

CANINE PARVOVIRUS GENE GROW IN *E.Coli* USING MOLASSES MEDIUM WHICH CAN BE USE AS A VACCINE.

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ABSTRACT: The Canine parvovirus gene is inserted in *E.coli* (DH5 α) strain. Grow the recombinant *E. coli* for the plasmid DNA using molasses medium to make the vaccine. During the initial studies I have found that the molasses can manipulate the C/N ratio as per requirement of cell. Molasses is one of the best alternatives as it is cheap and can be easily manipulated. During the experiment the O.D of culture and O.D of plasmid DNA was observed in respective of different optimization method. I observed that the O.D value has increase to i.e. 2.129, as it was earlier i.e. 0.393. The quality and quantity of the plasmid DNA was very good. It is possible to produce vaccine by molasses medium. The paper is opening a new face of study.

Keywords: Molasses, Canine parvovirus gene, *E.coli* DH5 α strains, Glucose, K₂HPO₄, Urea.

INTRODUCTION

Canine parvovirus (CPV) is a member of the parvovirus genus which was first recognized in 1978 (Appel *et al.*, 1985) and (Burtonboy *et al.*, 1979). It is a small nonenveloped, single-stranded DNA virus (Siegl *et al.*, 1990). It replicates autonomously, causes severe enteritis in dogs of all ages and myocarditis in pups less than 12 weeks of age. The amplified VP2 gene PCR product was characterized by nested PCR using primer set forward: 5'-TAC CAT GGTACA GAT CCA G-3'; reverse: 5'-CCT CTA TAT CAC CAA AGT TA-3' reported for CPV diagnosis (Uwatoka *et al.*, 1995). This gene of CPV is transformed in *e.coli*.

The advantages of using molasses as a medium is apart from the cost effectiveness of the product, molasses as a medium is manipulative to requisite micro-organisms, because molasses contain nitrogen in low amount and can be optimized by adding the cheapest source of the growth material. The naturally occurring source as like waste product molasses as substitute from the industry or from the natural plant.

To fulfill the demand of the growth components and other trace materials for achieving an optimum recombinant plasmid yield so in this project the effort is made to optimize the molasses medium. Molasses is made from young green sugar cane and is treated with sulfur dioxide, which acts as a preservative, during the sugar extraction process. Unsulfured molasses is made from mature sugar cane and does not require treatment with sulfur during the extraction process. To make molasses, which is pure sugar cane juice, the sugar cane plant is harvested and stripped of its leaves. Its juice is extracted from the canes, usually by crushing or mashing. The juice is boiled to concentrate and promote the crystallization of the sugar. The majority of sucrose from the original juice has been crystallized but black strap molasses is still mostly sugar by calories (WHF). Grow the *E. coli* in molasses as a medium. *Escherichia coli* (*E. coli*) are a bacterium that is commonly found in the lower intestine of warm-blooded animals. Most *E. coli* strains are harmless, but some, such as serotype O157:H7, can cause serious food poisoning in humans, and are occasionally responsible for costly product recalls (Dippold *et al.*, 2005). The harmless strains are part of the normal flora of the gut, and can benefit their hosts by producing vitamin K₂, or by preventing the establishment of pathogenic bacteria within the intestine (Hudault *et al.*, Sep. 2001). *E. coli* is Gram-negative, facultative anaerobic and non-sporulating. It can live on a wide variety of substrates. *E. coli* uses mixed-acid fermentation in anaerobic conditions, producing lactate, succinate, ethanol, acetate and carbon dioxide. Since many pathways in mixed-acid fermentation produce hydrogen gas, these pathways require the levels of hydrogen to be low, as is the case when *E. coli* lives together with hydrogen-consuming organisms such as methanogens or sulfate-reducing bacteria (Martinko *et al.*, 2006).

MATERIALS AND METHODS

Host Bacterial strain

Escherichia coli DH5 α (Promega, Madison) host strains were used for transformation with recombinant plasmids.

Standard markers

DNA molecular weight markers used were DNA / *Eco*R1 double digest (MBI fermentas, MD), &1Kb DNA ladder (MBI fermentas, MD).

Medium Molasses as a medium.

Chemical & Plastic ware

All chemicals used in the study were either AnalaR or molecular biology grade from sigma (MO), Promega (Madison), Qiagen (Valencia, CA), MBI fermentas (MD), Gibco (MD), and New England Biolabs (MA), Bangalore Genei (Bangalore, India), JRH Biosciences Lenexa), Plastic wares and other consumables were from Axygen, TRP, Nunc, Greiner, Tarsons, Corning or Borosil.

Sugar estimation method

5gm of molasses was accurately weighed and dissolved in 60ml of distilled water. Then added 5ml of 1:1HCl with proper mixing. The solution was heated up to 60 $^{\circ}$ C for 5min in a water bath. The temperature was then raised to 70 $^{\circ}$ C for min. Then the sample was cooled immediately under running tap water. After that sample was neutralized by adding NaOH solution using phenolphthalein as an indicator. then final volume of the sample was made up to 500ml by distilled water. then 5ml each of Fehling's A and B were mixed and diluted by adding 30 to 50ml of DW. After that the inversed molasses sample was taken in the burette and Fehling's mixture was titrated against it.

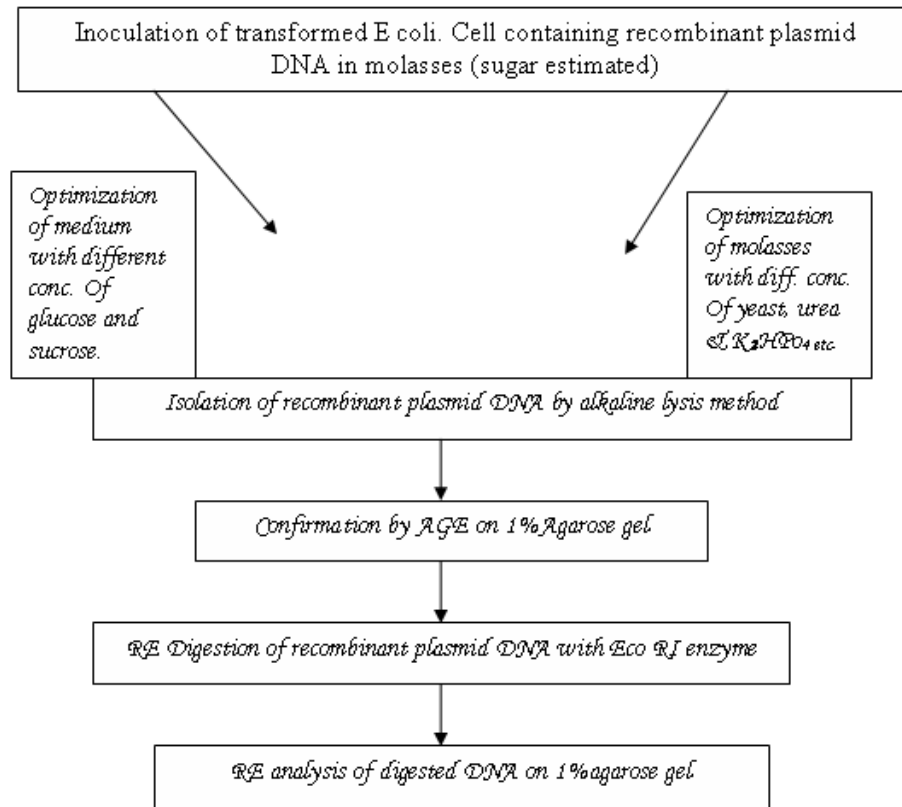
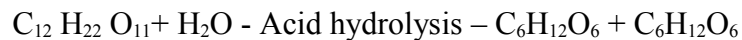


Fig. No.1:-Flow diagram showing the detailed methodology used in the present work.

A few drops of methylene blue were added and used as indicator to get end point. then the end point is arrived when blue solution to brick red precipitate.

Acid Hydrolysis

1. When sucrose is hydrolyzed it forms a 1:1 mixture of glucose and fructose gives glucose and fructose.



Calculations

$$\begin{aligned} &\text{Volume of inverted sugar for titration of 10ml Fehling's Solution} \\ \text{\% of invert sugar} &= \frac{\text{Fehling's factor} \times \text{Volume of invert sugar} \times 100}{\text{Weight of sample} \times \text{titer volume}} \\ \text{\% of invert sugar} &= \frac{0.02 \times 500 \times 100}{5 \times 18} = 11.11\% \end{aligned}$$

Isolation of plasmid DNA

Small-scale plasmid DNA isolation was done following the alkali lysis method (Birnboim and Dolly, 1979). Briefly individual colonies were inoculated in 5 ml molasses with appropriate antibiotic and were kept in orbital shaking incubator overnight. 3ml of overnight grown broth culture was pelleted in a micro centrifuge tube at a12000g for 30 seconds and resuspended in 300 μ l of buffer P1 by vortexing. To this bacterial suspension, 300 μ l of buffer P2 was added, mixed and gently and incubated at room temperature for 5 minutes. Then, 300 μ l of chilled buffer P3 was added and the lysate was mixed gently and kept on ice for 5 minutes. After centrifugation at 12000 g for 19 minutes, the supernatant was transferred gently to another microfuge tube. The supernatant was extracted once with phenol:chloroform mix (1:1v/v) by mixing vigorously and centrifugation at 12000 g for 15 minutes. The upper aqueous phase containing the plasmid DNA was collected and precipitated with 0.8 volume of isopropanol. The pellet obtained by centrifugation at room temperature at 12000 g for 30 minutes was washed with 70% ethanol and air dried. The plasmid DNA was dissolved in 40 μ l of TE buffer.

Restriction endonuclease digestion and analysis of digested DNA

The plasmid DNA (5 to 10 μ g) was incubated overnight with appropriate 1X restriction endonuclease buffer and 3 to 5 U restriction endonuclease at 37°C in a water bath. The digested DNA was mixed with 1X loading dye and run on 1% agarose (SRL, Mumbai) gel using Tris acetate EDTA (TAE) running buffer system containing ethidium bromide (0.5 μ g/ml) and visualized under UV-transilluminator and photographed (Sambrook, 2001).

Reaction mixture for RE digestion

r-plasmid taken from (a)-Molasses+ Yeast (5gm) +Nacl (1gm)

Enzyme taken - EcoR1

(a)-r-plasmid DNA	4 μ l
(b)-restriction enzyme	1 μ l
(c)-assay buffer (10x)	1 μ l
(d)-nuclease free water	4 μ l

Total	10 μ l
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Agarose gel electrophoresis

Agarose, type 1 gelling temperature(40-45°C) from SRL, (Mumbai) was used to perform majority of gel electrophoresis in concentrations of 0.8% to 1.2% for the separation of DNA fragments ranging from 0.4 Kb. Appropriate amount of agarose was mixed with 1X TAE buffer and melted in a microwave oven. When the molten gel had cooled to 45°C, Ethidium bromide was added to final concentration of 0.5 μ g/ml. The gel was mixed thoroughly by gentle g and then poured into the gel casting tray fitted with the comb. The gel was allowed to solidify and the comb was removed. The DNA sample were mixed with gel loading dye 1X (Final concentration) and loaded into the wells. The gel was run at a voltage of 1.5 V/cm for 1-2 hours and the bands were visualized under UV light and photographed (Sambrook, 2001).

RESULTS

In order to reduce the cost of the product the aims of yields of plasmid DNA from recombinant *E. coli* using molasses.

Table: 1 Showing the Different optimization Method for Molasses.

S.No	SAMPLE(ml)	pH(B*G*)	pH(A*G)	OD(B*G*)	OD(A*G*)
1.	Molasses(50ml)+Yeast(.323gm)	7.4	5.0	0.31	1.3
2.	Molasses(80ml)+Yeast(3gm)+Nacl(1 gm)	7.3	5.0	0.37	2.0
3.	Molasses(80ml)+Yeast(5gm)+Nacl(1 gm)	7.4	5.0	0.41	2.1
4.	Molasses(100ml)+Urea(.33gm)+Nacl(1gm)	7.3	6.0	0.85	1.4
5.	Molasses(100ml)+Urea(.33gm)+K ₂ HPO ₄ (1%)+Nacl(1 gm)+Yeast(3gm)	7.2	6.5	0.91	2.2
6.	Molasses(100ml)+Urea(0.5gm)+Yeast(3gm) K ₂ HPO ₄ (2%)+Nacl(1gm)	7.5	7.0	0.45	1.5

O.D. --- Optical Density A*G* ---- After growth. B*G* ---- Before growth.

Digestion of the product we have form that the bands and so obtained was in molasses Medium.

Sugar Estimation:-

The above result shows that the Molasses medium contains 11.11% sugar content.

Plasmid DNA isolation:-

- 1% Agarose gel electrophoresis of isolated plasmid DNA from E.coli cells grown in molasses containing different conc. of yeast & Nacl shown clear bands (fig.no.2).
- 1% Agarose gel electrophoresis of isolated plasmid DNA from E.coli cells grown in molasses containing different conc. of yeast, Nacl, Urea, & K_2HPO_4 shown (fig.no.3).

when added urea, k_2HPO_4 in the molasses then RNase activity was reduced.

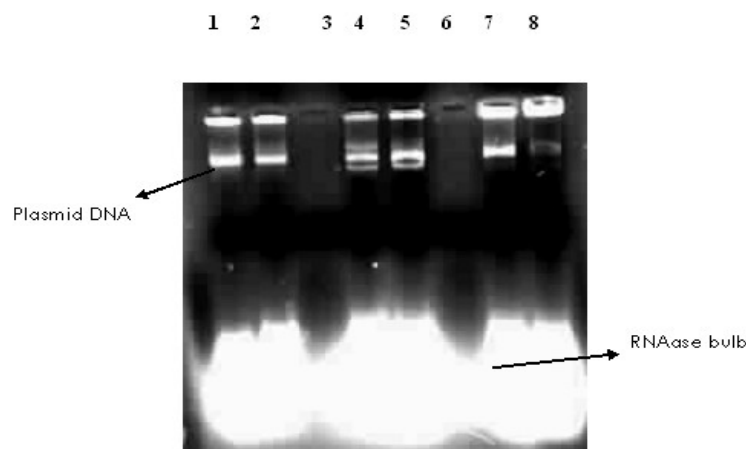


Fig. No.2:- 1% Agarose gel electrophoresis of isolated plasmid DNA.

Lane1, 2:- Plasmid DNA isolated from E.coli cells grown in Molasses containing 3gm yeast & 1%Nacl.

Lane4, 5,7,8:- Plasmid DNA isolated from E.coli cells grown in Molasses containing 5gm yeast & 1%NaCl.

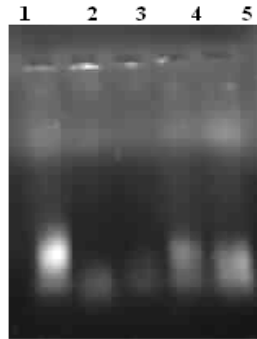


Fig. No 3:- 1%Agarose gel electrophoresis of isolated plasmid DNA.

Lane1 :- Plasmid DNA isolated from *E.coli* cells grown in molasses containing 5gm yeast &1%Nacl.

Lane2, 3:- Plasmid DNA isolated from *E.coli* cells grown in Molasses containing 3gm yeast, 0.5gm urea, 2% K_2HPO_4 & 1% Nacl.

Lane4, 5:- Plasmid DNA isolated from *E.coli* cells grown in Molasses containing 0.323gm yeast.

Restriction Enzyme Analysis

1% Agarose gel electrophoresis of R.E. digested recombinant plasmid DNA from *E.coli* cells grown in molasses containing 5gm yeast & 1% Nacl, shown clear bands of recombinant plasmid DNA. It cleared that restriction enzyme of *Eco. R1* cut at 2 sites in DNA. (Fig.no.4).

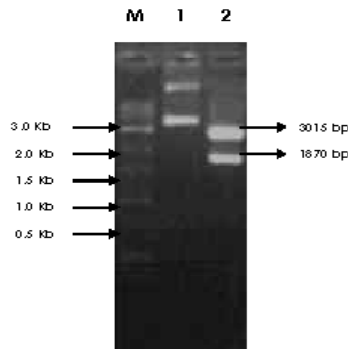


Fig.No 4:- R.E. Analysis on 1% Agarose gel.

Lane M- 1 Kb DNA Ladder.

Lane 1- Undigested r-Plasmid

Lane 2- Plasmid DNA isolated from *E.coli* cell grown in molasses containing 5gm yeast &1%Nacl (Digested with *EcoR1*)

DISCUSSION

During the experiment the O.D of culture and O.D of plasmid DNA was observed in respective of different optimization method. I observed that the O.D value has increase to i.e. 2.129, as it was earlier i.e. 0.393. The quality and quantity of the plasmid DNA was very good.

Efforts made to make the production cost effective:

I have also tried to grow the recombinant *E. Coli* for the plasmid DNA using molasses medium to make the vaccine. During the initial studies I have found that the molasses may be a good media where one can manipulate the C/N ratio as per requirement of cell.

Further primary studies suggested that by using Urea and K_2HPO_4 in the molasses results in low RNA activity in Agarose electrophoresis, if repeated experiments have same result then it is suggested that the use of RNAse enzyme can be restricted to inhibit RNA activity, during plasmid DNA isolation.

CONCLUSION

The above experiment shows that it is possible to produce vaccine in molasses. Molasses is one of the best alternatives as it is cheap and can be easily manipulated. The Carbon: Nitrogen ratio of molasses can be easily manipulated. This particular technology holds the key for the future, as it reduce the cost of production of DNA vaccines and making it available to everyone. Further study ahead it is possible to use this technology for production of vaccine for human uses.

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