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PREPARATION AND PURIFICATION OF DNA FROM BACTERIAL CELLS; CHARACTERIZATION OF PLASMID DNA

M Praveen, G Adarsh, T Ramesh, M Ramesh*
Genetic Engineering Laboratory, Department of Biotechnology, JNTU Kakinada-UCEV, Andhra Pradesh, India

***Corresponding Author:** Dr. Ramesh Malothu, Assistant Professor and Head, Department of Biotechnology, JNTU, Kakinada-UCEV, Vizianagaram, Andhra Pradesh, India.

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Abstract: The use of genetic material to deliver genes for therapeutic purposes has been practiced for many years. With the advancement in genetic engineering, foreign genes of industrial applications can be inserted into cloning vector for mass production in various host cells. *Escherichia coli* is an extremely important model organism in modern biological engineering, the suitable growth media is essential for the optimal expression of the genes in *E. coli*. The present study aims at isolation and purification of genomic DNA from *E. coli*, the characterization of pBR322 plasmid DNA. Bacterial culture conditions were optimized in shake – flask cultures based on optimal temperature, inoculum size and medium composition. Solutions and methods are disclosed for the effective, simple isolation of DNA from bacterial cells. High bioprocess recovery and product quality were primarily associated with the complete removal of total cellular RNA impurity. The process was demonstrated without the use of animal-derived RNase. High-molecular-weight (HMW) RNA and other impurities were removed by selective precipitation using calcium chloride at an optimal concentration. The optimal conditions for the growth of *Escherichia coli* were shown maximum absorbance as 7.5 at 37°C temperature, 1% inoculum size using TB medium composition. The purified genomic DNA had concentration as 73.5 µg/ml and purity 1.8. The 0.5M $CaCl_2$ was optimal concentration for removal of RNA. The plasmid DNA pBR322 was confirmed by comparing the band to 4.36 Kb, purity of plasmid was 1.85 and it contains 96.8% of super coiled DNA. The contaminants like chromosomal DNA, RNA, host cell proteins and mycoplasma were absent in the plasmid DNA.

Keywords: *E. coli*, DNA, Plasmid, pBR 322, Restriction Digestion, HPLC, RT-PCR.

INTRODUCTION

Many vital techniques, such as molecular cloning and over expression of cloned genes, were initially developed in *E.coli* and are still simpler and more effective in the bacterium. It is easy to grow under laboratory conditions, and research strains are very safe to work with. As compared with many bacteria, *E. coli* grows quickly, which allows many generations to be studied in a short time. In fact, under ideal conditions, *E. coli* cells can double in number after only 20 minutes. The bacterium is still a primary resource in many modern laboratories. The suitable growth media is essential for the optimal expression of the genes in *E.coli*. Various growth media and conditions (such as pH, temperature, O_2 availability, carbon and nitrogen sources) influence not only the growth of bacteria but also the amount of gene expression. Complex media such as LB (Luria Bertani) medium and TB (Terrific Broth) have been successfully used to grow *Escherichia coli* in small-scale cultures.

The isolation and purification of DNA is a key step for most protocols in molecular biology studies. To concentrate nucleic acids for re-suspension in a more suitable buffer, solvents such as ethanol (75-80%) or isopropanol (40-50%) are commonly used in the presence of salt to precipitate nucleic acids. Ethanol is more preferred because less salt will co-precipitate and the pellet is more easily dried. High bioprocess recovery and product quality were primarily associated with the complete removal of total cellular RNA impurity. High-molecular-weight (HMW) RNA is removed by selective precipitation using calcium chloride at an optimal concentration. Here we demonstrated without the use of animal-derived products (e.g. RNase, lysozyme) to produce DNAs. The purified plasmid DNA also contains very low levels of contaminants such as chromosomal DNA, RNA, host cell proteins, mycoplasma and endotoxins. These were characterized by using different analytical methods.

MATERIALS AND METHODS

Escherichia coli bacterial strain (ATCC 8739) was obtained from NCIM Pune. Plasmid DNA pBR322 was obtained from Invitrogen life technologies. Nru I was also purchased from Invitrogen. All required medium components and chemicals were purchased from S D FINE CHEMICALS Limited or Sigma Aldrich.

Preparation of bacterial cells

Luria Bertani Broth, Miller is used for the routine cultivation and maintenance of *Escherichia coli* strain ATCC 8739. Culture medium was prepared by dissolving

3.75 grams of LB broth in 150ml of distilled water with the help of magnetic stirrer and pH was measured. 100ml Transferred into 1000ml conical flask for inoculation and 50ml transferred into 500ml conical flask for pH and O.D. (Blank) measurements, then closed with cotton plugs tightly, kept for sterilization. Sterilization was done by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Then sterilized flasks were cooled down to room temperature.

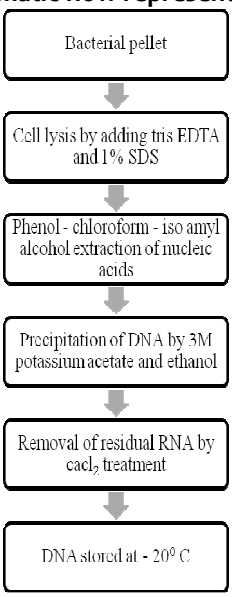
The conical flasks were kept in orbital shaker carefully by maintaining required temperature and rpm. Here the following table shows the studied parameters and variables in shake flask cultures. Remaining parameters were kept constant.

Table.2.1: Studied parameters and variables in shake flask cultures

S. No	Temperature	Inoculum%	Medium composition
1	35°C	0.5	LB
2	37°C	1	TB
3	40°C	2	SOB

Isolation and purification of genomic DNA:

Schematic flow representation



Concentration of DNA sample (µg/ml) = 50 x A_{260} x dilution factor
Purity of DNA = A_{260}/A_{280} and A_{260}/A_{230}

Characterization of pBR322 plasmid DNA:

The purified plasmid DNA also contains very low levels of contaminants such as chromosomal DNA, RNA, host cell proteins, mycoplasma and endotoxins. These were characterized by using different analytical methods. Here in the following table represents recommended assays for assessing the purity and impurity limits.

Restriction digestion of plasmid DNA:

The reaction mixture was taken in the following order in a micro centrifuge tube for 20 µl digest.

- Plasmid DNA - 3 µl
- Nru I - 2 µl
- 10X Nru I Buffer - 2 µl
- 0.1% BSA - 2 µl
- Sterile de – ionized water - 11 µl

The reaction mixture in the tube was gently flicked and spun down in microfuge for a few seconds. Then the tube was placed in heat block at 37°C for two hours. After reaction was completed, placed on ice and prepared 1% agarose gel in 1X TAE or TBE buffer.

Table.2.3: Test assays for characterization of pBR322 plasmid DNA

Test	Analytical method	Approval limits
Plasmid Identity	Restriction digestion and AGE	Confirmation
Quantitation of plasmid DNA	UV - Absorption	1.7 – 2.0
Topology of plasmid DNA	HPLC or AGE	Super coiled > 95%
Chromosomal DNA	RT - PCR	Absent
RNA	Visual inspection after AGE	Absent
Host cell proteins	Protein assay	< 3 µg/ml
Mycoplasma	PCR and AGE	Absent

Quantitation of plasmid DNA:

The concentration and purity of plasmid DNA samples were determined by using NanoVue plus spectrophotometer. The absorbance was measured at wave length between 230 nm – 320 nm.

- Concentration = A_{260} x dilution factor x 50 µg/ml
- Purity = A_{260}/A_{280} and A_{260}/A_{230}

Topology of Plasmid DNA HPLC:

Different isoforms of DNA were separated by high performance liquid chromatography. Injection method and column conditions are shown as follows:

- Column : TSKgel DNA – NPR, 4.6mm x 7.5 cm
- Sample : pBR322 plasmid
- Injection volume: 10 µl
- Elution :Buffer A: 20Mm Tris, pH 9.0; Buffer B: 20Mm Tris, pH 9.0 with 1M NaCl
- Gradient : Linear gradient from 50% to 65% Buffer A to Buffer B.
- Flow rate : 1mL/min
- Detection : UV@ 260nm
- Column temperature : 25°C
- Sample Temperature : Ambient
- The sample was run through the TSKgel DNA – NPR Anion Exchange Column, and then it was analyzed by using system generated chromatogram.

Detection of chromosomal DNA:

Preparation of reaction mix:

- dNTPs - 2 µl
- Taq Polymerase - 1 µl

- PCR buffer - 5 µl
- SyBr dye - 1 µl
- Forward primer - 1 µl
- Reverse primer - 1 µl
- Sterile water - 8 µl

Setting up the reaction:

- PCR vials were placed in the PCR vial holder.
- 19 µl of reaction mix was added to all the sample vials and to that 1µl of diluted samples were added.
- To the negative template control (NTC) sample 20 µl of reaction mix was added.
- The PCR program was set up and samples were run according to the following conditions:

PCR cycle:

- Initial denaturation -95°C 3 min
- Denaturation -94°C 30 sec
- Annealing -55°C 45 sec
- Elongation -75°C 1 min
- From step 2 to 4 were repeated for 30 cycles
- Final Extension -72°C 10 min
- Final hold -4°C - ∞

Detection of RNA:

The RNA contamination was determined by using visual inspection after agarose gel electrophoresis.

Host cell proteins:

The presence of host cell proteins in the DNA sample was determined by using E. coli HCP, ELISA Kit Cat. No. 800- 130- ECP.

Mycoplasma:

The presence of mycoplasma in the DNA sample was determined by using EZ – PCR Mycoplasma Test Kit.

RESULTS AND DISCUSSION

Bacterial culture conditions:

The optimal temperature for *E. coli* growth was observed at 37°C, resulted higher O.D values. The O.D values were decreased at 35°C and 40°C. This may be due to the enzymes becomes denatured at higher and lower temperatures.

Table 3.1.1: Effect of temperature on *E.coli*growth

S. No	Temperature	O.D (600 nm)
1	35° C	0.8
2	37° C	2.0
3	40° C	1.2

The optimal inoculum size for the growth of *E.coli*was 1 ml (1%). From the present results it is evident that change in inoculum size greatly effects the lag time, increase of inoculum size decreases the lag time and it influences on media composition but doubling time was not affected. So O.D value at 1% is in increasing tendency, 0.5% inoculum shown less O.D and 2% does not shown any increase in O.D levels.

Table 3.1.2: Effect of inoculum percentage on *E.coli* growth

S. No	Inoculum %	O.D (600 nm)
1	0.5	2.4
2	1.0	4.0
3	2.0	2.7

Experimental conditions were kept constant like temperature 37°C, 1% inoculum size and studies conducted on various media compositions. Among those TB broth have shown better O.D values than SOB and LB broth. This may be due to the presence of two phosphates and glycerol in its composition.

Table 3.1.3: Effect of the media composition on *E.coli*growth

S. No	Medium composition	O.D (600 nm)
1	LB	3.6
2	TB	7.0
3	SOB	4.2

Isolation and purification of genomic DNA:

Various concentrations (0.3M – 1.0M) of $CaCl_2$ were studied for removal of residual RNA without degrading genomic DNA. For residual RNA removal 0.5M $CaCl_2$ was optimized. At 0.3M $CaCl_2$ RNA was not completely removed and with increasing concentrations of $CaCl_2$ to 0.7M and 1.0M the DNA was also getting degraded. The isolated and purified genomic DNA was used as a standard for host cell genomic DNA identification in plasmid DNA characterization. A_{260}/A_{280} ratio must range in between 1.7 – 2.0 indicates the purity of DNA. If A_{260}/A_{280} ratio is lower than 1.7, it indicates the presence of protein content and organic solvents. If A_{260}/A_{280} ratio is ≥ 2.0, it indicates the presence of RNA content in the sample.

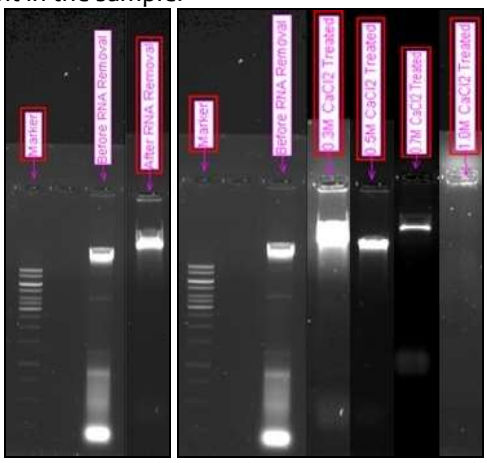


Fig 3.2: Genomic DNA before and after RNA removal with Different molar concentrations of $CaCl_2$ treatment

Characterization of pBR322 by analytical methods:

Plasmid identity: Plasmid identity method was confirmed by the restriction digestion method using Nru I enzyme. pBR322 plasmid DNA having 4361 base pairs in its length. So the plasmid was confirmed, after restriction digestion and gel run by comparing the band at 4361 base pairs.

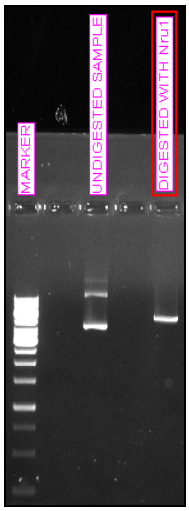


Fig 3.3.1: Plasmid identity

Quantitation of plasmid DNA: Plasmid DNA was quantified by using the Nano spectrophotometer. We got the results from the experiment was concentration 153 µg/ml and purity 1.85. Identification of impurities are based on the A_{260}/A_{280} ratio, from the experiment it was in between 1.7 – 2.0, this indicates the purity level of the plasmid.

Topology of plasmid DNA: Topology of the plasmid DNA was analyzed by using HPLC column TSKgel DNA – NPR. We were clearly identified the separation of super coiled, open circular and linear forms of plasmid (Topological forms of plasmid DNA). Based on our results, these were as supercoiled plasmid 96.80%, open circular 1.48 and linear 1.72. If the open circular content is more it indicates that the denaturation of plasmid DNA. (See figure 3.3.3).

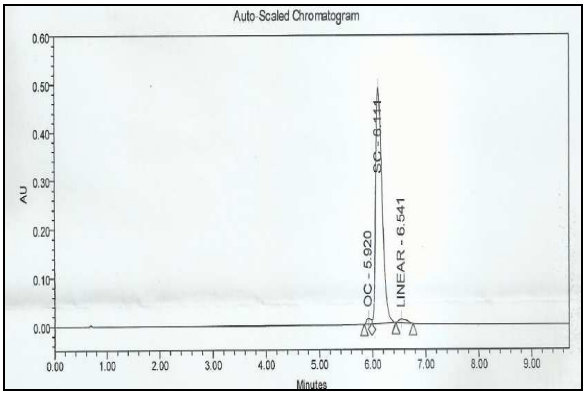


Fig 3.3.3: Topology of plasmid DNA

Chromosomal DNA detection: The contamination check of plasmid with chromosomal DNA was carried out by using RT – PCR. Here we used in house prepared and purified (explained in earlier chapters) genomic DNA as a standard to identify the chromosomal contamination in plasmid DNA pBR322. As per the results, (see figure 3.4) it was clearly shown that the plasmid DNA was not contaminated with chromosomal DNA.

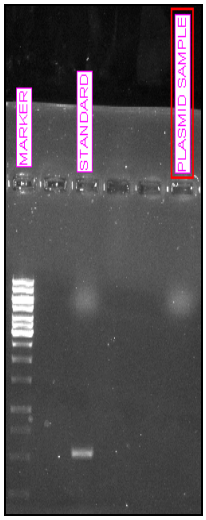


Fig 3.4: chromosomal DNA detection

RNA detection:

RNA detection was carried out by using agarose gel electrophoresis and A_{260}/A_{280} ratio was 1.90. (See figure 3.5) The gel indicates that the pBR322 plasmid DNA does not contain RNA. A_{260}/A_{280} ratio also indicates the absence of RNA, if it is > 2.0 indicates the presence of RNA.

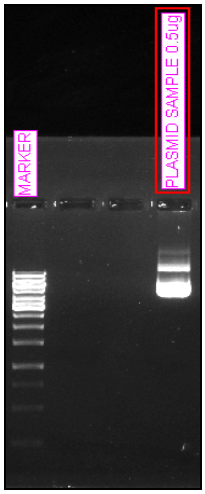


Fig 3.5: RNA detection

Host cell proteins:

Host cell proteins detection was carried out by using *E.coli* HCP, ELISA Kit method. As per standard norms host cell proteins limits are < 3 µg/ml. we got the result from the experiment was 0.36µg/ml, as per standard norms the host cell proteins in plasmid DNA are within the limits.

Mycoplasma:

The detection of mycoplasma in pBR322 was carried out by using EZ- PCR mycoplasma test kit based on RT - PCR technique. The PCR product was loaded onto the gel and it indicates that the absence of mycoplasma in pBR322. This is compared with the mycoplasma standard provided in kit. (See figure 3.7).

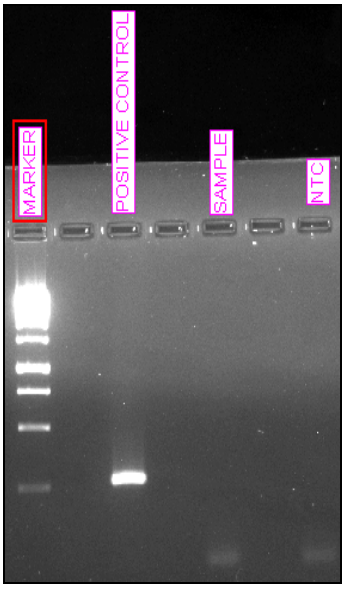


Fig 3.7: mycoplasma detection

CONCLUSIONS

From the experimental results we conclude that a rich buffered medium, terrific broth (TB), yielded more biomass at temperature 37°C and inoculum size 1% compared to LB and SOB medium compositions. The high quality and quantity of current method for isolation and purification of genomic DNA from bacterial cells is not only simple, but also cost effective. Here we demonstrated procedure without the use of animal derived enzymatic substances (such as proteinase K, RNase and lysozyme). From the experiments conducted we found that, the 0.5M $CaCl_2$ was optimal concentration for removal of RNA and other impurities. The purified genomic DNA had concentration as 73.5 µg/ml and purity 1.8. The characterization of pBR322 plasmid DNA was analyzed by various analytical methods. From the conducted experimental assays, the plasmid identity was confirmed and through quantitation we found that the purity was 1.85. The plasmid contains super coiled DNA more than 95% and host cell proteins were less than 3 µg/ml. The remaining contaminants such as chromosomal DNA, RNA and mycoplasma were absent in the plasmid DNA.

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