JOURNAL OF SCIENCE AND TECHNOLOGY
ISSN: 2229-8460 e-ISSN: 2600-7924

JST

Vol. 16 No. 2 (2024) 10-19
https://publisher.uthm.edu.my/ojs/index.php/jst

# Construction of Novel Plasmids for Eradicating Dengue Disease Using Recombinant Yeast Microbiota

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DOI: https://doi.org/10.30880/jst.2024.16.02.002

**Article Info**

Received: 19 May 2024
Accepted: 17 November 2024
Available online: 21 November 2024

**Keywords**

Gene expression, paratransgenesis, dengue, cell surface, vaccine

**Abstract**

With several dengue vaccines available that vary in effectiveness, paratransgenesis could provide an additional method for managing and possibly eradicating the dengue disease. The research in this work has focused on production of anti-dengue Dicer2/DCR2 and R2D2 proteins in yeast which are responsible for viral RNA degradation through siRNA pathway. In line of this work, pPICZ A_Pir1_Dcr2 and pPICZ A_Pir1_R2D2 novel plasmids are designed that would enable expression and genetic immobilization of our target proteins in the cell surface of yeast. The genetic cassette of the constructed plasmids contains AOX1 promoter, PIR1 gene and 6xHis genetic tag. The drosophila melanogaster (fruit fly) genes Dcr2 and R2D2 are then inserted into the designed genetic cassette of pPICZ A_Pir1_Dcr2 and pPICZ A_Pir1_R2D2 plasmids for construction of pPICZ A_Pir1_Dcr2 and pPICZ A_Pir1_R2D2 final plasmids. Both of the final plasmids are designed in such a way that would enable precise control of gene expression of our target enzymes in yeast and could be transformed with Pichia caribbica, Pichia ohmeri and Pichia guilliermondii. The transformed yeasts can then be introduced into mosquitoes Aedes aegypti either directly using a baited trap or encapsulated/seeded/oviposited recombinant yeasts into water to contaminate aquatic juvenile forms of mosquitoes.

## 1. Introduction

Dengue is the most rapidly spreading mosquito-borne virus globally, with its incidence increasing 30-fold over the last 50 years due to geographic expansion into new regions and, more recently, from urban to rural areas [1]. In Bangladesh, dengue fever is endemic, with seasonal surges, particularly during the monsoon. This year has witnessed an alarming rise, starting in late April. According to the WHO, as of 23 October 2023, Bangladesh has recorded 255,046 cases and 1,255 deaths across all 64 districts, making it the most severe dengue outbreak in the history of country [2]. Globally, dengue affects approximately 3.9 billion people in 129 countries, causing an estimated 40,000 deaths each year, primarily transmitted by Aedes mosquitoes [3]. These mosquitoes belong to one of three genera Anopheles, Aedes, and Culex responsible for most human vector-borne diseases, such as malaria (Anopheles), Zika virus, chikungunya, and dengue (Aedes), and the West Nile virus (Culex) [4, 5].

Dengue presents a significant public health challenge as no specific treatment is available [6]. Current management focuses on symptom relief, including rest, hydration, and pain relievers like acetaminophen. In severe cases, hospitalization for supportive care and intravenous fluids may be necessary [6]. Despite the availability of vaccines, their effectiveness varies. Two vaccines—Dengvaxia® (CYD-TDV) and Qdenga® (TAK-003)—are currently licensed. Dengvaxia® is recommended for individuals aged 9-45 years in certain countries, while Qdenga® is recommended for children aged 6-16 in areas of high transmission [7, 8]. Research continues

to explore novel treatments and vaccines [9]; however, until an effective cure or universal vaccine is developed, vector control remains essential in combating dengue transmission [10, 11]. Aedes aegypti, the primary vector of dengue, harbors a variety of symbiotic microorganisms, including bacteria and yeasts, within its salivary glands, midgut, and reproductive organs [12]. Common bacterial taxa include Serratia, Klebsiella, Asaia, Bacillus, Enterococcus, Enterobacter, Kluyvera, and Pantoea, with most genera found in the mosquito midgut, and some, like Asaia and Pantoea, in the ovaries and eggs [13, 14]. Pichia species, particularly Pichia caribbica, Pichia ohmeri, and Pichia guilliermondii, have been isolated from Aedes mosquitoes, with Candida also found in the midgut and ovaries [15, 16].

The use of paratransgenesis, which involves genetically modifying symbiotic microbes to block pathogen transmission, has emerged as a promising tool for mosquito control. This approach offers potential advantages over traditional chemical methods, such as pesticides, which carry risks of resistance, ecosystem disruption, and harm to non-target organisms [17, 18]. By introducing genes into mosquito-associated microbes that inhibit viral replication, the transmission of dengue could be significantly reduced. Among the symbionts found in Aedes aegypti, certain bacteria such as Asaia and Pichia species have shown potential for paratransgenesis due to their presence in key anatomical regions like the midgut and reproductive organs, which are critical for virus propagation and transmission [19].

The key objective of this study is to construct yeast plasmids expressing genes that utilize the siRNA pathway to inhibit the dengue virus. Specifically, the genes Dcr2 and R2D2 were selected for their roles in the siRNA mechanism. Dcr2 (Dicer2) is a ribonuclease enzyme that processes viral double-stranded RNA into small interfering RNAs (siRNAs), while R2D2 is a protein that aids in the loading of siRNAs onto the RNA-induced silencing complex (RISC), a critical step in antiviral defence [20]. By expressing these genes in symbiotic yeast plasmids, we aim to disrupt viral replication within the mosquito host and reduce dengue transmission. The primary goal of this study is to construct yeast plasmids that express genes of interest using the siRNA pathway to target and eliminate the dengue virus [21]. The constructed plasmids possess additional properties like expressing proteins that are genetically immobilized on the yeast cell wall and are designed to allow precise regulation of gene expression. These qualities enhance the functionality of the plasmid, making it a versatile tool for paratransgenesis-based dengue control.

During this study, one of the main challenges was the size of the anti-dengue genes (Dcr2 and R2D2), which are larger than the commercial plasmid selected for insertion. The complete size of the Dcr2 gene is approximately 3.9 kb, and R2D2 is around 1.8 kb [22]. To overcome this, the genes were fragmented using restriction enzymes and inserted sequentially into the plasmid. While co-transfection of two plasmids carrying individual genes is a common approach to overcome size limitations, in this work, gene fragmentation was employed due to the specific design constraints and the need for a unified expression system. Further references and literature suggest that while this method is less commonly used, it has been successfully applied in other contexts of recombinant DNA technology [22].

## 2. Materials and Methods

### 2.1 pPICZ A Plasmid

The pPICZ vector is widely used in molecular biology and biotechnology for expressing recombinant proteins in the yeast *Pichia pastoris*. This methylotrophic yeast is favored for heterologous protein synthesis due to its strong and tightly regulated methanol-inducible promoter, known as the *AOX1* promoter. This promoter allows precise control of gene expression, essential for optimizing protein yield. The vector contains a Zeocin™ resistance gene for selection, a polylinker region for easy gene insertion, a secretion signal for extracellular protein production, termination signals for mRNA synthesis, and a yeast origin of replication for autonomous replication within *Pichia* cells [22].

**Table 1** *pPICZ vectors information*

| Feature | Details |
|---|---|
| Promoter | *AOX1* |
| Product Type | Pichia Expression Vector |
| Antibiotic Resistance | Zeocin™ (ZeoR) |
| Shipping Condition | Room Temperature |
| Protein Tag | His Tag (6x), c-Myc Epitope Tag |
| Cloning Method | Restriction Enzyme/MCS |

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### 2.1.1 *AOX1* Promoter

The alcohol oxidase I (*AOX1*) promoter is essential for methanol metabolism, encoding the alcohol oxidase enzyme. Its expression is tightly regulated in response to various carbon sources. While methanol induces *AOX1* expression, carbon sources such as glucose and ethanol repress its activity [23]. This regulation makes the *AOX1* promoter an excellent choice for expression in *Aedes aegypti*, as it allows for controlled expression of anti-dengue genes in a method that aligns with the vector control strategies targeting dengue transmission. Controlled expression ensures that the introduced genes can be activated in response to specific environmental conditions, thus minimizing potential toxicity or metabolic burden on the mosquito host [24].

### 2.1.2 Myc Tag

The Myc tag (EQKLISEEDL), derived from the c-*Myc* oncogene, and is a short peptide that plays a critical role in protein identification and purification. In this study, the Myc tag was incorporated into the recombinant proteins to facilitate their tracking and purification. By utilizing specific antibodies that bind to the Myc tag, researchers can effectively isolate the target proteins from complex mixtures, thereby enhancing the overall efficiency of protein purification [25].

### 2.1.3 6XHis Tag

A polyhistidine tag, often referred to as His tag (6xHis), is typically located near the protein's N- or C-terminus and contains a sequence of at least six histidine residues. His tag enables the isolation of the target protein from complex mixtures, as it binds to nickel or cobalt ions in affinity chromatography. This technique is widely used to purify recombinant proteins expressed in *Pichia pastoris*, facilitating their subsequent analysis and characterization [26].

### 2.1.4 Primer Design

A specific set of primers has been developed to assist in plasmid construction, each tailored for amplifying targeted DNA fragments. The primer pairs, including Dcr2_For1, Dcr2_Rev1, Dcr2_For2, Dcr2_Rev2, Pir1_For, Pir1_Rev, and R2D2_For and R2D2_Rev1, have been selected to amplify regions of interest for subsequent assembly into the intended plasmid. Each primer pair serves a specific function within the molecular cloning process.

**Table 2** *List of primers used in this work*

| Primer | Sequence | Length h | Melting Temperature (°C) | GC Content t (%) |
|---|---|---|---|---|
| Pir1_For | ATCTTTACTACTTTATGCATAGTTGTCCTATCTTCT | 36 | 59 | 44 |
| Pir1_Rev | AAAGTTTGTTTATTTTTTTATACTATATACATTATTATTTCTA | 43 | 53 | 45 |
| Pir2_For | GCGCGCCTTCGAAATCTTTACTACTTTATGCATAGTTGT | 39 | 65 | 41 |
| Pir2_Rev | ACTATATACATTATTATTTCTAGGTACCGGCGCGC | 35 | 62 | 40 |
| Dcr2_For1 | CACTCTGTCACCGGCTTCTTT | 21 | 59 | 63 |
| Dcr2_Rev1 | GAATCGTGTGAGTACACGGAACA | 23 | 58 | 52 |
| Dcr2_For2 | CATGTATTTAAATCTCCAAACACCAAGA | 28 | 55 | 45 |
| Dcr2_Rev2 | CTTGGGTCGAAATAAATATTTTTATACAATTG | 32 | 54 | 45 |
| R2D2_For | CATCGTGACGTGTCGATTATG | 21 | 56 | 52 |

Amplified Genes by Primer Pairs:
Pir1_For and Pir1_Rev: Amplifies the *Pir1* gene.
Pir2_For and Pir2_Rev: Amplifies a second *Pir* gene variant.
Dcr2_For1 and Dcr2_Rev1: Amplifies the *Dcr2* gene (*Dicer2*), essential for siRNA processing.
Dcr2_For2 and Dcr2_Rev2: Further amplifies segments of the *Dcr2* gene to ensure full coverage for plasmid assembly.
R2D2_For and R2D2_Rev1: Amplifies the *R2D2* gene, which aids in siRNA loading onto the RISC complex.

### 2.1.5 PCR Amplification of Fragments

The primer design for the *Pir1, Dcr2,* and *R2D2* segments considered several factors. The initial set of restriction sites for *Pir1* amplification comprised Bst B1 and SfiI. The resulting Pir1_PCR_FRAGMENT1 exhibited a GC content of 44% for the forward primer and 41% for the reverse primer. Pir1 was subsequently amplified again using Bst B1 and Acc65I, yielding Pir1_PCR_FRAGMENT2. Due to the size of the *Dcr2* gene, two fragments were generated: Dcr2_PCR_FRAGMENT1 and Dcr2_PCR_FRAGMENT2. The first fragment had reverse primers with a KpnI restriction site and a 52% GC content, alongside forward primers featuring a 63% GC content and a SfiI restriction site. The second fragment comprised reverse primers with a 45% GC content that spanned the final 32 nucleotides and an ApaI restriction site, while forward primers began at 3203 bp with a 45% GC content and a KpnI restriction site. Additionally, *R2D2* was amplified to create R2D2_PCR_FRAGMENT using forward primers with a 52% GC content and an Acc65I restriction site and reverse primers with a 44% GC content and an ApaI restriction site. Together, these fragments facilitate the plasmid assembly process.

The PCR reaction followed the program below:
Cycle 1: 5 min at 95°C; 1.5 min at 55°C; 43 s at 72°C
Cycle 2 (5 times): 45 s at 95°C; 1.5 min at 55°C; 45 s at 72°C
Cycle 3 (30 times): 45 s at 95°C; 1.5 min at 56°C; 45 s at 72°C
The size of the PCR fragment was confirmed by gel electrophoresis.

### 2.1.6 Insertion of the Amplified Fragments into pPICZ A Plasmid

The insertion of the amplified fragments into the pPICZ A plasmid was performed several times during the plasmid assembly process. The initial plasmid, pPICZ A_Pir1, was generated by inserting the Pir1_PCR_FRAGMENT, which contained BstB1 and SfiI restriction sites. The *Dcr2* fragments were added in two stages: first, Dcr2_PCR_FRAGMENT1 (with SfiI and KpnI sites) was introduced into pPICZ A_Pir1 to create pPICZ A_Pir1_Dcr2 (fragment1). The second stage involved the insertion of Dcr2_PCR_FRAGMENT2, which has KpnI and ApaI restriction sites, resulting in the final plasmid, pPICZ A_Pir1_Dcr2. The sizes of the generated plasmids were verified through gel electrophoresis. Additionally, in a separate set of constructs, pPICZ A was modified by inserting Pir1_PCR_FRAGMENT2 into it to create pPICZ A_Pir1, and subsequently, the R2D2_PCR_FRAGMENT was introduced into this plasmid to form the pPICZ A_Pir1_R2D2 construct.

### 2.1.7 Ligation

In genetic engineering and molecular biology, ligation is essential because it makes it easier to integrate DNA fragments and create recombinant molecules. The pPICZ A_Pir1_Dcr2 Plasmid is made using the fragments Pir1_PCR_FRAGMENT, Dcr2_PCR_FRAGMENT1, and Dcr2_PCR_FRAGMENT2. For research on gene function, protein expression, and gene cloning, this recombinant plasmid is an invaluable tool. Similar to this, ligating the segments Pir1_PCR_FRAGMENT2 and R2D2_PCR_FRAGMENT results in the assembly of the pPICZ A_Pir1_R2D2 Plasmid.

**Table 3** *List of Plasmid construct in this work*

| Plasmid | Description | Marker | Reference |
|---|---|---|---|
| pPICZ A_Pir1_Dcr2 | Plasmid encoding total Dcr2 fragment under the control of AOX1 promoter | BleoR (Confer resistance tobleomycin, phleomycinand zeocinTM) | This work |
| pPICZ A_Pir1_R2D2 | Plasmid encoding R2D2 under the control of AOX1 promoter | BleoR (Confer resistance tobleomycin, phleomycin and zeocinTM ) | This work |

## 3. Results and Discussion

The study of paratransgenesis offers significant implications for controlling vector-borne diseases, including malaria. Our primary focus is on the genetic engineering of yeast for therapeutic protein production, paralleling efforts to combat malaria through genetically modified organisms [27]. Specifically, manipulating symbiotic bacteria such as *Wolbachia* in mosquitoes can prevent the development of *Plasmodium* parasites, responsible for malaria. This highlights the potential of genetic modifications in various organisms for disease prevention [28].

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Following the successful insertion of plasmids into yeast cells, cultured cells produce target proteins encoded by inserted sequences. The gel electrophoresis results shown in Fig. 1(a-d) are derived from in silico simulations using SnapGene software [29]. While these results provide preliminary insights into fragment sizes, experimental validation is needed. Fig. 2(a-b) illustrates the expression of the Pir1 and Dcr2 proteins in the pPICZ A_Pir1_Dcr2 plasmid, and Pir1 and R2D2 proteins in the pPICZ A_Pir1_R2D2 plasmid, regulated by cellular machinery of yeast responding to growth medium signals.

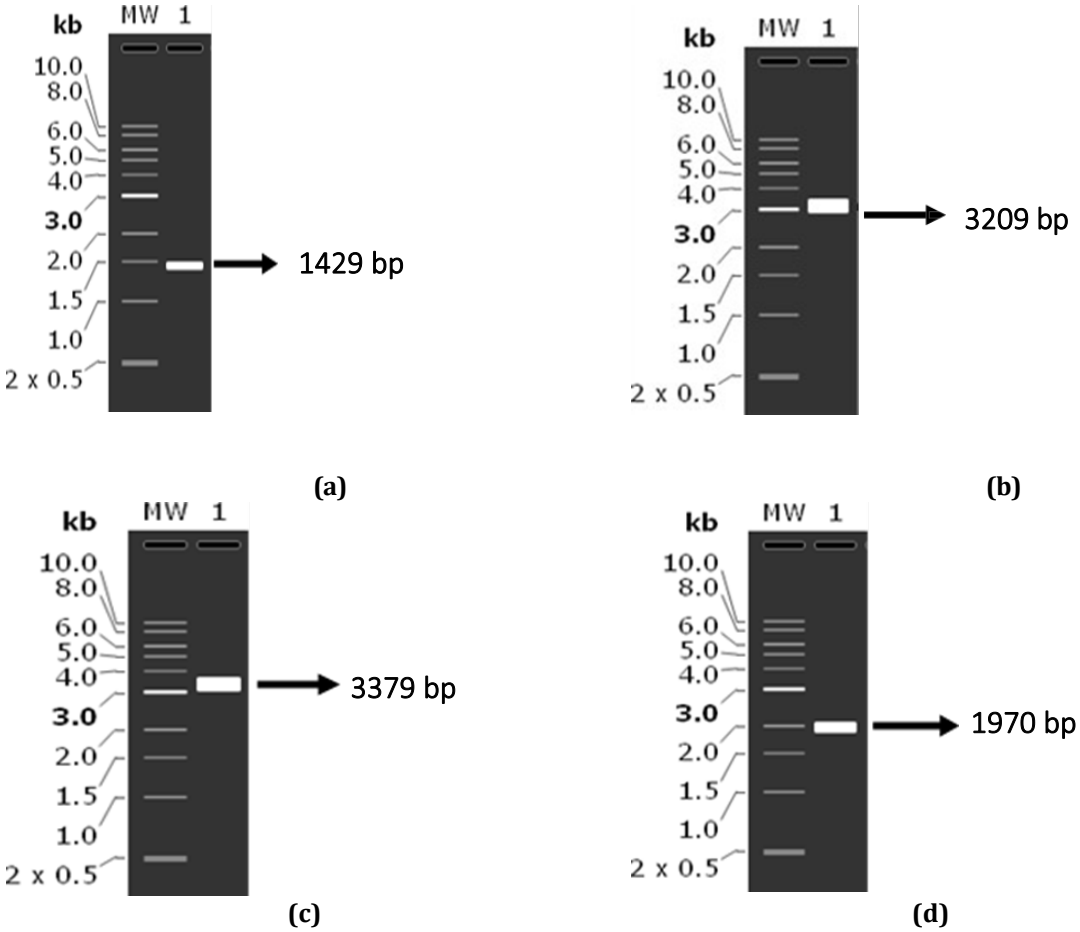
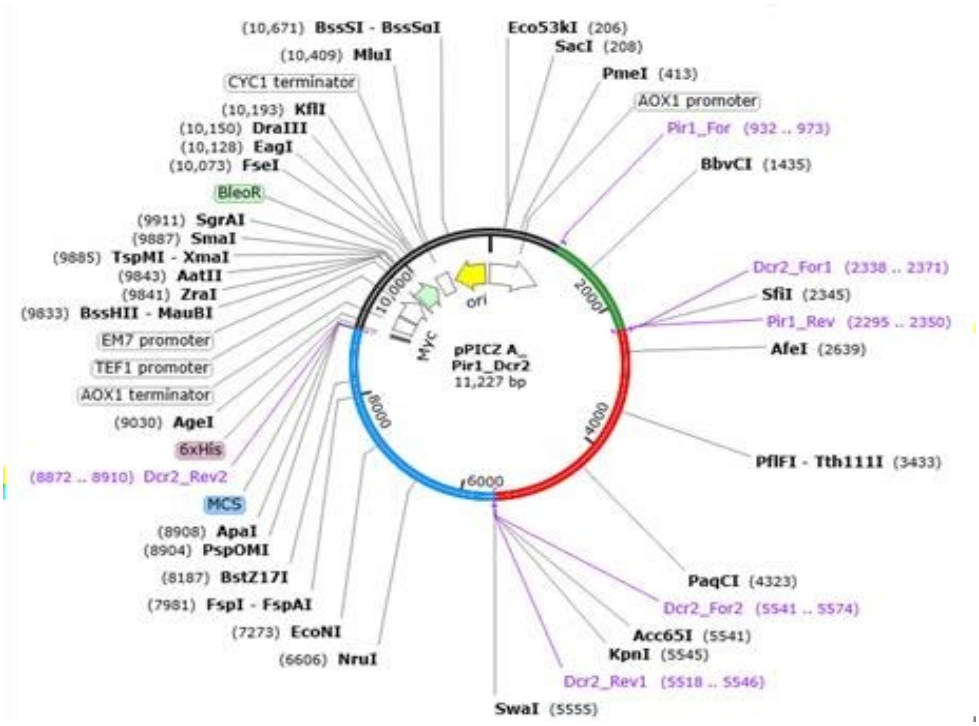
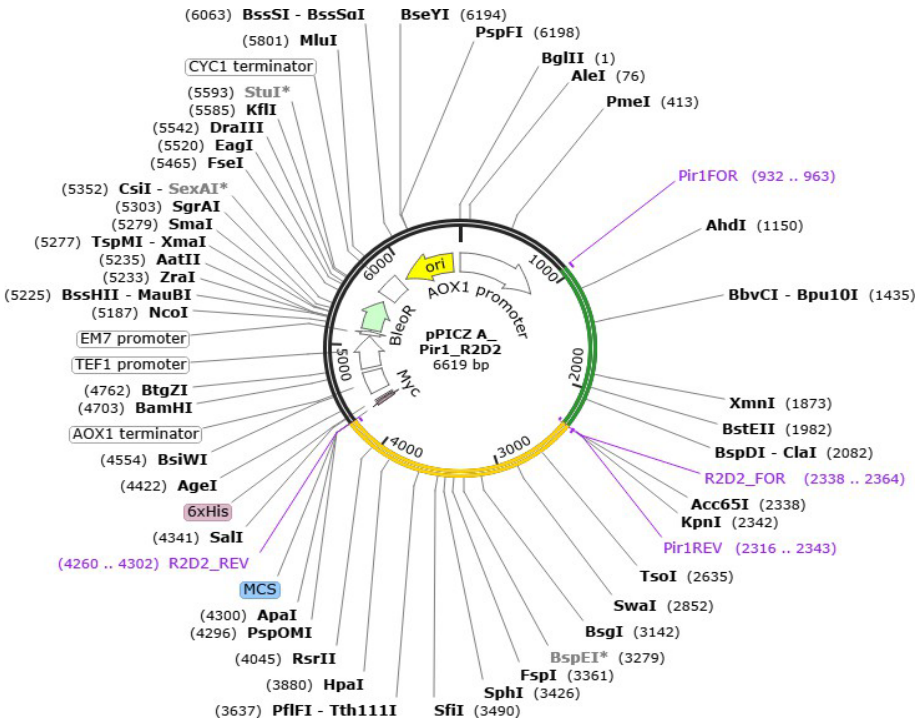



**Fig. 1** *Gel simulated gel electrophoresis of (a) Pir1_PCR_FRAGMENT (size: 1429 bp), (b) Dcr2_PCR_FRAGMENT1 (size: 3209 bp), (c) Dcr2_PCR_FRAGMENT2 (size: 3209 bp), and (d) R2D2_PCR_FRAGMENT (size: 1970 bp). Images generated using SnapGene software*




**(a)**




**(b)**

**Fig. 2** *Genetic map of pPICZ A_Pir1_Dcr2 Plasmid and pPICZ A_Pir1_R2D2 Plasmid. The genetic map of pPICZ A_Pir1_Dcr2 Plasmid is created by inserting of these three fragments (Pir1_PCR_FRAGMENT, Dcr2_PCR_FRAGMENT1 and Dcr2_PCR_FRAGMENT2) where green – Pir; red - Dcr2 fragment1 and blue - Dcr2 fragmen2t. Similarly, the genetic map of pPICZ A_Pir1_R2D2 Plasmid is created by inserting of these two fragments (Pir1_PCR_FRAGMENT2, R2D2_PCR_FRAGMENT) where green - Pir1; yellow - R2D2*

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The produced effectiveness of protein against illnesses like dengue fever, transmitted by mosquitoes, warrants further investigation. Paratransgenesis has several advantages over traditional vector control methods; for instance, modified symbiotic bacteria can colonize various insect strains, making them a versatile tool in disease control [16]. Additionally, generating transformed microbes is typically simpler and more cost-effective than modifying mosquitoes. However, this approach is still in its infancy, requiring further study to maximize its effectiveness [22].

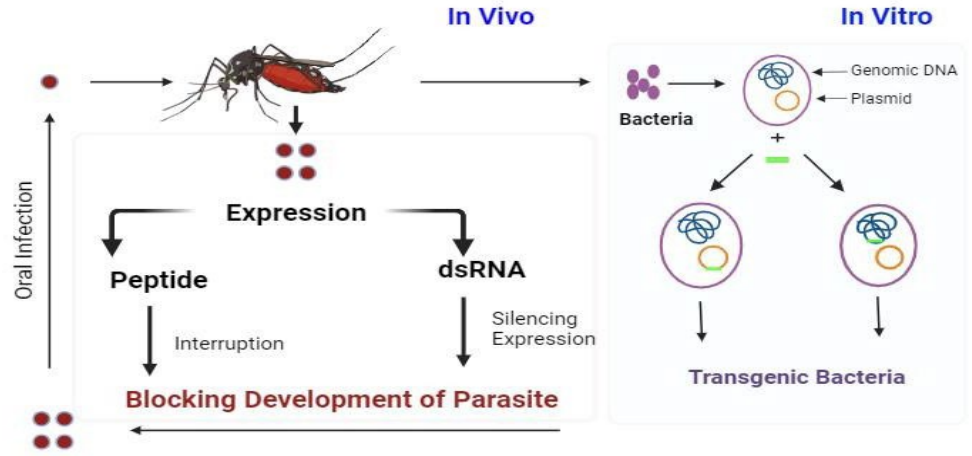


**Fig. 3** *Whole process of paratransgenesis*

Fig. 3 and Fig. 4 provide broader context for paratransgenesis as a strategy against diseases like malaria. While our work focuses on yeast engineering, the principles of genetic modification and potential applications of produced proteins in disease prevention are critical. Fig. 3 outlines the paratransgenic approach, and Fig. 4 emphasizes role of *Wolbachia* in altering mosquito populations to hinder malaria transmission [30].

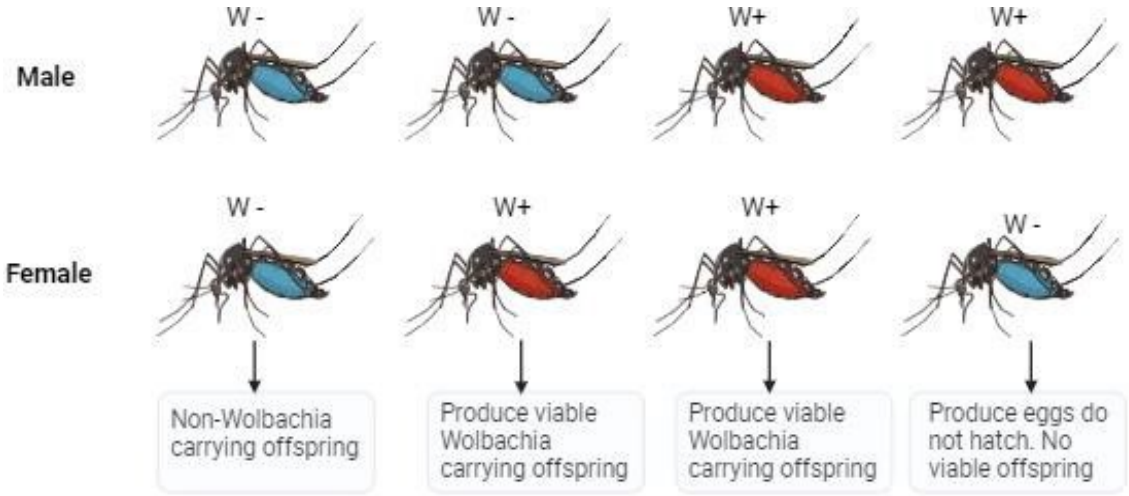


**Fig. 4** *Cytoplasmic incompatibility of mosquitos*

Furthermore, the manipulation of symbiotic bacteria such as Wolbachia allows female carriers to produce live eggs, enabling transfer from mother to offspring. Releasing males carrying Wolbachia into specific areas can decrease Aedes aegypti populations, as their eggs will not hatch, showcasing the versatility of genetic modifications for disease prevention. Transgenic mosquitoes expressing antibodies or RNA interference pathways targeting disease-causing microorganisms represent another promising method, although concerns about long-term efficacy and gene dissemination among wild populations remain [22].

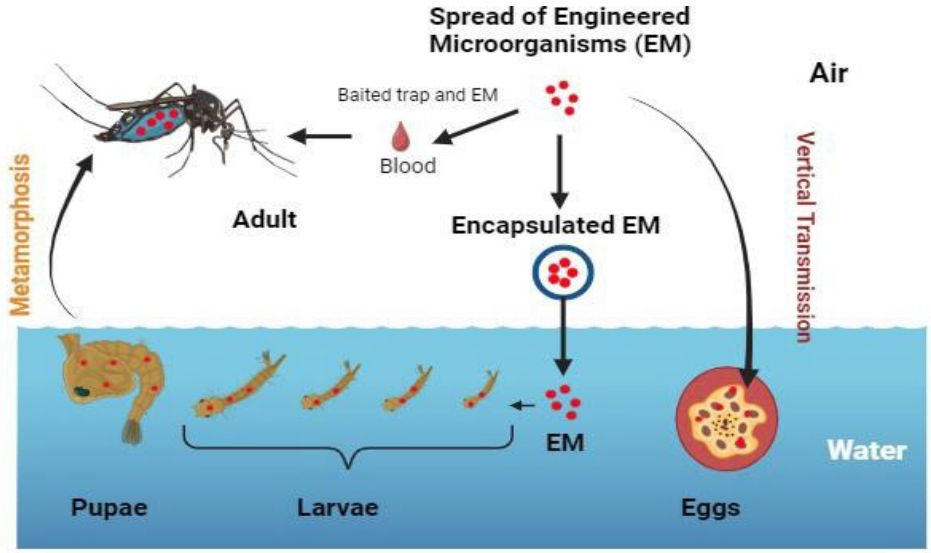


**Fig. 5** *Spread process of engineered yeast microorganisms*

Fig. 5 illustrates the transmission of genetically modified organisms (GM) to wild mosquitoes. Engineered microorganisms (EM) can be supplied to winged adults via baited traps or introduced into water for aquatic juveniles. Female carriers can vertically transmit EM, infecting eggs placed on land. Ideally, the bacterial species should develop through various life stages or spread horizontally within the host, allowing EM to exist both permanently and cyclically in the environment [30].

## 4. Conclusion

This study presents a novel approach to dengue management through the use of engineered bacteria, offering several advantages over traditional strategies like insecticides and mosquito nets. Unlike insecticides, which indiscriminately kill beneficial insects, engineered bacteria can be specifically targeted at mosquitoes that carry the dengue virus. Additionally, these bacteria may persist in the environment longer than pesticides, which degrade quickly [31]. However, there are critical concerns that need to be addressed before widespread implementation. Potential ecological impacts on mosquito populations and the environment must be carefully considered [32]. Furthermore, there is a risk that the dengue virus could evolve resistance to the antiviral compounds produced by these modified bacteria, potentially reducing their effectiveness over time [18]. Despite these challenges, developing plasmids for the midgut microbiota of mosquitoes represents an innovative strategy for dengue control. With further research and optimization, this approach could become a powerful tool in combating this serious disease. In this study, genetic cassettes were developed for the surface display of the Dcr2 or R2D2 proteins in the cell wall of Saccharomyces cerevisiae. The plasmids created facilitate the introduction of any target gene, allowing for the monitoring of expression via a regulatable promoter [33]. The produced proteins play a crucial role in degrading viral RNA through the siRNA pathway, utilizing established yeast protein synthesis methods. Depending on the presence of a binding site for the target protein, the *S. cerevisiae* cell wall can express the protein, or it can be secreted via the secretory pathway of cell. Ultimately, this research aims to streamline the insertion of target genes into genetic cassette of a novel plasmid [30], making it a valuable resource for future studies. The findings contribute to the development of efficient plasmids and enhance understanding of the process, paving the way for innovative solutions in disease management.

## Acknowledgement

The authors acknowledge the support of Research and Innovation Center, Khulna University, Khulna and The Ministry of Education, Bangladesh for supporting this research work.

## Conflict of Interest

Authors declare that there is no conflict of interests regarding the publication of the paper.

## Author Contribution

*The authors confirm contribution to the paper as follows:* ***study conception and design:*** *Rima Akter; Sheikh Shahjada Sangit, Sk Amir Hossain****; data collection:*** *Sheikh Shahjada Sangit;* ***analysis and interpretation of***

***results:*** *Rima Akter; Sheikh Shahjada Sangit; Sk Amir Hossain;* ***draft manuscript preparation:*** *Sheikh Shahjada Sangit. All authors reviewed the results and approved the final version of the manuscript.*

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