NEW PLASMID CONSTRUCTS FOR PRODUCTION OF YEAST BASED ORAL VACCINES

ABSTRACT

In order to avoid conventional vaccines, there is an urgent need to look for new ways of vaccine generation that can cut down the cost, time, and the need for special requirements during storage and distribution. The development of oral peptide/protein-based vaccines could be an ideal alternative. Five expression plasmids are designed in this work for the production of *Saccharomyces cerevisiae*-based oral vaccines. Therefore, in this work genetic cassette with different organizations of the required genetic components for efficient gene expression is constructed in the pYES2 plasmid. The construction of five new plasmids for surface display of heterologous recombinant proteins in *S. cerevisiae* would enable easy insertion of the gene of interest into an optimized genetic cassette consisting of a strong host promoter *GAL1*, HA-tag for detection of recombinant enzymes, *GAS1* gene with/without binding sequence coded for GAS1 cell wall protein and signal sequence for directing the protein into the secretory pathway. After the construction of the genetic cassettes, E gene coded for envelope protein of Chikungunya and Dengue virus is inserted in the genetic cassettes. Genetic models pYES2HAGAS1EgeneChikungunya, pYES2HAGAS1EgeneDengue, pYES2GAS1EgeneChikungunyaHA, and pYES2GAS1EGeneDengueHA are constructed with different genetic organizations to display envelope protein of Chikungunya and Dengue virus in the cell surface of *S. cerevisiae*. In the pYES2SignalEGeneDengueHA construction, envelope protein will be secreted by *S. cerevisiae* into the media. The constructed vectors are specialized to regulate the expression and surface display of heterologous recombinant proteins in *Saccharomyces cerevisiae* and will be suitable for large-scale oral vaccine production.

Keywords: Cell surface display system, Genetic cassette, Heterologous recombinant protein, Oral vaccine

1. INTRODUCTION

Cell surface display of recombinant proteins has become a crucial tool in biotechnology and biomedical applications, enabling the expression of proteins on the cell surface [1, 20]. The effectiveness of this technique depends on the cell system used and the specific anchoring methods for the displayed proteins. To meet the demands for scalable, cost-effective production, a platform that is inexpensive, safe, and adaptable is essential [2].

Oral vaccines, a promising application of cell surface engineering, have seen advancements in display and evaluation methods. However, the current methods for producing Chikungunya and Dengue vaccines face significant challenges [3]. There is an urgent need to develop a new strategy for manufacturing safe and effective oral vaccines for these viruses. Yeast display technology presents a potential solution [16]. Traditional vaccine platforms have struggled to provide adequate protection against Chikungunya and Dengue viruses. Thus, a novel platform using *Saccharomyces cerevisiae* for surface display may offer a viable approach for developing effective oral vaccines [4, 23].

Chikungunya (CHIKV) and Dengue (DENV) viruses, transmitted by Aedes mosquitoes, have become prominent in tropical and subtropical regions [5, 6]. Dengue is particularly widespread in Argentina, where the recombinant tetravalent vaccine

Dengvaxia® has been approved but is not part of the national vaccination schedule. Chikungunya outbreaks, while less frequent, can lead to chronic rheumatism, and there are currently no approved vaccines or effective antiviral therapies [7, 17]. Again, oral vaccines offer benefits such as avoiding injection-related side effects and being more acceptable to those with needle phobia. However, challenges include lower immunogenicity and complex production processes [8]. This study aims to develop an oral vaccine using yeast display technology, where the envelope proteins of Chikungunya and Dengue viruses are expressed on the surface of *Saccharomyces cerevisiae*[9].

Using the pYES2 plasmid, various genetic cassettes have been constructed, including a signal sequence (GAS1) for protein secretion [10], a cell wall anchoring protein (GAS1) for N-terminal immobilization, the envelope proteins of Chikungunya and Dengue viruses, and a genetic tag (HA) [18]. Five different plasmid constructs were tested to identify the most effective configuration for protein expression and surface display [11, 24]. The functionality of these constructs was assessed through Western blot analysis, confirming the expression of the target proteins on the yeast cell surface [12, 25]. Subsequently, the proteins will be isolated and used to develop an oral RNA vaccine (RT-PCR) [5]. This study proposes a yeast-based platform for the development of oral vaccines against Chikungunya and Dengue, aiming to overcome the limitations of current vaccine technologies and provide a rapid, scalable solution [13].

2. MATERIAL AND METHODS

2.1 pYES2HAGAS1CHIKUNGUNYAE1GENE Plasmid Construction

To construct the plasmid pYES2HAGAS1CHIKUNGUNYAE1GENE, in Table 1, the process involved several steps using Snap Gene Software for design and analysis. Initially, specific restriction sites (HindIII, SacI, NotI, SphI) were selected in the pYES2 plasmid for gene insertion. Primers for the HA tag, GAS1 gene, and Chikungunya E1 gene were designed with corresponding restriction sites for PCR amplification. For the HA tag, HindIII and SacI restriction sites were incorporated into the forward and reverse primers, respectively. PCR conditions included an initial denaturation at 95°C, followed by cycles of denaturation, annealing, and extension, with fragment size confirmed by gel electrophoresis [19, 26].

Table 1.List of primers used in pYES2HAGAS1CHIKUNGUNYAE1GENE

Primer	Sequence	Length (Bp)	Melting Temperature (°C)	Gc Content (%)
HA Forward Primer	AAGCTTGACATCATCATCACCATCAT	26	55	38
HA Reverse Primer	GAGCTCCTCACTGATGATTCGCGTCTA	27	61	52
*GAS1*Forward Primer	GCAGCAGAGCTCATGTTGTTTAAATCCC	28	59	46
GAS1 Reverse Primer	GCAGCGGCCGCTTAAACCAAAGCAAAACCG	30	65	57
Chikungunya Virus E1 Gene Forward Primer	GCGGCCGCTACCCATTCATGTGG	23	62	65
Chikungunya Virus E1 Gene Reverse Primer	GCATGCCACACTTGCCTTTCTTGCT	25	59	52

Similar procedures were followed for the GAS1 gene using SacI and NotI sites, and for the Chikungunya E1 gene using NotI and SphI sites. The amplified HA tag was inserted into the pYES2 plasmid to create the pYES2HA plasmid. Next, the GAS1 gene was inserted into pYES2HA to generate pYES2HAGAS1. Finally, the Chikungunya E1 gene was inserted into

pYES2HAGAS1 to produce the final plasmid, pYES2HAGAS1CHIKUNGUNYAE1GENE. Each insertion step was verified by gel electrophoresis to ensure successful plasmid construction.3.2 Plasmid 2 pYES2HAGAS1EGeneDengue plasmid construction [27].

2.2 pYES2HAGAS1EGeneDengue Plasmid Construction

For constructing the plasmid pYES2HAGAS1DengueVirusEgene shown in Table 2, Snap Gene Software was utilized to design primers and identify restriction sites. The pYES2 plasmid was chosen with restriction sites HindIII, BamHI, NotI, and SphI for inserting the HA tag, GAS1 gene, and Dengue virus E gene. Primers for the HA tag included HindIII and BamHI sites, while primers for the GAS1 gene featured BamHI and NotI sites. For the Dengue virus E gene, NotI and SphI sites were used. PCR was conducted to amplify each gene segment, and the PCR products were verified using gel electrophoresis. The HA tag was first inserted into the pYES2 plasmid to generate the pYES2HA plasmid.

Table 2.List of primers used inpYES2HAGAS1EGeneDengue plasmid

Primer	Sequence	Length (Bp)	Melting Temperature (°C)	Gc Content (%)
HA Forward Primer	AAGCTTGACATCATCATCACCATCATGG	28	58	43
HA Reverse Primer	GGATCCCTCACTGATGATTCGCGTC	25	60	56
GAS1 GENEForward Primer	GCAGGATCCGCAATGTTGTTTAAATCCCTTTCAAAG	36	63	42
GAS1 GENEReverse Primer	GCGGCCGCTTAAACCAAAGCAAAACCGAC	29	64	55
*E GENE*Forward Primer	GCGGCCGCTGCGATGCGTAGGAGTAGGA	28	68	68
*E GENE*Reverse Primer	GCATGCTGTGAAGCCCAGAAACAGAGTG	28	62	54

Next, the GAS1 gene was inserted into pYES2HA to form pYES2HAGAS1. Finally, the Dengue virus E gene was incorporated into pYES2HAGAS1, resulting in the final plasmid, pYES2HAGAS1DengueVirusEgene. Each step of the cloning process was confirmed by gel electrophoresis [19, 20].

2.3pYES2*GAS1EGeneChikungunya*HA Plasmid Construction

In Table 3, for the construction of the pYES2GAS1ChikungunyaE1GeneHA plasmid, we utilized Snap Gene Software to design primers and analyze restriction sites. We first inserted the GAS1 gene into the pYES2 plasmid using HindIII and SacI restriction sites, generating the pYES2GAS1 plasmid. Next, the Chikungunya virus E1 gene was amplified with primers incorporating SacI and NotI sites and inserted into the pYES2GAS1 plasmid to create pYES2GAS1ChikungunyaE1Gene.

Table 3.List of primers used inpYES2*GAS1EGeneChikungunya*HA Plasmid

Primer	Sequence	Length (Bp)	Melting Temperature (°C)	Gc Content (%)
*GAS1*Forward Primer	GCAGCAGCAAAGCTTATGTTGTTTAAATCCCTTTC	25	60	40
*GAS1*Reverse Primer	GAGCTCTTAAACCAAAGCAAAACCGAC	27	58	44
*CHIKUNGUNYA VIRUS E1 GENE*Forward Primer	GAGCTCTACCCATTCATGTGGGGC	24	60	58
*CHIKUNGUNYA VIRUS E1 GENE*Reverse Primer	GCGGCCGCCACACTTGCCTTTCTTGCTGGC	30	69	67
HA Forward Primer	GCGGCCGCGACATCATCATCACCATCATGG	30	67	60
HA Reverse Primer	GCATGCCTCACTGATGATTCGCG	23	58	57

Lastly, the HA tag was amplified using primers with NotI and SphI sites and inserted into the pYES2GAS1ChikungunyaE1Gene plasmid, resulting in the final pYES2GAS1ChikungunyaE1geneHA plasmid. Each step was validated by 1% agarose gel electrophoresis to confirm successful insertion and plasmid construction [19,27].

2.4 pYES2GAS1EgeneDengueHAPlasmid Construction

To construct the pYES2GAS1EgeneDengueHA plasmid in Table 4, we utilized Snap Gene Software to design primers and analyze restriction sites for cloning. We began by inserting the GAS1 gene into the pYES2 plasmid using HindIII and BamHI restriction sites. The GAS1 gene was amplified with primers designed to include these restriction sites, and the successful insertion was confirmed through gel electrophoresis. Next, the Dengue virus E gene was inserted into the pYES2GAS1 plasmid using BamHI and NotI sites [29, 30].

Table 4.List of primers used inpYES2GAS1EgeneDengueHA

Primer	Sequence	Length (Bp)	Melting Temperature (°C)	Gc Content (%)
*GAS1*Forward Primer	AAGCTTGCAGCAGCAATGTTGTTTAAATCCCTTTC	35	62	40
*GAS1*Reverse Primer	GGATCCTTAAACCAAAGCAAAACCGACACC	30	61	47
*E GENE*Forward Primer	GGATCCCATCCTACGGAATGCGATGC	26	62	58
*E GENE*Reverse	GCGGCCGCTGTGAAGCCCAGAAACAGAGTG	30	68	63

Primer				
HA Forward Primer	GCGGCCGCGACATCATCATCACCATCATGG	30	67	60
HA Reverse Primer	GCATGCCTCACTGATGATTCGCGTC	25	60	56

2.5 pYES2SignalEGeneDengueHAPlasmid Construction

To construct the pYES2SignalEGeneDengueHA plasmid, firstly, the Signal sequence, Dengue virus E gene, and HA tag were inserted into the pYES2 plasmid using Snap Gene software for design and restriction site analysis. For the Signal sequence, primers with HindIII and BamHI restriction sites were designed and PCR was performed. Gel electrophoresis confirmed the successful amplification. Next, the Dengue virus E gene was inserted into the pYES2Signal plasmid using BamHI and NotI restriction sites, with PCR confirmation via gel electrophoresis. Finally, the HA tag was added to the pYES2SignalEGeneDengue plasmid using NotI and SphI restriction sites. The entire construct was verified by gel electrophoresis, resulting in the final pYES2SignalDengueVirusEgeneHA plasmid [27, 29].

Table 5.List of primers used inpYES2SignalEGeneDengueHA

Primer	Sequence	Length (Bp)	Melting Temperature (°C)	Gc Content (%)
Signal Sequence Forward Primer	AAGCTTGCAGCAGCAGCAATGTTGTTTAAATCCCTT	36	63	42
Signal Sequence Reverse Primer	GGATCCTCATTCAAAGCCTTCATACATTCGG	31	60	45
E GENE Forward Primer	GGATCCCATCCTACGGAATGCGATGC	26	63	58
E GENE Reverse Primer	GCGGCCGCTGTGAAGCCCAGAAACAGAGTG	30	68	63
HA Forward Primer	GCGGCCGCGACATCATCATCACCATCATGG	30	67	60
HA Reverse Primer	GCATGCCTCACTGATGATTCGCGTC	25	60	56

Similarly, the E gene was amplified and validated by gel electrophoresis. Finally, the HA tag was introduced into the pYES2GAS1EgeneDengue plasmid using NotI and SphI sites. The insertion was verified by gel electrophoresis, resulting in the final pYES2GAS1DengueVirusEgeneHA plasmid [28].

3. RESULTS AND DISCUSSION

3.1 pYES2HAGAS1ChikungunyaE1gene Plasmid construction

The pYES2HAGAS1ChikungunyaE1gene plasmid was constructed by incorporating the HA tag, GAS1 gene, and Chikungunya virus E1 gene into the pYES2 plasmid, which contains essential elements such as the GAL1 promoter, CYC1 terminator, URA3, and AMPR genes. The pYES2 plasmid's size (5856 bp) was confirmed using 1% agarose gel electrophoresis. The HA tag (480 bp) was amplified by PCR and inserted into the plasmid, forming the pYES2HA plasmid (6318 bp), confirmed by HindIII and SacI digestion. The GAS1 gene (1703 bp) was amplified, inserted into pYES2HA, resulting in the pYES2HAGAS1 plasmid (7941 bp), with restriction digestion yielding fragments of 6258 bp and 1683 bp.

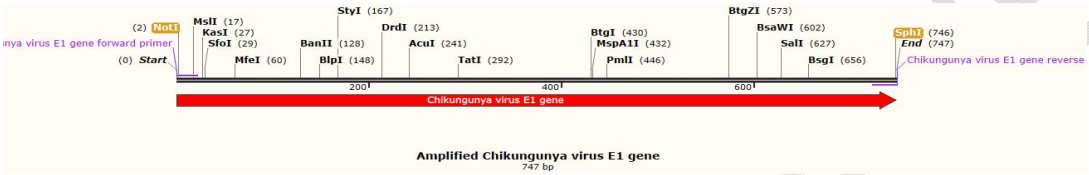

Fig. 1. Restriction map of amplified PCR fragment of E gene of Chikungunya Virus

Fig. 2. Genetic map of pYES2HA*GAS1ChikungunyaE1Gene* plasmid(GAL1 promoter-Green, CYC1 terminator, ori-Yellow, URA3-light red, AMP^{R} -light blue, HA-tag -Dark orange, *GAS1* gene-Yellow, *Signal peptide* – Light blue, *Chikungunya E1 Gene* - Red) and The gel Electrophoresis of pYES2HAGAS1ChikungunyaE1gene plasmid is constructed by using 1% Agarose gel and the MW is a 1 kb DNA Ladder.

The final step involved inserting the Chikungunya E1 gene (red) into pYES2HAGAS1, generating the pYES2HAGAS1ChikungunyaE1gene plasmid (8670 bp), confirmed by NotI and SphI digestion, producing fragments of 7926 bp and 744 bp. Each step was verified through PCR and agarose gel electrophoresis.

3.2 pYES2HA*GAS1EGeneDengue* plasmid construction

The construction of the pYES2HAGAS1EgeneDengue plasmid began with the amplification and insertion of the HA tag (480 bp) into the pYES2 plasmid, which includes the GAL1 promoter, CYC1 terminator, origin of replication, URA3, and AMPR genes. This insertion was verified using 1.2% agarose gel electrophoresis with a 1 kb DNA ladder. The GAS1 gene (1700 bp) and its signal peptide were then amplified and inserted into the plasmid, confirmed by 1% agarose gel electrophoresis, resulting in a pYES2HAGAS1 plasmid of 7944 bp. Restriction digestion of pYES2HAGAS1 with BamHIand NotI produced fragments of 6254 bp and 1690 bp. Next, the Dengue virus E gene (1489 bp, green) was inserted into the pYES2HAGAS1 plasmid, forming the pYES2HAGAS1Egene plasmid.

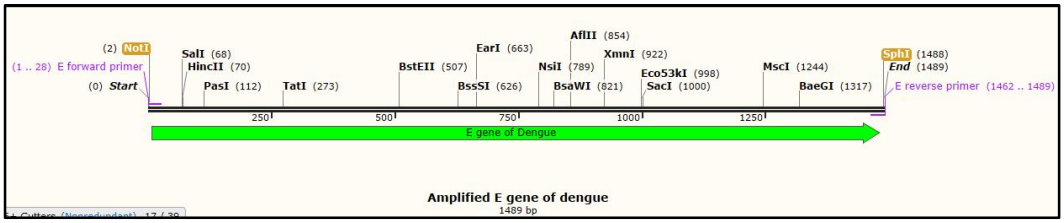

Fig. 3. Restriction map of amplified PCR fragment of *E gene of Dengue Virus*

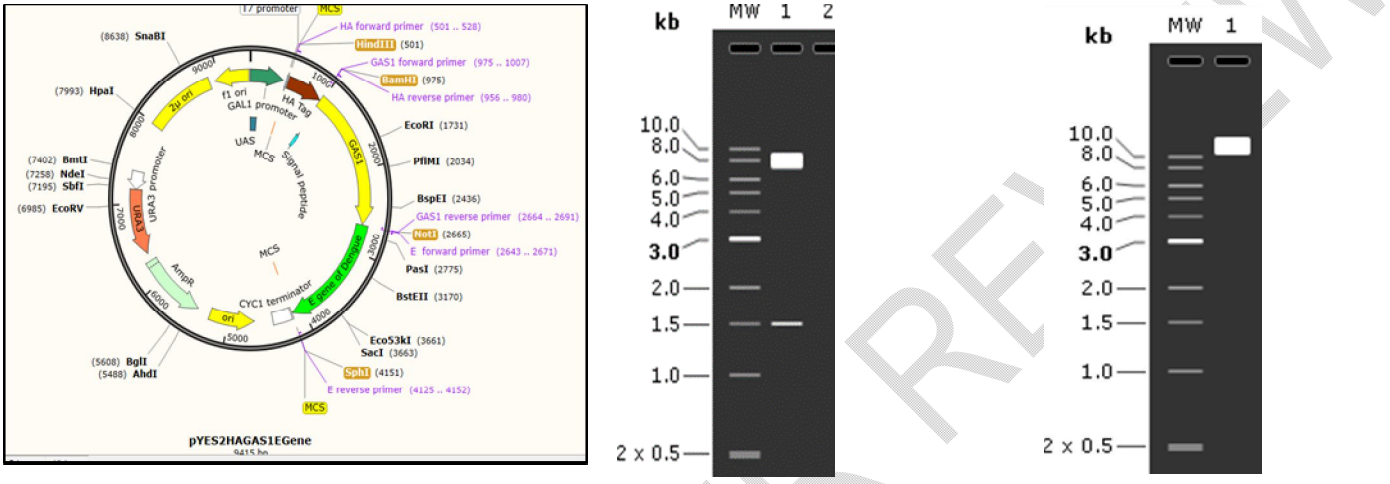

Fig. 4. Genetic map of pYES2HA*GAS1EGene* plasmid (GAL1 promoter-Green, CYC1 terminator, ori-Yellow, URA3-light red, AMP^R -light blue, HA-tag -Dark orange, *GAS1* gene-Yellow, *Signal Peptide*-Light Blue*, Dengue E Gene* – Green) and *Gel Electrophoresis of pYES2HA*GAS1Egeneplasmid*.*

This was confirmed via gel electrophoresis, showing a size of 9415 bp. Digestion with NotI and SphI restriction enzymes generated fragments of 7929 bp and 1486 bp, confirming successful plasmid construction.

3.3pYES2*GAS1EGeneChikungunya*HA plasmid construction

The construction of the pYES2GAS1EgeneChikungunyaHA plasmid involved the amplification of key components via PCR, including the HA tag, GAS1 gene, and Chikungunya virus E1 gene. The HA tag, GAS1 gene (yellow, 1701 bp), and signal peptide (light blue) were inserted into the pYES2 plasmid through restriction digestion and ligation at specific sites. The E1 gene (red, 747 bp) was also inserted. Gel electrophoresis of the resulting pYES2GAS1ChikungunyaE1gene plasmid using a 1% agarose gel and 1 kb DNA ladder confirmed a plasmid size of 8206 bp. Digestion with SacI and NotI produced fragments of 7470 bp and 736 bp.

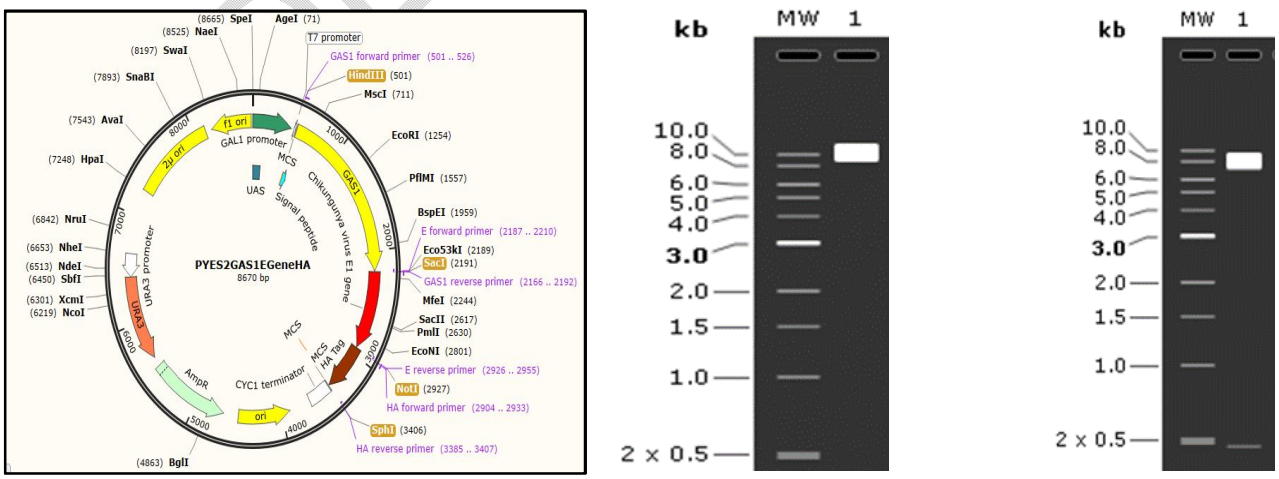

Fig.5. Genetic map of pYES2GAS1EGeneChikungunyaHA plasmid
(GAL1 promoter-Green, CYC1 terminator, ori-Yellow, URA3-light red, AMP^R -light blue, *GAS1*-Yellow, Signal Peptide-Light blue, *E gene of Chikungunya*-Red, HA-tag -Dark orange) and Gel Electrophoresis of pYES2*GAS1EGene*HA plasmid.

For the pYES2GAS1EgeneHA plasmid preparation, the amplified HA tag (dark orange, 482 bp) was confirmed via 1.2% agarose gel electrophoresis. Restriction analysis of pYES2GAS1EgeneHA with NotI and SphI resulted in fragments of 8191 bp and 479 bp. Finally, gel electrophoresis of the complete pYES2GAS1EgeneHA plasmid showed a size of 8670 bp.

3.4pYES2*GAS1EgeneDengue*HA plasmid construction

The pYES2GAS1EgeneDengueHA plasmid was constructed by amplifying and inserting the HA tag, GAS1 gene, and Dengue virus E gene into the pYES2 plasmid. The GAS1 gene (1701 bp, yellow) and signal peptide (light blue) were confirmed via 1% agarose gel electrophoresis. Restriction analysis with HindIII and BamHI produced fragments of 5838 bp and 1695 bp, with a total plasmid size of 7533 bp. The Dengue virus E gene (1495 bp, red) was then inserted, yielding a pYES2GAS1Egene plasmid of 8963 bp.

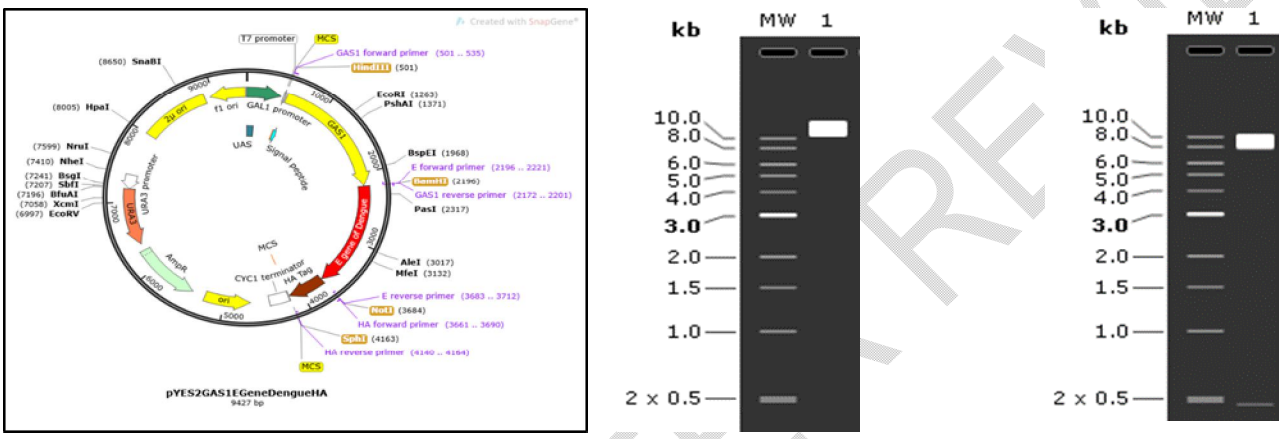

Fig. 6. Genetic map of pYES2*GAS1EGene*HA plasmid. (GAL1 promoter-Green, CYC1 terminator, ori-Yellow, URA3-light red, AMP^R -light blue, HA-tag -Dark orange, GAS1 gene-Yellow, Signal peptide – Light blue, Dengue *E Gene* – Red) and Gel Electrophoresis of pYES2GAS1EgeneHA plasmid.

Digestion with BamHI and NotI resulted in fragments of 7475 bp and 1488 bp. The amplified HA tag (482 bp, dark orange) was confirmed by 1.2% agarose gel. The final pYES2GAS1EgeneHA plasmid was 9427 bp, and digestion with NotI and SphI produced fragments of 8948 bp and 479 bp.

3.5.pYES2SignalEGeneDengueHA plasmid construction

The construction of the pYES2SignalEgeneDengueHA plasmid involved amplifying and inserting components such as the HA tag, GAS1 signal peptide, and Dengue virus E gene into the pYES2 plasmid using restriction enzymes. The GAS1 signal peptide (350 bp, yellow) was confirmed via gel electrophoresis using 1% agarose gel. The pYES2Signal plasmid (6182 bp) was analyzed after HindIII and BamHI digestion, yielding fragments of 5838 bp and 344 bp. The Dengue virus E gene (1495 bp, red) was inserted, resulting in the pYES2SignalEgene plasmid (7612 bp).

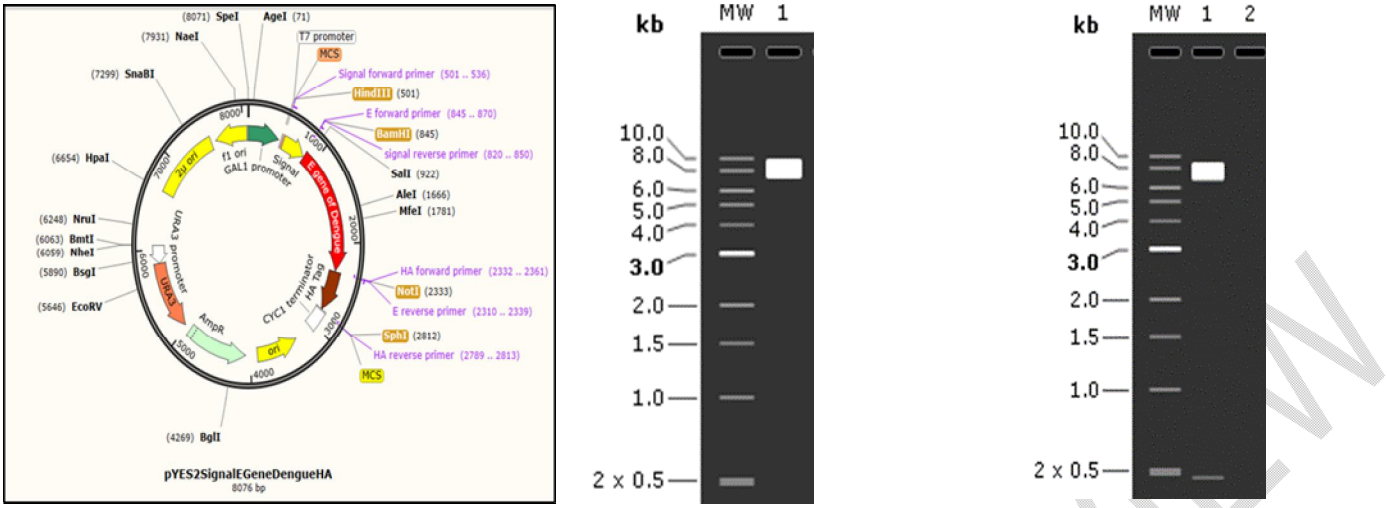

Fig. 7. Genetic map of pYES2*SignalEGene*HA plasmid (GAL1 promoter-Green, CYC1 terminator, ori-Yellow, URA3-light red, AMP^R -light blue, HA-tag -Dark orange, *Signal peptide* of *GAS1* gene-Yellow*, Dengue E Gene* – Red) and *Gel Electrophoresis of pYES2*SignalEgene*HA* plasmid.

Digestion with BamHI and NotI produced fragments of 6124 bp and 1488 bp. The HA tag (482 bp) was amplified and inserted into the plasmid, confirmed by 1.2% agarose gel electrophoresis. The final pYES2SignalEgeneHA plasmid (8076 bp) was verified through gel electrophoresis, and digestion with NotI and SphI produced fragments of 7597 bp and 479 bp.

Various plasmids were constructed using SnapGene software to develop oral vaccines based on viral envelope proteins [14]. The HA tag, GAS1 gene, and envelope proteins from Dengue and Chikungunya viruses were inserted into the pYES2 plasmid through restriction and insertion methods using enzymes such as HindIII, BamHI, SacI, NotI, and Sph [13, 15]. Gel electrophoresis was used to confirm the size of the inserted components. This genetic framework serves as a model for future lab work, allowing for the insertion of target genes to facilitate vaccine production [17]. Although the current work is software-based, it lays the foundation for laboratory experiments aimed at constructing accurate plasmids for oral vaccine development.

4. CONCLUSION

Cell surface display systems have emerged as powerful tools for engineering and displaying a wide range of proteins. Over the past twenty years, significant progress has been made in developing new expression systems and techniques for the efficient display of heterologous proteins. Traditional laboratory plasmids often lack optimization for diverse applications, which has led to the creation of new plasmids with enhanced genetic cassettes. In this study, we constructed plasmids designed for displaying envelope proteins from Chikungunya and Dengue viruses on the surface of *Saccharomyces cerevisiae* (yeast). The plasmids feature various genetic fragment orientations to evaluate their impact on protein expression levels. By selecting plasmids that exhibit the highest expression, we can identify the most effective constructs. Plasmids with binding sites enable protein expression in the yeast cell wall, while those without facilitate secretion into the media. This work provides a framework for constructing plasmids that can be easily adapted for inserting any target gene. The developed plasmids will support future research and vaccine production by simplifying the construction process and offering a reliable model for creating effective plasmids.

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