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DNA construct instability in bacteria used for Agrobacterium mediated plant transformation

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Abstract: *The use of plasmid in the production of genetically modified (GM) crops is highly essential in research and in commercial production of GM plants. However plasmid instability constitutes a major problem in the use of recombined microorganisms in the production of GM crops. In this study we evaluated the stability of p8114 carrying a gene coding for a transcription factor (TFIIIA) driven by Cassava Vein Mosaic Virus (CsVMV) promoter and an nptII selectable marker driven by 35S promoter in the T-DNA. The plasmid was amplified in E.coliDH5α strain on Luria Broth (LB)agar supplemented with 100 µg/ml kanamycin. The colonies were confirmed by Restriction Fragment Length Analysis (RFLA) and by DNA sequencing. The confirmed colonies were stored as glycerol stock at -80^{0}C and as DNA extracts in TE buffer at 4^{0}C. Agrobacterium strains LBA4404, EHA 105 and AGL1 were also transformed with DNA from the confirmed colonies. Plasmid stability was evaluated after 3 months. Sixteen to hundred percent level of instability was observed in E.colicolonies stored at -80^{0}C and 50% level of instability in plasmid transformed into Agrobacterium strain LBA4404. Agrobacterium strain LBA4404 showed a higher level of stability 75% compared to EHA 105 (0%) and AGL1 (50%).*

Key words: *Plasmid instability, genetically modified crops, Escherichia coli, Agrobacterium tumefaciens,*

I. Introduction

Development of genetically modified plant both as a proof of concept or for product development depends essentially on the production of heterologous protein encoded by the gene of interest. However the production of the desired protein depends on the expression of the target gene cloned in an expression vector whether plasmid or phage. Plasmid instability as a result of insertion, deletion or plasmid rearrangement could lead to loss of target gene and as a consequence lead to the transfer of incomplete or altered genetic sequence which will produce aberrant gene product[1]. Lack of quality control of such plasmid constructed for the production of economically important food and industrial crops could result to waste of resources, time and labor in the production of plants deficient of the desired gene or protein product.

Gene of interest are often amplified from source and cloned into a vector in the form of plasmid or phage for multiplication and transfer into target cells. Plasmids are extra-chromosomal pieces of DNA that possess an autonomous origin of replication and have the ability to exist independently in bacteria cells[2]. They usually have genes that code for specific traits such as antibiotic resistance which confers characteristic traits on cells harboring them. Based on theseselectable markers, plasmids are essential for expression of foreign DNA in heterologous host including prokaryotes and eukaryotes. Essential features for plasmid desired for genetic engineering of plants include high copy number which amplifies the concentration of transcription template, presence of several unique restriction sites that facilitates insertion of several genes coding for desired traits, relatively manageable size that imposes less metabolic burden on the host and facilitates transfer to target cells during transformation and of course genetic stability that ensures that the desired product is formed[3, 4].

Plasmid stability can be affected by both cellular and environmental conditions which includes, the vector type and host genotype, origin and size of foreign DNA, increased metabolic burden and host culture conditions [5]. Schmidt et al.[6] showed that plasmid stability in E.coli was increased in a cloning vector system pEG1 constructed on the basis of pBR322 derivative by cloning parB locus of R1 plasmid to alkaline phosphatase gene (phoA) promoter. They concluded that the combination of parB locus and phoA promoter facilitated plasmid stabilization as a result of post-segragational killing of plasmid-free cells during growth. Differential host plasmid stability has also been reported. Al-Allafet al. [7] reported of the DNA structural change in a recombined plasmid harboring an HIV based cassette "50-Olig2cDNA-IRES-dsRed2-30" in recA1 E.coli strain Stbl2 during large-scale bacterial cultivation. However the same plasmid when introduced in an,E.coli strain Stbl3 did not exhibit any form of structural change [7].

Also implicated for plasmid instability is the presence and transcription of specific sequences located on plasmid expression vectors. Chiang and Bremer [8] showed that plasmid stability was enhanced by deleting a region within the tetpromoter of pBR322[8,9]. There could probably exist such other sequences that affect the

stability of other plasmids [3]. DNA size is another factor that could affect the stability of plasmid in recombinant bacteria. Warnes and Stephenson [10] showed that insertions of DNA fragment greater than 8 kb affected plasmid stability[10,11]. Importantly also is the competition between plasmid positive (P^+) and plasmid negative (P^-) cells which could lead to the depletion of P^+ cells due to greater growth difference between the two types of cells. This could however be controlled by the incorporation of selective pressure that enriches the survival of cell carrying the recombined plasmid.

In this study, the stability of recombined plasmid in bacteria used in the production of genetically modified cassava was investigated to determine the frequency of plasmid instability and therefore develop quality control strategies for ensuring that bacteria host employed in this process contains plasmid with the intact coding sequence required to achieve the targeted product as well as saving time, labor and cost.

II. Material and Methods

2.1. p8114 vector construction.

Gene expression cassette for TFIIIAdriven by cassava vein mosaic virus (CsVMV) promoter with AtuORF23 terminator was amplified using specific primers AAATCTAGAGGTGACTGACTGAACTAAACC (sense) and ATTAAGCTTGCAACCTGGGACTCCCAT (antisense). A 3.6-kb fragment was excised and ligated to zero-blunt vector from Invitrogen following the manufacturer's instruction. Two microliters of the ligation reaction was used to transform DH5α competent cells and plated out on Luria Broth (LB) agar medium containing 50 mg/l kanamycin. The resultant clones were screened using Ecor1 and Xma1 restriction enzyme in a 50 µl reaction containing. Positive colonies and pCambia 2300 were first digested in a 30 µl reaction with Xma1 restriction enzyme for a better resolution of the insert from zeroblunt backbone and then in a 50 µl reaction with Xba1 and Hind III restriction enzymes. The 3.6kb TFIIIA fragment and 8.9kb pCambia 2300 fragment were gel cleaned and ligated in a 10 µl reaction using T4 DNA ligase from NEB (USA) following the manufacturer's instruction. Positive colonies were confirmed with Xba1/Hind III restriction reaction and designated p8114 (Fig. 1). Agrobacterium LBA4404 was transformed with the positive colony and reconfirmed by back transforming in E.coli.

2.2. DNA sequencing

DNA was extracted from overnight grown E.colicultures using QiagenDNeasy plasmid mini kit (Qiagen, CA, USA) according to manufacturer's instruction. The DNA samples were prepared for sequencing by diluting the DNA to a concentration of 50 ng/µl. Five microliters of 5 µM sense and antisense primers were separately added to the DNA samples and sent for sequencing at GENEWIZ (South plainfield, NJ, USA). Sequence information obtained was edited and contiged using Lasagene (DNA star) program. Sequence identities were confirmed by searching for homology in NCBI database.

2.3. Restriction Fragment Length Analysis (RFLA) of ZFN constructs

DNA extracted from glycerol stock of E.coliDH5α cultures stored at -80^{0}C were screened using Xho1, BamH1 and Bgl II restriction enzymes. The digestion reaction was carried out in a 20 µl volume reaction with 1 µg DNA concentration and appropriate buffer systems for the restriction enzymes as specified by NEB. The reaction was incubated at 37^{0}C for 1 hour. The DNA fragment were analyzed by separation on 1% agarose gel electrophoresis and visualized by means of an alpha imager gel documentation system (Cell Bioscience Inc. CA, USA).

Three different Agrobacterium strains; LBA4404, EHA 105, and AGl1 were transformed with p8114. Electro-competent strains of theseAgrobacterium strains were transformed by electroporation using Gene pulser®II (Bio-Rad, CA, USA) and incubated at 28^{0}C for 4 hours. Successful transformants were selected on a selection medium containing 30 µg/ml rifampicillin, 30 µg/ml streptomycin and 100 µg/ml kanamycin (LB/RSK) plate incubated at 28^{0}C for 2 to 3 days. Two colonies each from the three strains were grown overnight and DNA extracted using QiagenDNeasy plasmid mini kit (Qiagen, CA, USA) according to manufacturer's instruction. The extracted DNA samples were used to transform E.coliDH5α competent cells from invitrogen (CA, USA). These cells were plated out on a LB media supplemented with 100 µg/ml kanamycin selection (LB/Kan)plate.Ten colonies were selected from each of the two DNA samples obtained from the three different Agrobacterium strains.

The DNA was digested with Bgl II and Sac1 restriction enzymes as described above. Restriction fragment were analyzed on 1% agarose gel as described above.

Fig 1: Schematic representation of p8114: A binary vector construct carrying transcription factor (TFIIIA) gene driven by cassava vein mosaic virus (CVMV) promoter fused to a neomycin phosphotransferase II gene driven by enhance cauliflower mosaic virus (CaMV) - 35S promoter

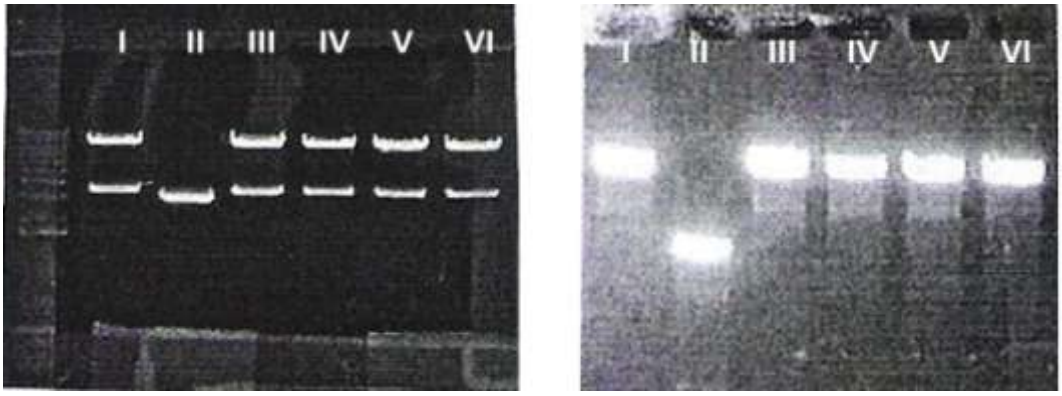

Fig 2: Restriction fragment length analysis (RFLA) of colonies harboring TFIIIA construct stored as glycerol stock at -80^{0}C. (A) Restriction pattern obtained with Xba1/HindIII double restriction reaction with four our of the five colonies showing the expected fragment sizes of 8718 bp and 3614 bp derived from vNTI restriction fragment analysis. (B) SacI restriction pattern of TFIIIA construct derived from same colonies with none of the five colonies showing the expected 9238 bp and 3098 bp fragment sizes expected from vNTI restriction fragment analysis. Colony showed a different construct size from the other four constructs

Fig 3: Restriction fragment length analysis (RFLA) of colonies transformed with TFIIIA construct stored at -4^{0}C. Sample I show expected fragment sizes of 8718 bp and 3614 bp for Xba1/HindII double restriction determined from vNTI restriction fragment analysis. (II) shows expected fragment sizes of 9238 bp and 3094 bp of SacI restriction determine from VNTI.

III. Result

3.1.Plasmid stability in E.coli and Agrobacterium

DNA extracted from E. coli DH5αglycerol stocks stored at -80^{0}C and analyzed by RFLA using Xba1/Hind III double restriction reaction and Sac1 restriction reaction are shown in Figure 2. Five out of the six colonies analyzed gave the expected 8718 and 3614 bps while one of the colonies remained uncut with Xba 1/ Hind III double restriction (Fig. 2A). For the Sac 1 restriction, surprisingly all six colonies did not give the expected 9238 and 3094 bps (Fig. 2B). The DNA sample stored at 4^{0}C were also analyzed with the same restriction enzymes and gave the expected fragments of 8718 and 3614 bps with Xba1/Hind III restriction and 9238 and 3094 bps with Sac I restriction (Fig. 3). This original DNA was previously confirmed by sequencing after cloning and stored at 4^{0}C.

From the result obtained it is suspected that certain level of rearrangement might have occurred in the glycerol stock. To confirm this, the TFIIIA expression cassette was amplified with sense and antisense primer sets derived from TFIIIA coding sequence and sequence information was obtained from Genewiz sequencing company (New Jersey, USA). Sequenced data returned, showed the presence of unidentified sequences within the T-DNA region of the TFIIIA cassette when compared to the original sequence obtained after cloning (data not shown). Several factors could be attributed to this phenomenon such as presence of transposable element, stability in bacteria or storage temperature.

To further investigate the stability of p8114 construct and to confirm the likely effect of storage temperature on plasmid stability, the DNA stored at 4^{0}C was used to transform freshly prepared E.coliDH5α and Agrobacterium LBA4404 competent cells. Successful transformants were selected on LB/Kan media for

E.colicolonies and on LB/RSK media for Agrobacterium colonies. Nine E.colicolonies were randomly selected and DNA samples extracted were analyzed with Xho1, BamH1 and BglII restriction enzymes. All nine colonies gave the expected restriction fragments of 7327, 4127 and 878 bps for Xho1 restriction, 9856, 1287 and 1189 bps for BamH1 restriction and 9485, 1612 and 1235 for BglII restriction (Fig. 4). Two resistant Agrobacterium colonies were randomly selected and retransformed in E.colifrom which four colonies were randomly selected and analyzed with Xho1, BamH1 and BglII restriction enzyme (Fig.5). All four E.colicolonies derived from Agrobacterium colony 2 gave the expected restriction fragment sizes expected from the three restriction enzymes while ¾ colonies derived from Agrobacterium 1 showed unexpected extra band when restricted with Xho1, two showed unexpected fragment size with BamH1 and with BglII all the colonies showed certain level of variation from the expected fragment sizes.

3.2.Stability of recombined plasmid in Agrobacterium strains LBA4404, EHA 105 and AGL1

The stability of p8114 was further evaluated in Agrobacterium strainsLBA4404, EHA105 and AGL1. Two colonies of each strain were retransformed into E.colifor amplification. Twenty colonies, ten from each Agrobacterium colony amplified in E.coli were analyzed for LBA4404, EHA 105 and AGL1 Agrobacterium strains (Fig. 6). For the LBA4404 strain 25% of the colonies screened with BglII showed some level of rearrangement and 30% with Sac1 restriction (Fig. 6A). Hundred percent of the EHA105 colonies showed different restriction pattern from the control with both restriction enzymes (Fig. 6B) while 50% of AGL1 colonies differed from the control (Fig. 6C).

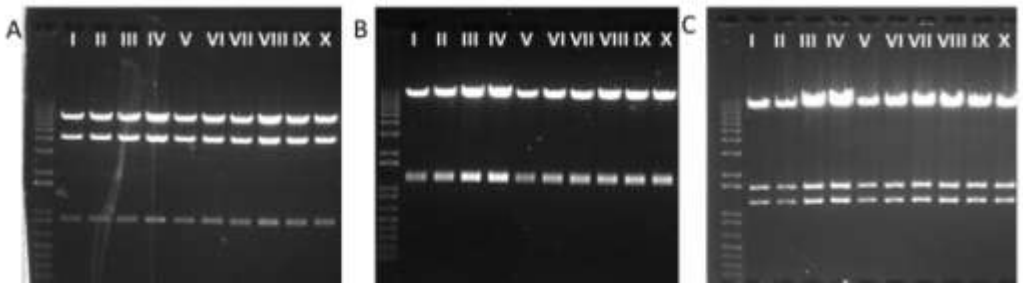

Fig 4: Restriction fragment length analysis (RFLA) of colonies derived from E.coliDH5α strain freshly transformed with TFIIIA construct. Nine colonies were randomly selected and screened with (A) Xho1, (B) BamH1 and (C) BglII restriction enzymes. All nine colonies gave the expected restriction pattern of 7327, 4127 and 878 bps for Xho1 restriction, 9856, 1287 and 1189 bps for BamH1 restriction and 9485, 1612 and 1235 for BglII restriction. All restriction patterns were confirmed with vNTI restriction fragment analysis and with the control sample X. The confirms the stability of TFIIIA construct in E.coliDH5α strain.

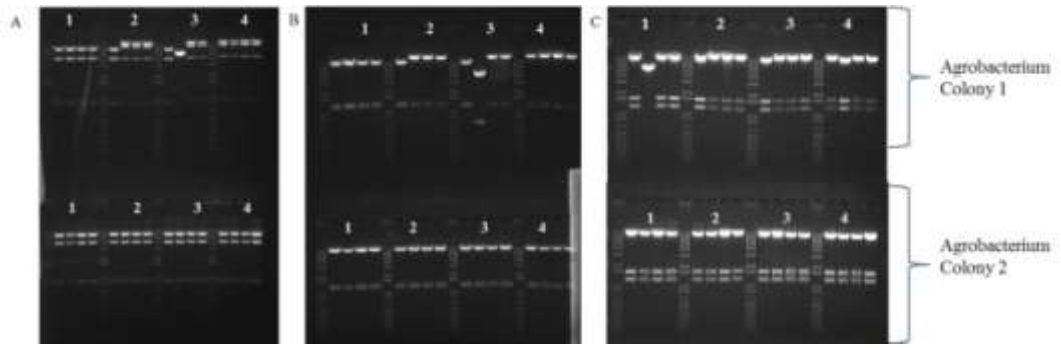

Fig 5: TFIIIA stability in two Agrobacterium LBA4404 back transformed in E.coli: Agrobacterium LBA4404 was transformed with TFIIIA construct and successful transformants selected on LB/RSK medium. DNA extracted from two randomly selected colonies were amplified in E.coliand screened for stability with (A) Xho1, (B) BamH1and (C) BglII restriction enzymes. All four E.colicolonies derived from Agrobacterium 2 gave the expected restriction fragment sizes expected from the three restriction enzymes while ¾ colonies derived from Agrobacterium 1 showed unexpected extra band when restricted with Xho1, two showed unexpected fragment size with BamH1 and with BglII all the colonies showed certain level of variation from the expected fragment sizes..

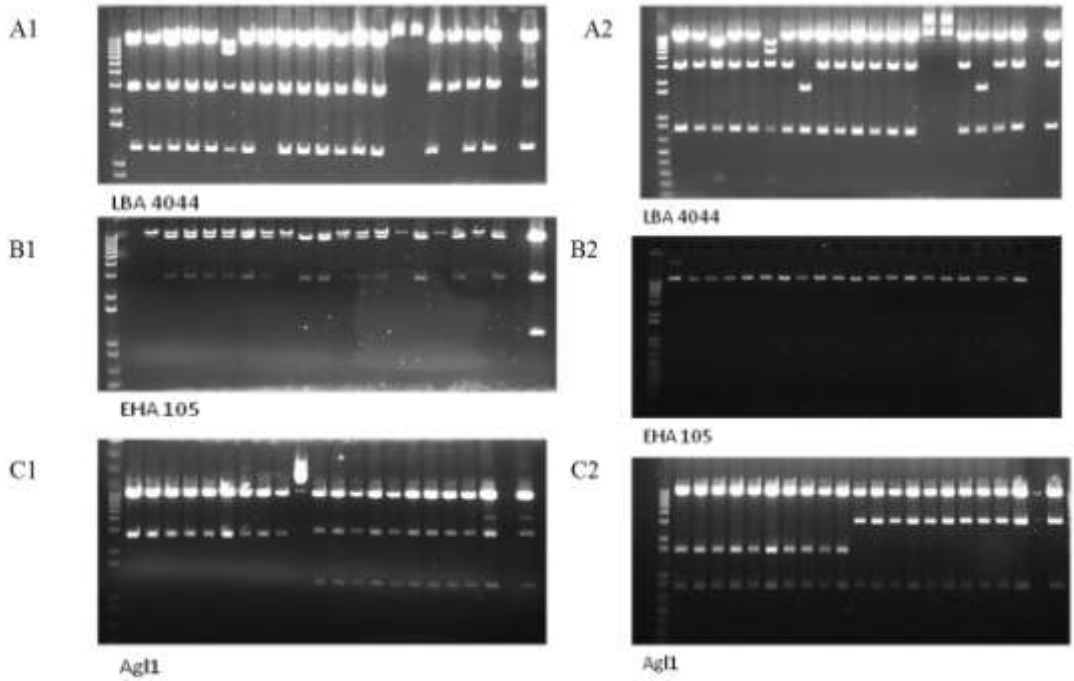

Fig 6: Restriction fragment length analysis of TFIIIA stability in different Agrobacterium strains**:**TFIIIA construct was transformed into Agrobacterium strains LBA4404, EHA105, and AGL1. After 3 days growth, DNA derived from these colonies were transformed into E.colifor amplification and analyzed with BglII and Sac I restriction analysis. LBA4044 Agrobacterium strain showed the least level of rearrangement, 25% and 30% with BglII and SacI while EHA105 strain showed 100% level of rearrangement with both enzymes and AGL1 50% rearrangement with both enzymes.

IV. Discussion

The TFIIIA construct stored at -80^{0}C showed 17% rearrangement when analyzed with XbaI/Hind III double digestion and 100% rearrangement with Sac 1 restriction enzyme. This result confirmed some level of inconsistency in the quality of the TFIIIA recombinant DNA molecule and raises a concern with respect to the effect of storage on the stability of the molecule. Nudelet al. [12] observed that the stability of expression of the tryptophan (thr) operon cloned into pBR322 and introduced into E. coli depended both on the genetic background of host as well as on environmental conditions. To investigate the effect of storage temperature on the stability of recombinant TFIIIA molecule, TFIIIADNA molecule stored at 4^{0}C was also analyzed using the same set of enzymes. RFLA gave the expected fragment sizes of 8718 and 3614 bps with Xba1/Hind III restriction and 9238 and 3094 bps with Sac 1 restriction (Fig. 2C). At this point the effect of storage on the stability of TFIIIA was suspected.

To investigate this hypothesis, new E. coli DH5α strain was transformed with the DNA stored at 4^{0}Cshowing the expected fragment length. All nine colonies screened did not show irregularities in the restriction fragment pattern and thus suggests that storage at -80^{0}C could have affected the stability of the TFIIIA construct. There is need for further studies on the effect of low temperature on construct stability using different constructs in different vector background. Instability of the TFIIIA molecule was shown to occur in both E. coli DH5α strain stored at -80^{0}C and in Agrobacterium LBA4404 strain in our study. This study is essential towards the success of biotechnological process that depends on the use of plasmid DNA for product development. It illustrates the importance of quality assurance of recombinant molecules before use in any genetic transformation or fermentation process.

The stability of TFIIIA DNA molecule was also investigated in three Agrobacterium strains LBA 4404, EHA 105 and AGL1 to determine the effect of host cell on the stability of the TFIIIA DNA molecule. Twenty E. coli colonies each derived from two colonies of LBA4404, EHA 105 and AGL1 Agrobacterium strains were analyzed. LBA4404 showed the least level of rearrangement among the three Agrobacterium strain with BglII (25%) and Sac I (30%) restriction enzymes , EHA105 showed the highest level of rearrangement (100%) with both restriction enzymes, while 50% of AGL1 showed level of rearrangement when analyzed with both enzymes. DNA sequencing result of the rearranged molecule confirmed the insertion of about 7 kb sequences originally not present in the molecule. This agrees with the findings of Nudelet al. [12] who showed that the observed changes in the expression of tryptophan operon was due to the insertion of a DNA molecule of approximately 5.6 kb. The data also showed that simply switching from LBA4404, as used in the present

studies, to EHA105 of AGL1 would not have solved the problems encountered here, and described above, related to recombination and rearrangements of the TFIIIA constructs.

V. Conclusion

In this study, we have shown that although Agrobacterium mediated transformation is widely employed for the production of GM crops and is preferred over direct DNA transfer methods such as microparticle bombardment mediated transformation, there is the likelihood of construct recombination in Agrobacterium before transfer to target cells. This study also confirms the importance of proper quality assurance of the recombinant DNA molecule prior to genetic transformation of plants to avoid waste of time and labor in recovering events without the desired trait. There is a need to optimize the application of direct DNA transfer methods such as microparticle bombardment, electroporation and polyethylene glycol for the transformation of most GM crops as the use of minimal cassette in the transformation of most crop have proven efficient in the production of quality transgenic crops with simple integration pattern, single copy number and absence of vector backbone integration.

References

[1]. N. Kuniechick, R. Munareto, T. Milech de Assunção, G. Volpato, J. Chies, C. PaivaNunes, and D. Santiago Santos. "Determination of the stability of recombinant plasmid containing the coding sequence of the human growth hormone in bioreactor."XSalão de IniciaçãoCientífica PUCRS, 2009, 2829-2831.

[2]. A. Guruatma, K. Jiaquot;. Mama Ji's Molecular Kitchen. ASU - Ask A Biologist. Retrieved September 10, 2015 from http://askabiologist.asu.edu/plasmids

[3]. P. Kumar, H. Maschke, K. Friehs, and K. Schügerl. Strategies for improving plasmid stability in genetically modified bacteria in bioreactors. Trends in biotechnology, 9(1), 1991, 279-284.

[4]. F. Hoffmann, and U. Rinas. Stress induced by recombinant protein production in Escherichia coli. In Physiological Stress Responses in Bioprocesses Springer Berlin Heidelberg 2004, 73-92.

[5]. M. Fakruddin, R. Mohammad Mazumdar, K. S. Bin Mannan, A. Chowdhury and M. N. Hossain, Critical factors affecting the success of cloning, expression, and mass production of enzymes by recombinant E. coli. ISRN biotechnology, 2013.

[6]. T. Schweder, I. Schmidt, H. Herrmann, P. Neubauer, M. Hecker, and K. Hofmann, An expression vector system providing plasmid stability and conditional suicide of plasmid-containing cells. Applied microbiology and biotechnology, 38(1), 1992), 91-93.

[7]. F. A. Al-Allaf, O. E. Tolmachov, L. P. Zambetti, V. Tchetchelnitski and H. Mehmet Remarkable stability of an instability-prone lentiviral vector plasmid in Escherichia coli Stbl3. 3 Biotech, 3(1), 2013, 61-70.

[8]. C. S.Chiang and H. Bremer, Stability of pBR322-derived plasmids. Plasmid, 20(3), 1988, 207-220.

[9]. L. Olavarrieta, P. Hernandez, D. B. Krimer, and J. B. Schvartzman. DNA knotting caused by head-on collision of transcription and replication. Journal of molecular biology, 322(1), 2002, 1-6.

[10]. A. Warnes and R. Stephensoin. The insertion of large pieces of foreign genetic material reduces the stability of bacterial plasmids. Plasmid 16, , 1986, 116-123.

[11]. C. H.Collins and A. J. Beale,(Eds.), Safety in industrial microbiology and biotechnology. Elsevier. 2013

[12]. B. C. Nudel, B. Vosman, , M. J. T. De Mattos, K. J. Hellingwerf, and O. M. Neijssel, Increased stability of recombinant plasmids by Tn1000 insertion in chemostat cultures of recombinantEscherichia coli GT123. Current Microbiology, 26(5), 1993, 281-286.