient centrifugation and anion exchange HPLC elution from alkaline cell lysates. The recombinant plasmids were purified from *E. coli* clones by anion exchange HPLC and $CsCl_2$ density gradient centrifugation. Purified samples were digested with restriction endonuclease and the ligated recombinant plasmids were used to transform *E. coli* HB101 strain. The $CsCl_2$ purified plasmid DNA exhibited higher degree of purity than HPLC purified plasmid. However, transformation efficiency was higher for HPLC purified samples; recombinant pUC8 in VE144 varied between 2×10^3 to 3.5×10^3 for crude sample, 2.52×10^3 to 4.2×10^3 for $CsCl_2$ density gradient purified sample and 7.5×10^4 to 1.15×10^5 transformants/μg of HPLC purified sample. The pBR322 in VE145 also showed transformation efficiency as 3.9×10^3 to 1.2×10^4 for crude sample, 5.6×10^4 to 6.3×10^5 transformants/μg of $CsCl_2$ purified and 4.5×10^5 to 5.04×10^6 transformants/μg of HPLC purified plasmid. Due to the higher transformation efficiency and less time consumption, plasmid purification by HPLC can imply major impact on recombinant DNA technology.

Key words: Plasmid purification, anion exchange HPLC, $CsCl_2$-EtBr density gradient centrifugation, transformation

INTRODUCTION

Plasmids are used as a cloning vehicle for recombinant DNA technology. A complete set of plasmid that is covalently closed circular plasmid will be used in further transformation for or particular inserts are used in analytical or preparative mode. For such work one can need plasmid DNA in very high purity. The conventional method of plasmid preparation was precipitation with polyethylene glycol which is tedious or banding them, in a $CsCl_2$ density gradient requires prolonged overnight ultra centrifugation at high speed and addition of ethidium bromide and caesium chloride ($CsCl_2$) has to be removed by organic extraction and dialysis, respectively. All of which might raise concerns about residues in the final product.

Plasmids can be purified by different HPLC methods. Purification by chromatography is considered as the method with highest resolution for producing plasmid DNA proficient for therapeutic applications (Urthaler *et al*., 2005a). The commonly used purification techniques are anion exchange (Hines *et al*., 1992; Eon-Duval and Burke, 2004), hydrophobic interaction (Diogo *et al*., 2000), reverse phase (Weiner *et al*., 1998) and size-exclusion chromatography (Whisenant *et al*., 1998). Most methods are based on plasmid isolation by standard procedures (Maniatis *et al*., 1989) yielding

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the cleared cell lysate and the resultant plasmid has to be separated by HPLC from the remaining contaminants of host cell origin such as proteins, small RNAs, residual chromosomal DNA and lipids (Schluep and Cooney, 1998).

Purified plasmid molecules play an essential role in gene therapy, genetic vaccination (Stadler *et al*., 2004) and in molecular biology laboratories to get stringent results. The avoidance of critical reagents such as animal-derived compounds (enzymes), organic solvents and detergents will significantly reduce the ill effects on patient and operator safety (Moreira *et al*., 2005). The purpose of the present study was to compare the biological activity of plasmid purified by $CsCl_2$ buoyant density gradient centrifugation and anion exchange HPLC elution from alkaline cell lysates.

MATERIALS AND METHODS

The study was conducted at Department of Molecular Microbiology, School of Biotechnology, Madurai Kamaraj University, India during January 2006 to June 2006.

Bacterial Strains and Plasmids

The *E. coli* clones VE 140, VE 141, VE 142, VE 143, VE 144 and VE 145 harboring plasmid pIC20R, pIC20R, pIC19H, pIC19H, pUC8 and pBR322 respectively, used in this study were obtained from School of Biotechnology, Madurai Kamaraj University, India. *E. coli* HB101, sensitive to ampicillin was used for transformation analysis (Table 1).

Plasmid Isolation from Bacteria

Plasmid DNA molecules were extracted by the alkaline lysis method of Maniatis *et al*. (1989). The isolated plasmids were suspended in 3 mL of TE buffer, pH 8.0. From this processed sample, 2 mL aliquots were taken for $CsCl_2$ density gradient purification and 1 mL was allotted for HPLC analysis in which 100 μL injections were made.

Plasmid Purification by $CsCl_2$-EtBr Density Gradient Centrifugation

All plasmid DNA were isolated by alkaline lysis method followed by caesium chloride-ethidium bromide density gradient ultracentrifugation by standard procedures (Maniatis *et al*., 1989). Two milliliter of plasmid DNA extracted by alkaline lysis method was purified by isopycnic banding in $CsCl_2$ with 4.4 g $CsCl_2$, 0.25 mL of 10% (w/v) EtBr and made up to 4 mL by TE buffer (1 mM Tris, 0.1 mM EDTA, pH 8.0) and centrifuged at 50 k for 14.5 h at 20°C. After centrifugation, the banded plasmids were removed and extensively dialysed against TE buffer (1 mM Tris, 0.1 mM EDTA, pH 8.0) and stored at -20°C. Purity of plasmids was checked by UV absorbance at A_{260}:A_{280} and 0.8% agarose gel electrophoresis.

Plasmid Purification by HPLC

The HPLC purification of plasmid DNA was performed by having Shimadzu liquid pump: LC-6AD, system controller: SCL-6B, UV-Vis detector: (195-700 nm)-SPD-6AV, data processor: CR-5A, column: DEAE 5PW, mobile phase: buffer A (25 mM Tris-HCl, 1 mM EDTA, pH 8.0) and

Table 1: Details of *E. coli* clones and recombinant plasmids used

E. coli clone	Insert DNA (size)	Plasmid (backbone size)	Cloning site of insert DNA
VE140	ICM15 (2.7 kb)	pIC20 R (2.7 kb)	*Bam*HI
VE141	ICM18 (2.7 kb)	pIC20 R (2.7 kb)	*Hind*III
VE142	TGM022 (2.5 kb)	pIC19H (2.7 kb)	*Eco*RI
VE143	TGM023 (2.5 kb)	pIC19H (2.7 kb)	*Eco*RI
VE144	CLV018 (2.7 kb)	pUC8 (2.69 kb)	*Mlu*I
VE145	CLV002 (2.7 kb)	pBR322 (4.36 kb)	*Pst*I

Buffer B (25 mM Tris-HCl, 1 mM EDTA, pH 8.0, 1 M NaCl), flow rate: 0.5 mL min^{-1} and wavelength: 260 nm. The 100 μL of crude plasmids were injected and different fractions were collected for all the peaks. The plasmids purified by $CsCl_2$-EtBr density gradient centrifugation were also injected at 10 μL volume and observed the peak with retention time. The fractions corresponding to the first major peak and second major peaks were collected separately and treated with 1/10th volume of 5 M ammonium acetate and 2.5 volume of 95% ethanol for precipitation. Precipitated pellets were washed with 75% ethanol and stored in 50 μL TE buffer (1 mM Tris, 0.1 mM EDTA, pH 8.0).

Analysis of Plasmid DNA

Concentrations of anion exchange-HPLC, $CsCl_2$-EtBr density gradient purified plasmids and crude plasmids were determined by taking UV absorbance readings at 260 nm and purity of the samples were determined by calculating the ratio of UV absorbance at 260/280 nm. Total yield of plasmids were calculated by multiplying the concentration by the volume of recovered eluate. The Samples were run on a 0.8% TBE agarose gel at 60 volts for 1 h, visually inspected under UV-trans illuminator to confirm their yield and purity and photographed.

Restriction Enzyme Digestion

Restriction enzymes and T_4 DNA ligase were obtained from Promega. The unpurified and purified plasmids pIC20R, pIC20R, pIC19H, pIC19H, pUC8 and pBR322 were digested with 2 units of *BamH*I, *Hind*III, *Eco*RI, *Eco*RI, *Mlu*I and *Pst*I respectively for 3 h at 37°C. After the digestion samples were kept in ice for 2 min, then kept at 65°C for 10 min and kept in ice. Digested products were checked on 0.8% agarose gel.

Transformation Experiment in *E. coli* HB101 Strain

The transformation experiments were done as reported by Cohen *et al*. (1972) and performed in triplicates. Fifty nanogram of restriction digested recombinant plasmids were ligated with the respective insert DNA molecule using T_4 DNA ligase, 10 mM ATP at 14°C for 6 h and the ligated recombinant plasmids (50 ng) were transformed to *E. coli* HB101 competent cells by heat shock method and plated on LB plates containing ampicillin (50 μg mL^{-1}) and incubated at 37°C. The positive and negative controls were maintained by having 50 and 0 ng of respective plasmid DNA. The colonies were counted and the transformation efficiencies were calculated as the number of transformed colonies obtained divided by the amount of plasmid DNA contained with in the spreading volume on the ampicillin plate.

RESULTS

Purification of Plasmids by HPLC and $CsCl_2$ Buoyant Density Gradient Centrifugation

The unpurified recombinant plasmids prepared from various strains were passed through DEAE-5PW column. The elution pattern monitored by absorption at 260 nm is shown in Fig. 1. The concentration and purity were analyzed by recording absorbance at 260 and 280 nm. Table 2 summarizes the plasmid concentration in different purification strategies and their recovery percentage.

Purity of the $CsCl_2$ density gradient purified recombinant plasmids were checked by anion exchange HPLC. The HPLC results showed that the plasmid pIC20R from VE140 clone have two peaks of retention time 6.202 and 27.248 min and $CsCl_2$ density gradient purified sample showed the peaks of 6.36 and 26.208 min. In pIC20R of VE141 injection, two peaks were observed (6.314 and 26.027 min) and $CsCl_2$ density gradient purified samples showed two peaks of 6.342 and 26.595 min. The pIC20H from VE142 showed two peaks (6.364 and 25.647 min) and its $CsCl_2$ density gradient purified counterparts showed two peaks of 5.9 and 26.487 min. In general all the clones exhibited similar kind of chromatogram i.e., first peak between 5.9 to 6.828 and second peak between 25.647 to 27.665 min and the fraction a, which corresponds to first

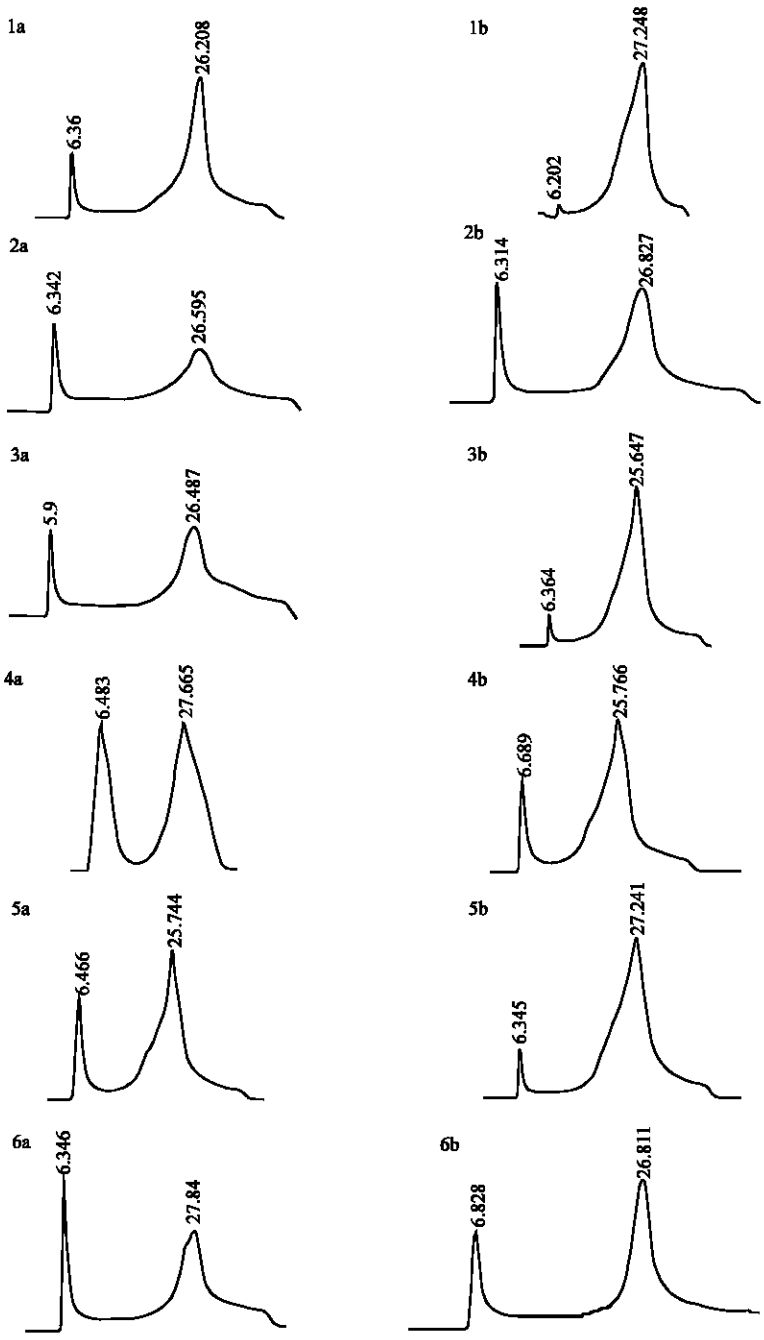

Fig. 1: Elution profile of recombinant plasmids from an anion-exchange HPLC column. 1, 2, 3, 4, 5 and 6 represents the recombinant plasmids from clones VE140, VE141, VE142, VE143, VE144 and VE145, respectively. (1a-3b) depicts the $CsCl_2$ density gradient purified samples and (4a-6b) represents the elution pattern of plasmid samples. The first peak eluted near the void volume denotes the contaminants and second peak denotes the elution of plasmids

Table 2: Concentration and purity of plasmids by UV absorbance at 260/280

E. coli clone	Concentration ($\mu g\ \mu L^{-1}$)			Purity level (A_{260}:A_{280})			Plasmid loaded in HPLC (μg)	Plasmid eluted from HPLC (μg)	Recovery (%)
	Crude plasmid	HPLC purified plasmid	$CsCl_2$ purified plasmid	Crude plasmid	HPLC purified plasmid	$CsCl_2$ purified plasmid			
VE140	1.80	0.82	1.41	1.91	1.77	1.79	42.50	32.00	75.29
VE141	2.67	1.05	1.35	1.98	1.73	1.82	162.30	123.50	76.09
VE142	1.23	0.93	1.35	1.56	1.71	1.77	81.25	61.50	75.69
VE143	3.26	2.25	4.85	1.32	1.72	1.76	89.44	63.00	70.44
VE144	4.60	2.58	3.10	1.39	1.75	1.73	274.61	230.00	83.76
VE145	1.47	0.94	1.27	1.42	1.71	1.75	94.12	73.50	78.09

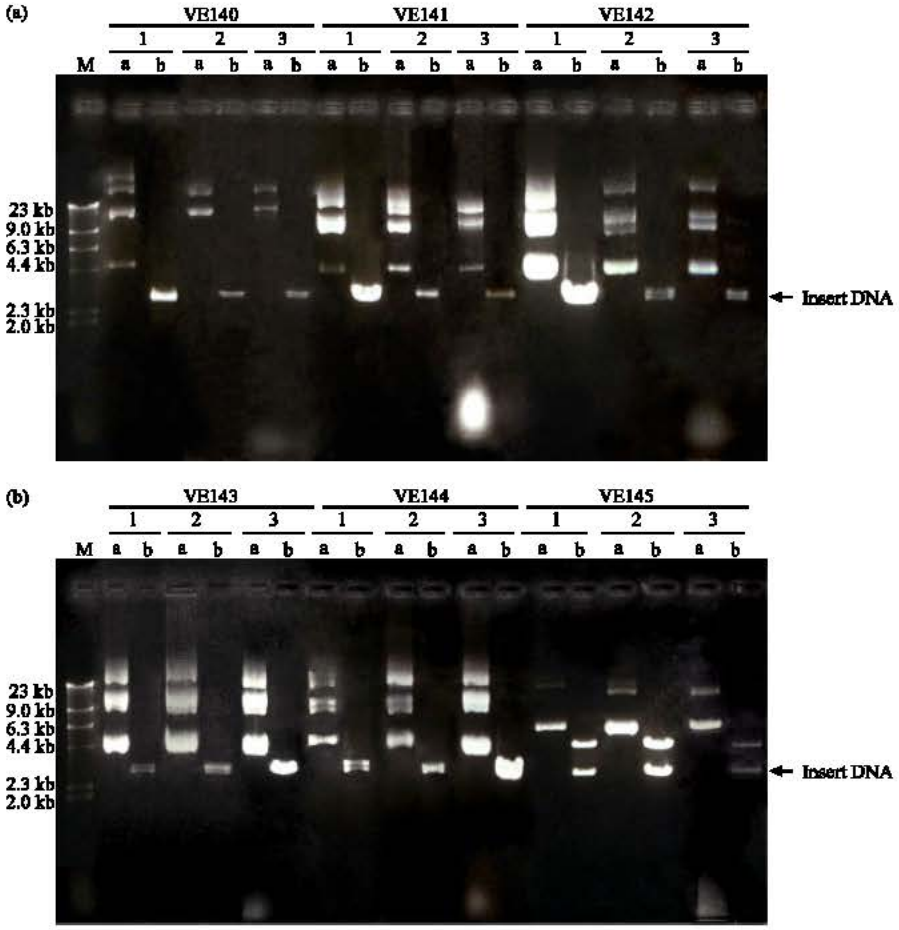

Fig. 2a and b: Restriction pattern of purified plasmids Lane M-λ *Hind*III size marker; 1, 2 and 3 represents HPLC, $CsCl_2$ purified and crude plasmids, (a) denotes undigested samples; (b) represents digested products of plasmids

peak is characterized by having maximum amount of protein, RNA and other contaminants. The fraction b of second peak represents the pure form of plasmid DNA and agarose gel electrophoresis of fraction b showed the presence of concatemeric, linear, nicked and supercoiled form of plasmid DNA.

Restriction Digestion Assay

All the recombinant plasmids were digested with respective restriction endonuclease as shown in Table 1. Agarose gel electrophoresis of digested samples showed the complete excision of insert DNA from plasmid backbone and there were no difference in the pattern of bands in the 0.8% agarose gel for all the crude, HPLC and $CsCl_2$ density gradient purified plasmid samples (Fig. 2a and b).

Transformation Assay

The transformation experiments were carried out for the two clones namely VE144 and VE145. Transformation efficiency of clone VE144 varied between 2×10^3 to 3.5×10^3 for crude sample, 2.52×10^3 to 4.2×10^3 for $CsCl_2$ density gradient purified sample and 7.5×10^4 to 1.15×10^5 transformants/μg of HPLC purified sample. The VE145 also showed similar transformation efficiency (i.e.,) 3.9×10^3 to 1.2×10^4 for crude sample, 5.6×10^4 to 6.3×10^5 transformants/μg of $CsCl_2$ buoyant density gradient purified plasmids and 4.5×10^5 to 5.04×10^6 transformants/μg of HPLC purified samples. The presence of plasmids in the Amp^r colonies of transformation assays were carried out and all the Amp^r colonies showed the presence of respective recombinant plasmids.

DISCUSSION

The traditional and newly developed DNA purification procedures have the goal to remove the toxic components and other cell debris from the cell extracts. A number of commercial kit-based DNA purification techniques have become popular but the large scale cost effective purification of DNA remains a technological challenge and make the DNA purification as the expensive one. Plasmid purification by anion exchange HPLC resulted in highly efficient plasmid molecules free from general contaminants of chromosomal DNA, RNA and proteins. The polynucleotides are negatively charged and they are independent of the buffer conditions in the anion-exchange chromatography (Urthaler *et al*., 2005b) and it may be the prime factor in yielding the biologically efficient and highly purified plasmid DNA by HPLC equipped with DEAE column. The HPLC purified plasmid DNA pools were devoid of residual contaminants and the percentage of recovery was in the range of 75.29 to 83.76. When compared to $CsCl_2$ buoyant density gradient centrifugation the plasmid purification by HPLC is a less tedious process and it can be employed both small and large scale purification of plasmid DNA molecules.

Transformation efficiency of HPLC purified samples was higher when compared to $CsCl_2$ buoyant density gradient purified plasmid DNA and both the preparations showed more or less similar transformation efficiency. However, the larger plasmids can be easily lost during $CsCl_2$ density gradient centrifugation (Corneau *et al*., 2001) but the HPLC system can separate the nucleic acids in order of size with the smaller fragments eluting first and can provide an effective separation of fragments as small as 10 bp to 50 kbp by ion exchange column and HPLC purification takes lesser time (about 30 min for separation) than $CsCl_2$ density gradient centrifugation. From the above results we can conclude that plasmid purification by anion exchange HPLC is best performed in transformation experiments and can be used for genetic engineering and other purposes.

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