Comparison of Plasmid yield between L.B. and using Molasses as a medium

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Abstract

During the initial studies I have found that the molasses may be a good alternative to costly L.B media where one 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. Escherichia coli is a bacterium that is commonly found in the lower intestine of warm-blooded animals. it is possible to produce vaccine by molasses medium. The paper is opening a new face of study.

Keywords

Molasses, L.B medium, *E.coli* DH5α strains, Sucrose, Glucose,K_2HPO_4,Urea..

1. Introduction

The need of comparison yields was made due to keeping in mind that the final product so obtained from the L.B Medium is costly, by using molasses. The cost of the product can be reduced as molasses medium is three time cheaper then L.B. medium. So in this project the efforts are made to use.

The advantages of the using molasses as an alternative to L.B Medium is apart from the cost effectiveness of the product, molasses as medium is manipulative to requisite micro-organism, because molasses contain the nitrogen in low amount and can be optimize 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 a optimum recombinant plasmid yields so in this project the affords as being made to optimize the molasses to commonly used L.B. 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 and also in Luria Bertani (L.B) 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 K_2, 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).

2. Materials and methods

2.1 Host Bacterial strain:-

Escherichia coli DH5α (Promega, Madison) host strains were used for transformation with recombinant plasmids.

2.2 Standard markers:-

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

2.3 Medium:-

- ♥ Luria Bertani (Hi media Co.)
- ♥ Molasses as a medium.

2.4 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), Banglore Genei (Bangloe,India), JRH Biosciences Lenexa), Plastic wares and other consumables were from Axygen, TRP, Nunc, Greiner, Tarsons, Corning or Borosil.

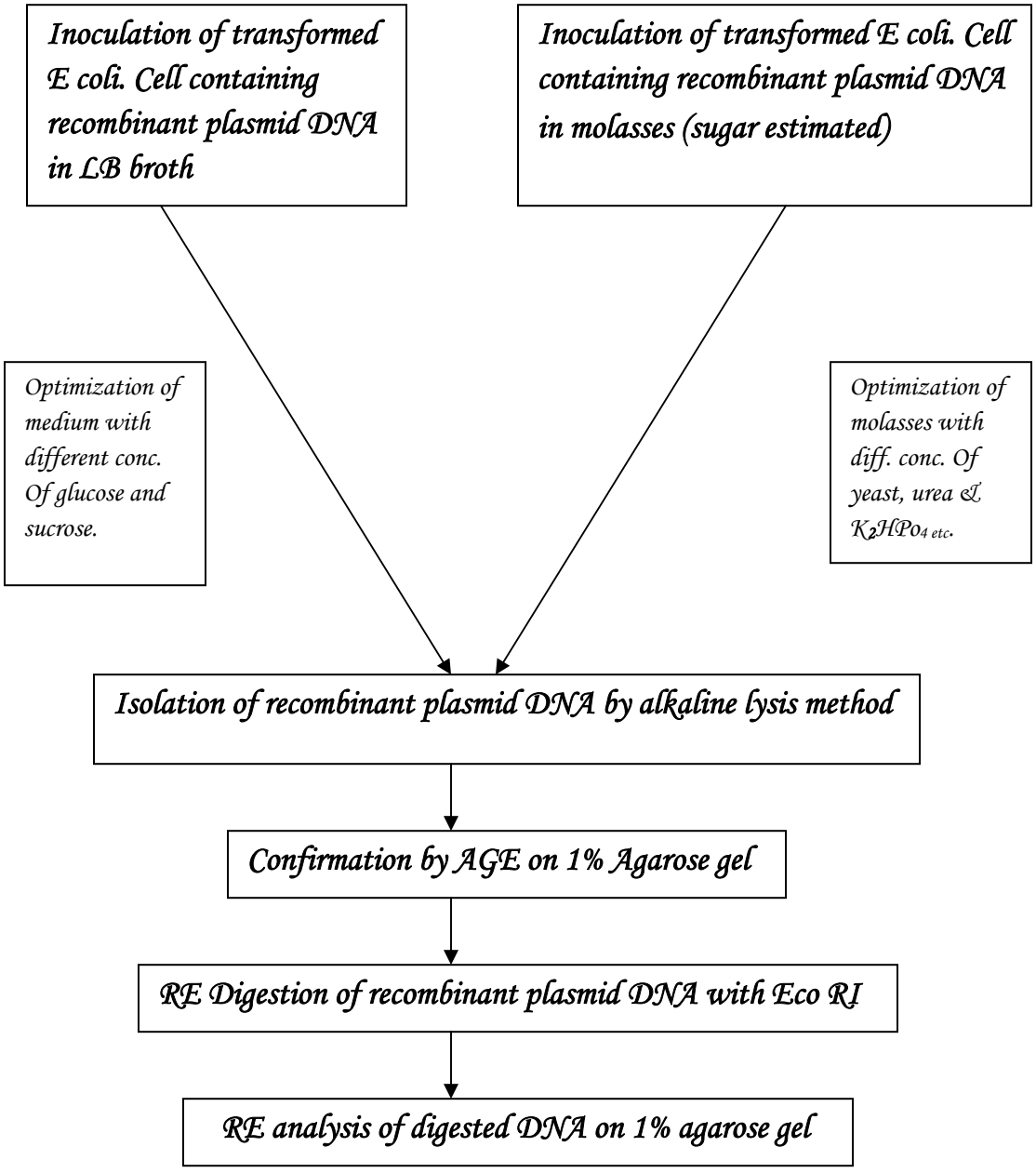

Fig. No.1:-Flow diagram showing the detailed methodology used in the present work.

2.5 Sugar estimation mathod:-

5gm of molasses was accurately weighed and dissolved in 60ml of distilled water. Then added 5ml of 1:1HCI with proper mixing. The solution was heated up to 60^0C for 5min in a water bath. The temperature was then raised to 70^0C 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.

A few drops of methylene blue were added and used as indicator to get and 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.

C12 H22 O11+ H2o - Acid hydrolysis – C6H12O6 + C6H12O6

Calculations

Volume of inverted sugar for titration of 10ml Fehling's Solution

$$\% \text{ of invert sugar} = \frac{\text{Fehling's factor} \times \text{Volume of invert sugar} \times 100}{\text{Weight of sample} \times \text{titer volume}}$$

$$\% \text{ of invert sugar} = \frac{0.02 \times 500 \times 100}{5 \times 18} = 11.11\%$$

2.6 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 LB broth 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 40µl of TE buffer.

2.7 Restriction endonuclease digestion and analysis of digested DNA

The plasmid DNA (5 to 10 µg) was incubated overnight with appropriate 1X restriction endonuclease buffer and 3 to 5 U restriction endonuclease at 37^{o} 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 µg/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)

(b)-L.B. + Sucrose (1%)

(c)-L.B. (100ml)

Enzyme taken - EcoR1

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

2.8 Agarose gel electrophoresis

Agarose, type 1 gelling temperature(40-45^{o}) from SRL, (Mumbai) was used to perform majority of gel electrophoresis in concentrations of 0.8% to1.2% for the separation of DNA fragments ranging from 0.4 Kb to 10Lb. Appropriate amount of agarose was mixed with 1X TAE buffer and melted in a microwave oven. When the molten gel had cooled to 45^{o} C, Ethidium bromide was added to final concentration of 0.5µg/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).

3. Result

In order to reduce the cost of the product the aims of yields of plasmid DNA from recombinant *E. coli* compared with using L.B. Medium and molasses.

On comparison using plane L.B. Medium the O.D. of the culture was----

S.N.	Medium(100ml)	pH(B*G*)	pH(A*G*)	O.D.(B*G*)	O.D(A*G*)
1-	L.B. Medium	7.2	5.0	0.41	1.4
2-	L.B.+0.25%Glucose	7.2	5.0	0.7	1.5
3-	L.B.+0.50%Glucose	7.0	4.5	.11	2.2
4-	L.B.+1%Glucose	7.2	5.0	.29	2.0
5-	L.B.+2%Glucose	7.5	6.5	.41	1.6
6-	L.B.+1%Sucrose	7.1	6.5	.31	2.2

Table no.:1 showing O.D. of L.B. medium.

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

1 2 3 4 5 6

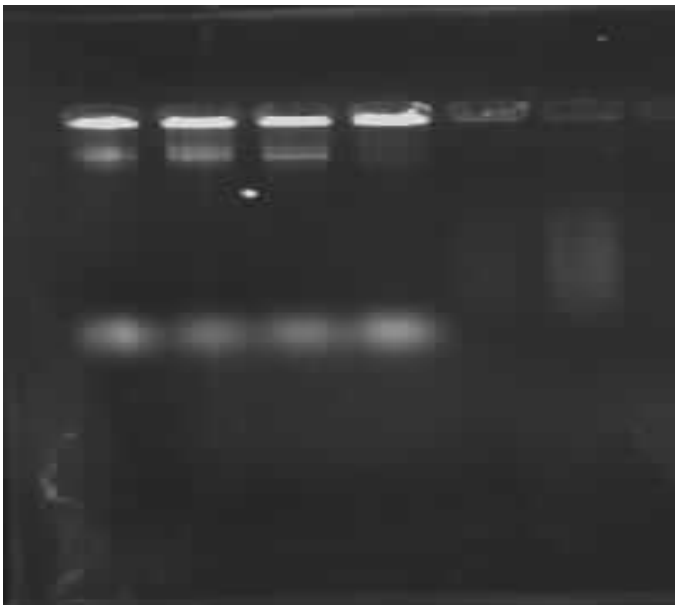

Fig.No.2:- 1% Agarose gel electrophoresis of isolated plasmid DNA.

Lane1:- Isolated plasmid DNA from E.coli cells grown in L.B. medium.

Lane2:- Isolated plasmid DNA from E.coli cells grown in L.B. containing 0.25% Glucose.

Lane3:- Isolated plasmid DNA from E.coli cells grown in L.B. containing 0.50% Glucose.

Lane4:- Isolated plasmid DNA from E.coli cells grown in L.B. containing 1% Glucose.

Lane5, 6:- Isolated plasmid DNA from E.coli cells grown in L.B. containing 2% Glucose.

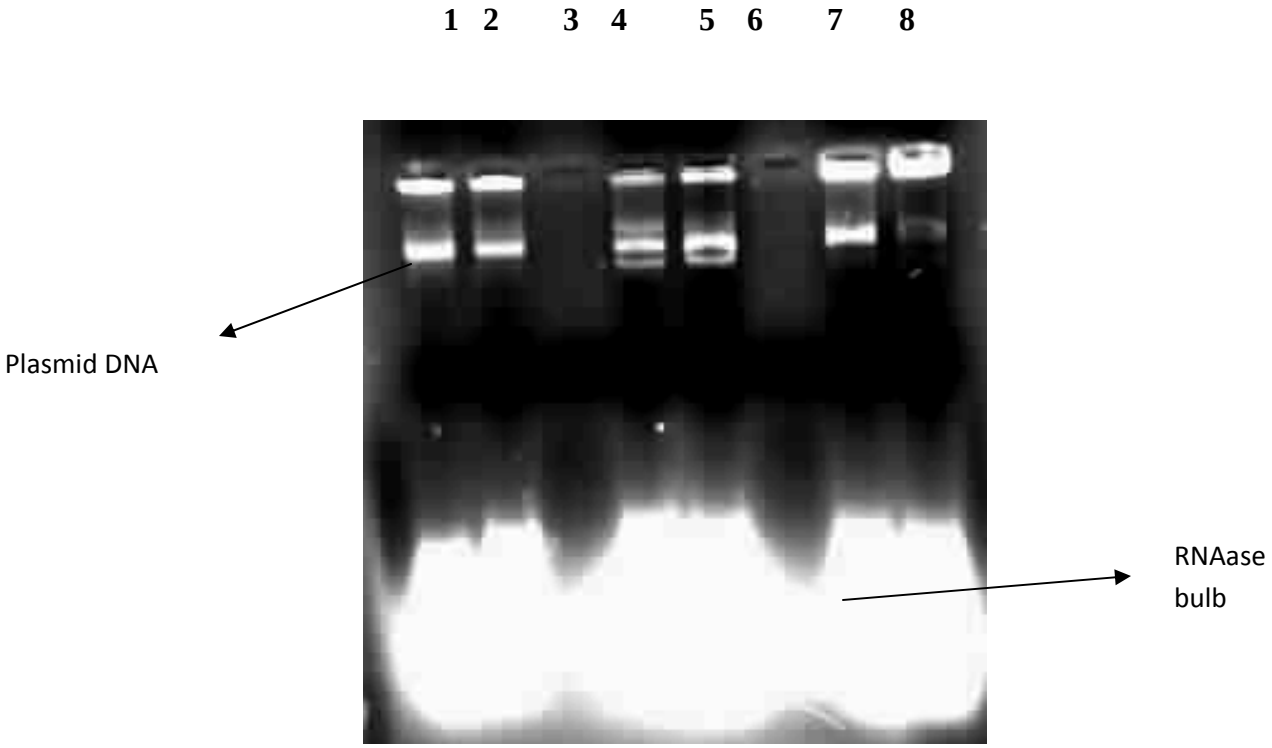

Fig. No.3:- 1% Agarose gel electrophoresis of isolated plasmid DNA.

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

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

Lane7, 8:- Plasmid DNA isolated from E.coli cells grown in L.B. containing 1% Glucose.

1 2 3 4 5

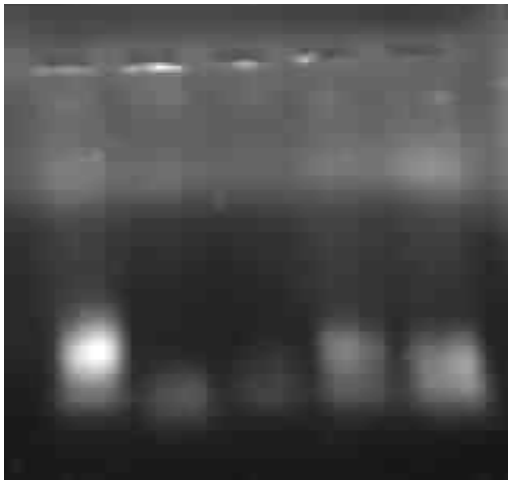

Fig. No.4:- 1%Agarose gel electrophoresis of isolated plasmid DNA.

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.

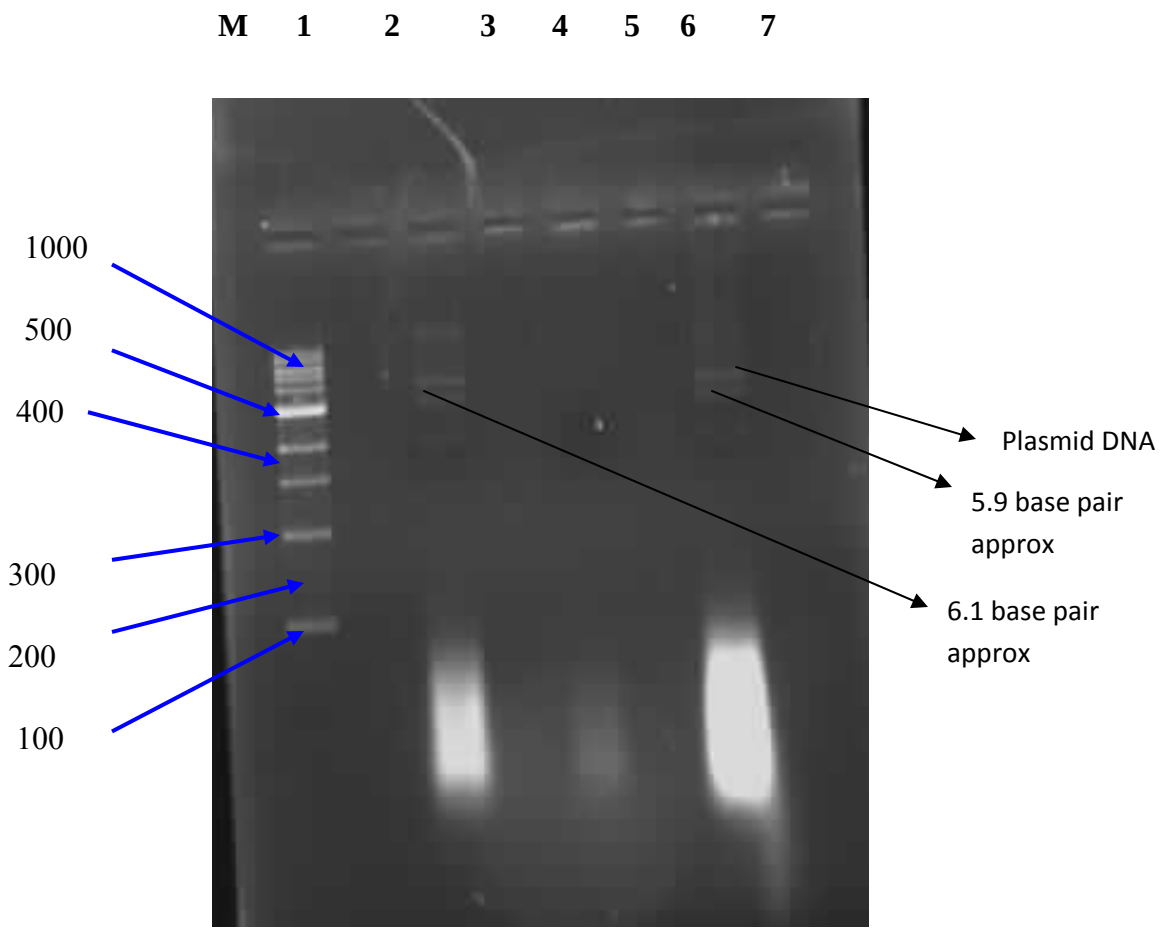

Fig.No.5:- R.E. Analysis on 1% Agarose gel.

Lane M- 1 Kb DNA Ladder.

Lane 2- Plasmid DNA isolated from E.coli cell grown in molasses containing 5gm yeast &1%Nacl.

Lane 4- Plasmid DNA isolated from E.coli cells grown in L.B. Containing 1 % sucrose.

Lane6- Plasmid DNA isolated from E.coli cells grown in L.B.

While using molasses without any supplement was---

S.N	SAMPLE(ml)	pH(B*G*)	pH(A*G)	OD(B*G*)	OD(A*G*)
1.	**Molasses(50ml)+Yeast(.323gm)**	**7.5**	**5.0**	**0.31**	**1.4**
2.	**Molasses(80ml)+Yeast(3gm)+Nacl(1gm)**	**7.4**	**5.0**	**0.37**	**2.1**
3.	**Molasses(80ml)+Yeast(5gm)+Nacl(1gm)**	**7.5**	**5.0**	**0.41**	**2.2**
4.	**Molasses(100ml)+Urea(.33gm)+Nacl(1gm)**	**7.4**	**6.0**	**0.85**	**1.5**
5.	**Molasses(100ml)+Urea(.33gm)+ K_2HPO_4(1%)+Nacl(1gm)+Yeast(3gm)**	**7.2**	**6.5**	**0.91**	**2.2**
6.	**Molasses(100ml)+Urea(0.5gm)+Yeast(3gm) K_2HPO_4(2%)+Nacl(1gm)**	**7.5**	**7.0**	**0.45**	**1.5**

Table: 2 showing the different optimization method for molasses.

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

Further increasing the nitrogen contain to the extent of the present in L.B. Medium that is 5% the yield difference is almost nill and after running the gel and digestion of the product we have form that the bands and so obtained was same as L.B. 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 L.B. containing different concentration of Glucose shown(fig.no.2)

  when increased the glucose concentration in L.B. up to a limit, then the DNA conc. was increased. **[0.25%&0.50% glucose conc. in L.B. ----------shown clear bands]**

  if glucose conc. increased after a limit then DNA concentration was reduced**. [1% & 2% glucose concentration in L.B. ---------- shown no bands of DNA]**.

- 1% Agarose gel electrophoresis of isolated plasmid DNA from E.coli cells grown in molasses containing different conc. of yeast & Nacl and in L.B. contaning 1% Glucose shown clear bands (fig.no.3).

- 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.4).

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

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, L.B. containing 1% Sucrose &L.B. shown 3 clear bands of recombinant plasmid DNA. Molasses medium show clear bands comparison of L.B. It cleared that restriction enzyme of *Eco. R1* cut at 2 sites in DNA. (fig.no.5).

4. 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. 1.293. 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 as an alternative to L.B media to make the vaccine or protein cost effective. During the initial studies I have found that the molasses may be a good alternative to costly L.B 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.

5. 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.

References

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