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PLASMID STABILITY AND MAINTENANCE OF COPY NUMBER USING NATURAL MARKER

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KEYWORDS

Plasmid Stability
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Transformation efficiency
Growth curve

ABSTRACT

Present study was conducted to study the plasmid stability with the help of natural plasmid isolated from the bacteria which lodges the ink gland of the sea squid and emits bioluminescence. Isolated bacterial strain was identified by using 16srRNA sequencing and its plasmid DNA was used for the experimental studies. The plasmid is found to be responsible for the bioluminescence. The stability of this plasmid was studied in shake flask method using the different sugar sources (Glucose, Sucrose, Lactose and Maltose) as fermenting medium. The cultures were incubated at different time intervals in which the plasmid stability was inferred by deducing the Bacterial Growth curve. The optical densities were read out and the growth pattern was obtained. Along with the growth curve, the transformation efficiency and CFUs (Colony forming Unit) was calculated for individual volumes of bacterial cultures. The transformation efficiency was found to be increased after every 12 hrs of incubation which indicated the improved plasmid stability. The effect of carbon sources was suggestively detected in the growth curve. Among the four sources of carbon, addition of Glucose exhibited highest Optical density value and inferred the enhancement in Plasmid stability.

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1 Introduction

DNA technology especially rDNA owes a great deal in producing recombinant proteins, these protein can be produced in bulk than many native proteins and moreover this, these can be easily controlled in quality wise. These recombinant proteins have heavy demand in the pharmaceutical sectors for producing vaccines, therapeutic proteins and functional enzymes (Andersen & Krummen, 2002). Over 150 human and veterinary based biopharmaceuticals have received approval and majority of these represent recombinant proteins in the form of recombinant antibodies (Friebs, 2004).

Plasmid stability is a common term which frequently used in recombinant protein industry. The success of the production of the recombinant protein largely depends on the stability of the plasmid. On successive generations there is a chance of the reducing copy number and thereby affecting the production of recombinants. Furthermore, the use of the antibiotic resistance genes as selectable markers in the plasmid is also a major concern, due to which the usage has been limited. Hence there is a need to search an alternative mode of plasmid stability selection. Here plasmid stability is a measure of how well a plasmid is maintained in a bacterial population in the absence of any selection for plasmid-encoded traits. Stability is typically evaluated by growing bacterial cells with the plasmid in a medium that does not select for the presence of the plasmid. After a known number of cell divisions (generations) the number of cells with and without the plasmid is counted. Growth rate affects several parameters that determine recombinant protein accumulation rate. Among them percentage of substrate utilized for cellular maintenance, RNA polymerase activity, ribosome number, plasmid stability, plasmid copy number, plasmid multimerization, and the distribution of cells in the cell-cycle phases are some common one (Escrion et al., 2001). Thus, it is possible to control recombinant protein production through growth rate and this growth rate can be manipulated by the manipulating nutrient availability. Namely, the main carbon or nitrogen source can be maintained at a predetermined concentration to obtain the desired growth rate. Such a manipulation can be achieved through fed-batch or continuous cultures (Bloquel, 2004).

Gene therapy has more valuable interest and more than 1,700 clinical trials have already been approved worldwide. Plasmid DNA production needs a specialized marker for screening after the process of transformation and during the amplification process (Colosimo et al., 2000). The plasmids once transformed into the host for propagation or expression are then checked for their stability. Stability refers to the ability of the plasmid to remain stable segregationally and the daughter cells should contain at least one copy of the plasmids. Plasmid stability is always correlated with the number of copies of a plasmid in the host bacteria. If plasmid cells are allowed to grow under non-selective conditions, there will be a serious threat to the plasmids of interest (Cooper, 1987). Infect plasmid exerts an extra pressure on the metabolic activity of a

bacteria and also effecting its growth rate. Hence it is always necessary to impose a selection pressure which favours the selection of bacteria containing the gene of interest (Doig, 2001).

Antibiotic resistance genes are often used as selection markers and in this plasmid containing cells are growing on the specific medium containing the respective antibiotic. But use of antibiotic resistance genes has been warned of serious threat to the nature owing to the chance of recombining with the homologous region in the hosts (El-Helow, 2000). If these antibiotic genes are used they might be spread into the wild pool contaminating the gene pool and making them resistant to the antibiotics. This might lead to future complications in relation to clinical significance. One major reason for considering this sort of gene markers is they might lead to an immune response and create allergy when used in the production of recombinant vaccines. Henceforth it will be importance for the rDNA sector if new innovations are made in the vectors will reduce the effect of cross contaminating the gene pool (Gardlik, 2011).

Limited information's are available on the stability of plasmids. According to Maskos, (2000) media composition plays a potential role in cell growth rate and it also helps in the maintaining plasmid copy number (Jones, 1980). Therefore, the possible effects of medium and its components should be evaluated for their role on the cell growth and plasmid productivity. Acetate accumulation has also been proved to be a major problem in medium design and also fermentation of recombinant *E. coli* at high density levels might inhibit the cell growth and protein expression (Summers, 1998). Among the different carbon sources used, glucose was preferably used for the production of plasmid pCMV-AP in *E. coli*. Glucose was found to be more useful for high plasmid yield both on the shake flask cultivation and pilot plant. Glycerol was also used as supplement to increase the yield of plasmid up to 70.6% (Summers, 1998).

In present study the plasmid used as a natural marker unlike the other available artificial markers. This proves to be an added advantage in the Therapeutics. The plasmid stability studies were done by shake flask method. The plasmids were also used for optimization by growing in various carbon sources such as Glucose, Lactose, Maltose, Sucrose and the growth rate was observed. The study provides an insight on the plasmid stability.

2 Materials and Methods

2.1 Dissection of sea squid

Defrosted squid was used for obtaining the ink gland. The squid was laid along the dorsal side down on a piece of wax paper in a dissecting tray. The squid was laid to a position in such a way that with its head to the left and its siphon up. The pen from the animal was removed from the dorsal side by

grasping it firmly with the fingers and pulled it free from the mantle. The mantle is now cut using a scalpel or dissecting scissors. The chromatophores which are of small freckle-like spots on the outer layer of the mantle were observed and peeled off a small piece of the skin that contains the spot.

2.2 Isolation of bacteria

The sea water complete (SWC) media (0.38M NaCl, 0.02M $MgCl_2$, 6H₂O, 0.25M Mg SO₄, 7H₂O, 8mM KCl, Peptone: 0.5g, Yeast extract: 0.3g, Glycerol: 0.3ml, Agar: 1.5g, Distilled water: 100ml) was prepared under sterile conditions. The ink squeezed from the ink gland was spread plated on SWC media. The plates were kept for incubation at 21° C for 24-48 hrs. Following the plates were observed under the UV illuminator. The colonies showing the presence of luminescence were further subcultured and stored at 4°C. The colonies were confirmed using the gram staining procedure.

2.3 16s rRNA sequencing

The colonies with peculiar variations in the morphology were made of pure culture and then sent for sequencing. The sequencing protocol followed was to be capillary based (Macrogen, Seoul, Korea).

2.4 Genomic DNA isolation

2ml of O/N culture was taken in a centrifuge tube and centrifuged at 5000rpm for 10 minutes. The supernatant obtained was discarded and the pellet was re-suspended in 300µl of lysis buffer (1M Tris HCl, 0.5M EDTA, 1% SDS) and gently vortexed. To the contents, 500µl of TE buffer (1M Tris HCl, 0.5M EDTA-2ml) and 500µl of chloroform were added and the tube is inverted gently upside down. The contents centrifuged at 9000rpm for 10 minutes. The upper aqueous layer was collected in a fresh eppendorf tube and added with 1/10th volume of 3M sodium acetate. After gentle mixing of the contents, 2 volumes of chilled ethanol were added along the walls of the tube. The precipitated DNA is now pelleted and washed with 70% ethanol for purity and finally re-suspend in 30-50µl of TE buffer (Sambrook et al., 1989).

2.5 Plasmid DNA isolation

Plasmid DNA was isolated by the method described by Sambrook et al., 1989. For this 2ml overnight luminescent culture was centrifuged at 14000rpm for 10 minutes and the pellet obtained was re-suspended in 200µl of freshly prepared solution I (50mM Tris, pH-8, 10mM EDTA, 100µg/ml RNase A, 50mM Glucose). The contents are mixed well and added with 200µl of solution II (200mM NaOH, 1% SDS) and 200µl of solution III (Potassium Acetate 3M). The contents were mixed by inverting the tube gently. The tubes were then centrifuged at 14,000rpm for 10 minutes and the supernatant obtained was transferred to a fresh set of tubes and the DNA was precipitated using 900 µl of 100% ethanol. The DNA

obtained was resuspended in 50µl of TE buffer with 5µl of freshly prepared RNase and the tubes were stored at -20° C until further use.

2.6 Transformation

Competent cells of *E.coli* were prepared using the calcium chloride method (50mM $CaCl_2$). The prepared cells were used for the transformation experiments. Briefly 100µl of competent cells were taken in two eppendorf tube and added with 5µl of plasmid DNA in one tube labelled as positive and the other was kept negative (no plasmid added). Both the tubes, positive and negative were incubated on ice for 20 minutes and later kept in hot water bath at 42° C for 2 min. following which the tubes were removed immediately and placed on ice for 10 minutes. 0.5ml of LB broth was added to both the tubes and was incubated at 37 °C for 1 hr. Meanwhile 2 plates (positive and negative) of LB agar were prepared. About 20µl of the samples was spread plated on their respective plates. The plates were then incubated at 37°C for 48 hrs in an inverted position and observed for the transformed colonies.

2.7 Shake flask cultivation

The culture obtained was further used for the stability studies by shake flask culture. The experiment was first carried out in 20ml of LB medium, this was followed by 100ml and 2 litres medium containing flask culture. Sugars were used as minimal medium to check the level of stability with different sugars (Glucose, lactose, Maltose and Sucrose). A single colony of isolated & identified bacterium was inoculated into 20ml LB medium contained in 250ml conical flask. The culture was incubated for 12hrs at 37 °C at 250rpm on rotatory shaker. After 12hrs of incubation 10ml of culture was diluted by inoculating into another 20ml LB medium. 0.5ml of 12hrs old culture was diluted with 4.5ml of LB broth and the absorbance was recorded at 600nm. Bacterial growth curve was analysed after every 12 hrs culture. Repeated inoculations were carried out into 20ml LB broth in a 250ml conical flask after every 12 hrs for growth curve. Meanwhile 4 plates containing LB agar along with the individual carbon source sucrose (1gm/L), glucose (1gm/L), lactose (1gm/L), maltose (1gm/L)) were prepared. 100µl of culture was spread plated on their respective plate. The plates were incubated at 37°C for 24-48 hrs and the numbers of colonies are counted by colony forming unit (cfu). The same procedure was repeated for 100ml and 2 litre flasks culture also.

The transformed colonies were counted using coulter counter (Digital colony counter (Optoprecision services, model: DCC-110) and the colonies were then checked for the transformation efficiency. Plates with more than 300 colonies cannot be counted and are designated too many to count (TMTC). Plates with fewer than 30 colonies are designated too few to count (TFTC). The number of bacteria (CFU) per millilitre or gram of sample was calculated by dividing the number of colonies by the dilution factor multiplied by the amount of specimen added to liquefied agar.

Transformation efficiency is the efficiency by which cells can take up extracellular DNA and express genes coded by it. This is based on the competence of the cells. It is calculated by dividing the no of successful transformants by the amount of DNA used during a transformation procedure. Transformants are cells that have taken up the DNA and which can express genes on the introduced DNA.

3 Results and Discussion

3.1 Isolation and identification of bacteria from squid

The bacteria were isolated from the ink gland of squid and cultured on the SWC media (Figure 1). The colonies exhibiting fluorescence under UV transilluminator were picked up and subcultured on separate SWC agar medium. Isolated bacteria were initially identified by the Gram staining and selected strains were taken for phylogenetic analysis by 16s rRNA sequencing (Figure 2). The sequence was found to be about 990bp. These sequences were taken for the sequence similarity with the identified organisms by using blast. The query was found to have 99% identity with Bacterium *Vibrio harveyi* strain IS01 16S ribosomal RNA gene partial sequence (Figure 3 & Figure 4).

Figure 1 Left: Figure showing the giant Sea squid. Right: Ink gland dissected from the squid.

Figure 2 A: Pure colonies of isolated bacteria on SWC Agar medium; B: Pure colonies of isolated Bacteria exhibiting fluorescence under UV transilluminator; C: Transformed colonies on LB agar; D: Transformed colonies exhibiting fluorescence under UV transilluminator; E: Transformed colonies without plasmid DNA (negative control) LB agar; F: Transformed colonies without plasmid DNA (negative control) LB agar; G: More colonies grown on LB agar with the sole carbon source Glucose comparative than all the carbon sources; H: Very few colonies grown on LB agar with the sole carbon source Maltose; I: Fewer colonies grown on LB agar with the sole carbon source Sucrose; J: Few colonies grown on LB agar with the sole carbon source Lactose.

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GGGGGTGACGGTATCGGAATTCTGGGGCGTAAGCGCATGCAGGTGGTTTGTAA
GTCAGATGTGAAAGCCCGGGGCTCAACCTCGGAATAGCATTGAAACTGGCAGA
CTAGAGTACTGTAGAGGGGGGTAGAATTTAGGTGTAGCGGTGAAATGCGTAGA
GATCTGAAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAGATACTGACACTC
AGATGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCG
TAAACGATGTCTACTTGGAGGTTGTGGCCTTGAGCCGTGGCTTTTCGGAGCTAACG
CGTTAAGTAGACCGCCTGGGGAGTACGGTTCGCAAGATTAAAACTCAAATGAATT
GACGGGGGCCCCGCACAAGCGGTGGAGCATGTGGTTTAATTTCGATGCAACGCGAA
GAACCTTACCTACTCTTGACATCCAGAGAACTTTCCAGAGATGGATTGGTGCCTT
CGGGAACCTCTGAGACAGGTGCTGCATGGCTGTCGTACGCTCGTGTGTAATGT
TGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTGTGTTGCCAGCACTTCGGG
TGGGAACCTCCAGGGAGACTGCCGGTGAAAACCGGAGGAAGGTGGGGACGACGT
CAAGTCATCATGGCCCTTACGAGTAGCTACACACGTGCTACAATGGCGCATACA
GAGGGCGGCCAACTTGCGAGAGTGAGCGAATCCCAAAAAGTGCGTCGTAGTCCG
GATCGGAGTCTGCAACTCGACTCCGTGAAGTCGGAATCGCTAGTAATCGTGGATC
AGAATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCAT
GGGAGTGGGCTGCAAAAAGAGTAGGTAGTTTAACTTCGGGAGGACGCTTACCA
CTTGTGGTTTCATGACTGGGTGATTAAAAAGGAACCCCCCATATATACAAAAAA
AGGGGGGAG

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Figure 3 Figure showing the 990bp sequence obtained by 16srRNA sequence of the bacterium isolated from the ink gland of the sea squid.

Figure 4 Left: Figure showing the blastn results of the sequence obtained by 16srRNA sequencing. Right: Figure showing the blastn score results showing the percent similarity to the nearest identical bacteria.

3.2 Isolation of genomic and plasmid DNA

Genomic and plasmid DNA was isolated and quantified by using UV spectrophotometer. The samples were then run on 0.8% Agarose gel together with 1kb ladder DNA for reference and checked for the purity. The concentration of the genomic and plasmid DNA obtained was of 2.4325 & 2.123µg/ml respectively.

3.3 Transformation

Transformation efficiency was calculated by dividing the number of successful transformants by the amount of DNA used during a transformation procedure. The concentration of the plasmid DNA is 20ng/µlitre. The amount of Plasmid DNA

used for the transformation is 5 µlitre. So the amount of DNA transformed is 100ng (20x5). The Volume of the culture plated for every 1ml is 25µl. Therefore the amount of DNA for 100µl is 10ng. The numbers of colonies observed on the plate were 65colony/plate. Plasmid stability was also reported Colosimo et al., 2004; Bloquel et al 2004 and found similar to the present study.

3.4 Shake flask for 20ml of culture

The shake flask method was carried out for 20ml of the culture and the growth was calculated by photometric assay by using calorimeter. The colony forming unit was calculated by counting the number of colonies grown on the individual plates.

Figure 5 Graph showing the number of colonies and transformed colonies of the transformed culture. All the values are the averages of triplicates. The dilution of the transformed culture was 1:10.

Figure 6 Graph showing the number of colonies and transformed colonies of the transformed culture for different incubation hours. The dilution of the transformed culture was 1:10.

A two way ANOVA between the different incubation period and carbon sources was conducted to compare the effect of carbon sources on plasmid stability. There was a significant effect of different carbon sources and incubation period remembered at the $p < 0.05$ level. The significance effect of the carbon source and incubation period was reported on the colony forming unit [F (3,12) = 88.4545, $p = 1.89\text{E-}08$] and [F(4,12)=23.8, $p=1.24512\text{E-}05$]. Furthermore, a significance effect of the carbon source and incubation on the Transformation Efficiency was also reported [F(3, 12) = 140.144, $p = 1.32\text{E-}09$] and [F(4, 12) = 20.8011, $p = 2.50374\text{E-}05$]. A significant amplification was reported in the colony forming units as well as in the transformation efficiency

with increasing the time for incubation period. Also among the different carbon sources added to the growth medium, glucose was reported best in raising the transformation efficiency (Figure 5 & Figure 6)

Similarly the experiment was carried out in 100ml culture and the growth was calculated by photometric assay by using calorimeter while the colony forming unit was calculated by counting the number of colonies grown on the individual plates. Result of the study are presented graphically against incubation time v/s CfU (Colony forming Units) & Transformation efficiency (Figure 7 & 8).

Figure 7 Graph showing the number of colonies and transformed colonies of the transformed culture. All the values are the averages of triplicates. The dilution of the transformed culture was 1:10.

Figure 8 Graph showing Colony forming unit and transformation efficiency. All the values are the averages of triplicates. The dilution of the transformed culture was 1:10.

A two way ANOVA between the incubation period and different carbon sources was conducted to compare the effect of carbon sources on plasmid stability. There was a significant effect of different carbon sources and incubation period remembered at $p < 0.05$ level. The significance effect on the colony forming unit between carbon source and incubation period [$F(3, 12) = 27.9444$, $p = 1.07E-05$] and [$F(4, 12) = 7.02381$, $p = 0.003740167$].

Further, it also showed a significance effect on the Transformation Efficiency between carbon source and incubation period [$F(3, 12) = 96$, $p = 1.18E-08$] and [$F(4, 12) = 17.6914$, $p = 5.69455E-05$].

Furthermore, a substantial increase in the number of colonies as the incubation period was increased. The CFU/ml was raised and the transformation efficiency was improved as the incubation hours were elevated. Addition of Glucose in the medium again showed an increase in the transformation efficiency.

3.5 Shake flask for 2 litres of culture

Like 20 and 100 ml culturing, shake flask method was also carried out for 2 litres culture and a graph was plotted against incubation time v/s CfU (Colony forming Units) & Transformation efficiency (Figure 9 & Figure 10).

Figure 9 Graph showing the number of colonies and transformed colonies of the transformed culture. All the values are the averages of triplicates. The dilution of the transformed culture was 1:10.

Figure 10 Graph showing Colony forming unit and transformation efficiency. All the values are the averages of triplicates. The dilution of the transformed culture was 1:10.

A two way ANOVA between the incubation hours and different carbon sources to compare the effect of carbon sources on plasmid stability and a significant effect of different carbon sources and incubation period was reported at the $p < 0.05$ level. The significance effect on the colony forming unit between carbon source and incubation period [$F(3, 12) = 30.5285$, $p = 6.73E-06$] and [$F(4,12) = 8.56694$, $p = 0.00166$]. And also showed a significance effect on the Transformation Efficiency between carbon source and incubation period [$F(3, 12) = 61.1344$, $p = 1.53E-07$] and [$F(4, 12) = 13.9355$, $p = 0.00018$]. There was a substantial increase in the number of colonies as the incubation period was increased. The CFU/ml was raised and the transformation efficiency was improved as the incubation hours were elevated. Addition of Glucose in the medium again showed an increase in the transformation efficiency. Similar type of plasmid study was conducted by Cooper (1987) & Friehs (2004) they reported similar type of results.

3.6 Growth Curve

After every 12 hrs of incubation, the sample culture was observed for the turbidity. It was then diluted and was read at 620nm calorimetrically. The optical density values were noted down and a graph was plotted against the incubation period (Figure 11).

The growth was accelerated after every 12 hrs with 2m, 100ml and 2 litre of culture. The carbon sources added into the growth medium. Among all, addition of glucose exhibited a rise in the Optical densities. Both the treatments are at par to

each other and are statistically different than the sucrose and glucose.

Conclusion

Markers used in Production of recombinant Proteins are crucial. Another factor which influences the production of genetically engineered proteins is the stability of the Plasmid. Nowadays, many artificial markers are used in the biotechnology industry. The marker helps in detecting the production of desired protein in the respective cell. It helps in detecting the success rate of the recombinant protein. Without a marker, it becomes difficult for a biotechnologist to detect the production of Recombinant protein involved. Use of Natural Marker in production of Recombinant protein has always proven to be an excellent choice. The Bacterial Plasmid isolated in the current study can be used as a marker as it exhibits fluorescence and being natural, it has potential applications in the field of Biotechnology. For further identification, the culture was sent for 16srRNA sequencing. The bacterium was found sharing 99% similarity with Bacterium 4D902 deposited in the website.

The shortcoming with the artificial Marker is the possibility of its integration into the host chromosome. This leads to the ethical tissues and limits the use of such Markers in the Biotechnology industry. Natural Marker proves to be of an advantage here. The Plasmid stability is another limitation for the production of recombinant proteins on a large scale. The protein expression decreases after every generation and this limits the use of recombinant protein in therapeutics.

Figure 11 Graph showing the rise in the Optical density values of the bacterial culture incubated at different time intervals and carbon sources.

In this project, the plasmid stability was improved by the addition of different carbon sources in a shake flask method. The effect of carbon sources was suggestively detected in the growth curve. Among the four sources of carbon, addition of Glucose exhibited highest Optical density value and inferred the enhancement in Plasmid stability. Furthermore, transformation efficiency increased after every 12 hrs of incubation which indicated the improved plasmid stability.

The extracted Plasmid has an additional advantage being natural and can be used as a marker. It will aid in dodging the ethical issues related to the implementation of Recombinant protein in therapeutics and Biotechnology industry. The fluorescence exhibited by Plasmid is easily detectable under UV transilluminator. By studying the effect of different carbon sources on the plasmid stability, it was shown that glucose addition enhances the Plasmid stability in the successive generations.

Conflict of interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

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