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Molecular Cloning and Recombinant Protein Expression of Hypoallergen Der p 2/1S in *Saccharomyces boulardii*

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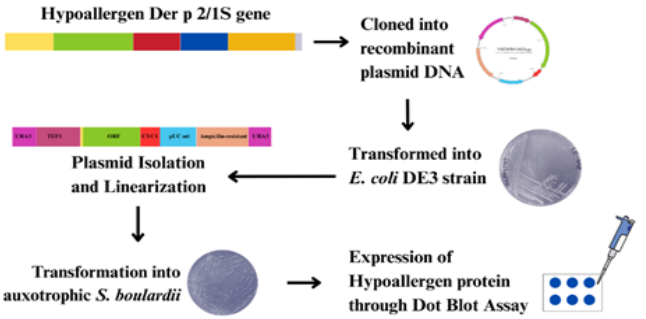
Graphical Abstract



Abstract

Der p 1 and Der p 2 are major allergens from the house dust mite *Dermatophagoides pteronyssinus* that trigger allergic sensitization of up to 90% of atopic patients. The recombinant mosaic hypoallergen Der p 2/1S derived from Der p 1 and Der p 2 allergens, has been reported to reduce IgE-reactivity and thus a good candidate for allergy prophylaxis. This study aimed to construct and express the recombinant Der p 2/1S hypoallergen protein in *Saccharomyces boulardii* (Sb) to generate a potential oral prophylactic agent for HDM allergies. The pYIP-rDerp2/1S plasmid, carrying codon optimized Der p 2/1S tagged with α-factor and his-tag, was successfully constructed and verified through Stu1 restriction enzyme digestion and DNA sequencing. Transformation into the *S. boulardii* genome was confirmed by PCR of the hypoallergen gene. Growth kinetics analysis revealed an extended lag phase in recombinant Sb-Derp2/1S compared to the control; however, both reached similar biomass yields. Dot blot immunoassay verified the successful expression of the His-tagged Der p 2/1S protein in transformed clones. These results demonstrate the stable genomic integration and functional expression of the recombinant. Future studies may focus on evaluating the immunological properties of the genetically engineered Sb expressing hypoallergen protein against allergic diseases.

Keywords: *Saccharomyces boulardii*, Der p 1, Der p 2, recombinant proteins, hypoallergen

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Introduction

The prevalence of allergic diseases caused by house dust mites (HDM) allergens continuously increases globally, affecting up to 50% of atopic individuals [1, 2, 3]. Group 1 and group 2 HDM allergens, especially those from *Dermatophagoides pteronyssinus* (Dp), Der p 1 and Der p 2, are significant triggers of allergic symptoms affecting up to 90% among Dp allergic patients [4, 5]. Allergic patients of various ages, from children to the elderly, could experience life-burdening symptoms such as eczema, atopic dermatitis, or airway diseases like asthma and rhinitis, as well as rhinoconjunctivitis, or in worst cases, the life-threatening anaphylactic shocks [6, 7].

Recombinant and native allergens such as Der p 1 and Der p 2 are used in allergy-specific immunotherapy (AIT) [8, 9]. Despite the convincing efficacy of AIT, it is associated with adverse side effects in some patients while others developed systemic side effects [8]. The clinical manifestation of these allergens has not been extensively evaluated, as this may cause life-burdening symptoms to patients undergoing AIT [10, 11].

Henceforth, hypoallergens control the mechanism of allergen immunotherapy. The construction of hypoallergen revolves around modifying the IgE-binding epitopes [12, 13] to reduce IgE-mediated immune response. Moreover, several in vitro studies have shown that hypoallergen constructs induce specific IgG without increasing allergen-specific IgE responses [13, 14]. As an example, the hypoallergen Der p 2/1S is a mosaic protein of Der p 1 and Der p 2 fragments with a documented reduction of IgE binding activity, providing potential application in the development of allergy prophylaxis and AIT [14].

As interesting as the production of hypoallergens for AIT, the potential protective role of probiotics in allergic responses has been shown in several studies in the past decade [13]. Probiotics could modulate immune responses in atopic individuals through the downregulation of Th2-biased responses observed in allergic reactions and the subsequent upregulation of Th1 responses [13]. Thus, probiotics became a strategic tool in AIT in conjunction with hypoallergen expression. *Saccharomyces boulardii* (Sb), a safe and FDA-approved probiotic as an oral health supplement, is currently used as a vehicle to express and deliver recombinant proteins due to its stability in delivering and secreting proteins to the gut [15, 16, 17]. Likewise, *Saccharomyces boulardii* is generally regarded as safe (GRAS) for consumption, thus ideal in in vivo animal and in clinical studies [16].

We describe in this study the cloning and expression of the hypoallergen Der p 2/1S in *Saccharomyces boulardii* which could be used in the development of an oral prophylaxis for allergic reactions as well as in AIT.

Materials and methods

Design, Construction and Cloning of pYIP-Derp2/1S Plasmid. The recombinant plasmid DNA carrying the hypoallergen Der p 2/1S was constructed using the Vector Builder (Chicago, USA). The 1074 bp hypoallergen Der p 2/1S nucleotide sequence which was codon optimized for *Saccharomyces* sp. S288c, was cloned into a Yeast Protein Expression Integrating Plasmid (pYIP) vector. The plasmid construct contains a translation elongation factor EF1 alpha promoter sequence (TEF1), Cytochrome c Isoform 1(CYC1) terminator, Kozak initiator sequence, pUC origin of replication, ampicillin resistance gene, and a URA3 gene. The open reading frame was constructed with an α-factor signal peptide at the N-terminus and a 6-histidine tag at the C-terminus. The recombinant plasmid DNA was transformed into an *E.coli* DE3 strain, and transformants were selected in LB Ampicillin plates.

Verification of Derp2/1s Insert in pYIP Plasmid. The *E. coli* transformant was cultured for 16 hours, and the recombinant plasmid DNA was isolated and purified using the ZymoPURE Plasmid MiniPrep Kit (ZymoResearch, USA) according to the manufacturer's centrifugation protocol. Polymerase chain reaction (PCR) was performed using designed primers that amplified only 1238 bp of the ORF containing the hypoallergen gene using a 20 bp forward primer, CCTCCGCATTAGCTGCTCCA, and a 25 bp reverse primer, ACTACCATGTTGACTAGCAGAATCA. The PCR master mix was prepared using 12.5 µL of 1x GreenTaq (Solarbio, Inc.), 1 µL of 0.4 µM Reverse Primer, 1 µL of 0.4 µM Forward primer, 2 µL of purified plasmid, and 10 µL of nuclease-free water. The PCR tubes were loaded into the thermal cycler with the following parameters: initial denaturation set at 90°C for 2 minutes, denaturation at 90°C for 1 minute, annealing at 55°C for 1 minute, elongation at 72°C for 1 minute, and final elongation at 4°C, with a total of 30 cycles. Ten microliters of PCR amplicons were resolved on 1.5% Agarose gel electrophoresis for 1 hour at 100V. A 1 kb Plus DNA Ladder (Vivantis) is used to determine the molecular weight of the plasmid. In addition, the recombinant plasmid was also verified by DNA sequencing (Vector Builder).

Der p 2/1S construct Transformation in S. boulardii. The purified plasmid was linearized by a StuI restriction enzyme, (New England Biolabs) through 1-hour incubation of 3 µL of plasmid DNA (1 µg/µL), 3 µL of StuI, 5 µL of NEB Buffer and 9 µL of nuclease-free water. The RE digestion product was transformed into auxotroph mutants of *S. boulardii*, M2 strain, (Sb-M2) using the optimized lithium acetate transformation method [18].

Overnight cultures of Sb-M2 were inoculated into a fresh medium of Yeast Peptone Dextrose (YPD) broth and incubated for 4-6 hours until the culture reached the 0.6-0.8 optical density at 600 nm. Approximate 4x107 CFU/mL of Sb-M2 were collected, then added with 240 µL of 50% polyethylene glycol (PEG), 50 µL of denatured salmon sperm, 32 µL of RE digested plasmid and 36 µL of 1.0 mM of lithium acetate (LiAc). The mixture was incubated for 30 minutes at 42°C, then 40 µL of 5% of dimethyl sulfoxide (DMSO) was added and boiled again for 30 minutes at 42°C in a water bath. The yeast cells were plated in a synthetic defined uracil-deficient agar media (SD-URA) at 30°C for 3-5 days. The growth of colonies confirmed the positive transformation of plasmid onto the Sb-M2, hence successful transformants were called Sb-Derp2/1S.

The colonies of the Sb-Derp2/1S were recultured onto a separate plate to obtain multiple clones for further use. The genomic DNA from the 72-hour culture of Sb-Derp2/1S was isolated using YeaStar Genomic DNA Kit (ZymoResearch, USA) according to the manufacturer's instructions. Then, isolated genomic DNA was amplified through PCR and resolved in a 1% agarose gel electrophoresis using the same parameters described earlier.

Detection of Recombinant Der p 2/1s Expression in S. boulardii by Dot Blot Assay. Cultures of Sb-Derp2/1S and Sb-expressing Rab7 clones were prepared. Cell lysis was performed using 120 µL of YD Digestion Buffer and 5 µL of Zymolyase (ZymoResearch) and incubated for 1 hour at 37°C, then followed by the addition of YD Lysis Buffer and 1 µL of 100 mM of phenylmethylsulfonyl fluoride (PMSF). The lysates were blotted onto a nitrocellulose membrane (Solarbio, Inc.) in duplicates. Blocking the blotted membrane was performed with 5% skimmed milk in 1X PBS-T for 1 hour at room temperature with gentle shaking. The detection was done using an anti-histidine antibody (Solarbio, Inc.) diluted for 1:2000 in a blocking buffer and then incubated for 16 hours at 4C in a shaker. Afterward, the membrane was washed three times with 1X PBS-T. The blots were then incubated for 1 hour with anti-Human IgG-HRP conjugated (Solarbio, Inc.) antibody diluted for 1:3000X in 1X PBS-T. Positive signals were detected using 3,3′,5,5′-tetramethylbenzidine (TMB) substrate with 1 hour incubation.

Growth Kinetic Analysis of Sb-transformant. Clones of Sb-Derp2/1S and Sb-M2 were cultured until they reached the optical density of 0.3 in 600 nm, then 1 mL of the cultures were inoculated into 50 mL SD-URA broth for Sb-Derp2/1S and uracil-sufficient synthetic defined broth (SD) medium for Sb-M2 in three replicates. They were incubated at 30°C at 200 rpm for 72 hours. The cultures were measured every 3 hours during incubation using a spectrophotometer at OD 600nm.

Results and Discussion

Der p2/1S Plasmid Construct for S. boulardii expression. The 1341 bp of ORF encoding Der p 2/1S hypoallergen was tagged with α-factor for recombinant protein secretion and a 6-histidine tag for purification of the expressed protein. This resulted in the formation of 5246 bp of pYIP-rDerp2/1S plasmid construct (Figure 1A) which demonstrates an effective strategy for recombinant protein expression for yeast strain. The plasmid contained regulatory elements necessary for stable expression in *S. boulardii*. The TEF1 promoter ensures strong and constitutive transcription, a CYC1 terminator for proper transcription termination, and the Kozak sequence for initiating eukaryotic translation. The construct included the URA3 gene for auxotrophic marker allowing for yeast transformation selection in uracil-deficient media and the StuI restriction enzyme site for linearization. It also contains pUC Ori to produce a high copy number of plasmid for plasmid maintenance and ampicillin resistance genes, facilitating the selection of *E. coli* transformant. The plasmid was further verified by linearization by the StuI restriction enzyme and resolved through gel electrophoresis (Figure 1B). The partial plasmid DNA sequence showed the TEF1 sequence, Kozak sequence, and the sequence of the open reading frame consisting of the α-factor, hypoallergen Der p 2/1S and the 6-histidine tag, which validated the accurate sequence of the plasmid (Figure 1B).

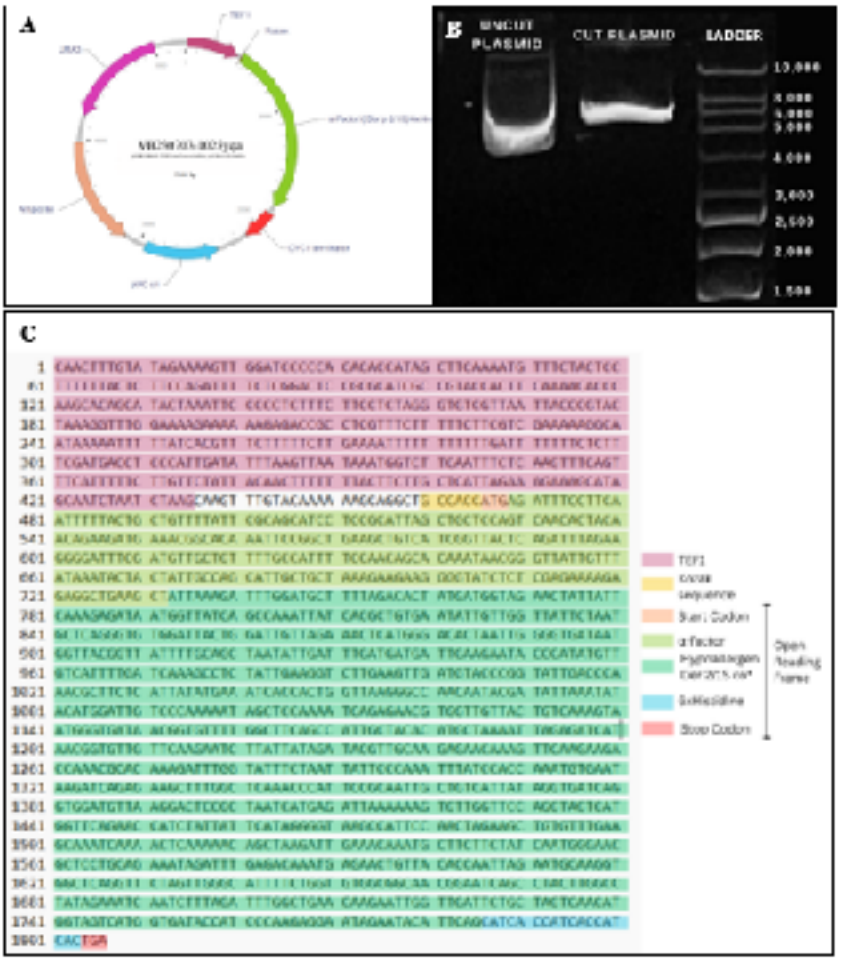

Figure 1. Schematic structure of the plasmid vector pYIP-rDerp2/1S construct
(A) with a total size of 5246 bp as confirmed through StuI linearization (B), and plasmid DNA sequence (C).

Using yeast integrating plasmids can overcome instability challenges, mainly the fluctuations in producing high copies of plasmids as well as plasmids that could be lost during cell division, issues associated with episomal plasmids [19]. Moreover, genetic engineering of yeast species traditionally used yeast integrating plasmids since it is suitable for large-scale production and long-term application [20].

Other studies use the TEF1 promoter more than other promoters, such as GAL1, since it is a strong constitutive promoter that drives levels of expression in yeast [21] and has a stronger activity even in glucose-exhausted phases during cultivation [17]. Moreover, the CYC 1 terminator provides mRNA stability through polyadenylation which is critical for protein translation.

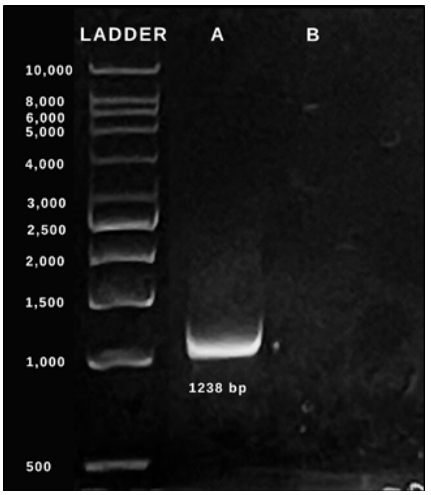

Figure 2. The 1238 bp of Der p 2/1S amplified from the positive transformant of *S. boulardii* confirms the positive integration of the plasmid and genome of the host.

While the Der p 2/1S sequence was codon optimized using *Saccharomyces cerevisiae,* the most widely characterized species of the genus *Saccharomyces* genetic manipulation and recombinant protein expression, the hypoallergen gene was successfully expressed in *S. boulardii* indicating shared codon usage between the two species [22].

Moreover, the linearization of the plasmid is a critical step, as it facilitates targeted integration into the yeast genome, since it integrates more efficiently into the yeast genome via homologous recombination, increasing the likelihood of stable expression [22].

Integration to S. boulardii genome. The plasmid linearization that cuts the URA3 gene using the StuI restriction enzyme facilitated the integration of the plasmid into the Sb-M2 genome via homologous recombination. The plasmid integration enabled the subsequent transformation of *S. boulardii* into recombinant Sb, hence Sb-Derp2/1S. Colonies on the plated SD-URA agar medium indicated a successful transformation. This was due to the URA3 gene allowing the strain to grow in a medium lacking uracil. PCR and gel electrophoresis confirmed the presence of Der p 2/1S hypoallergen gene in the positive transformants, Sb-Derp2/1S, but not in the genomic DNA of control, Sb-expressing Rab7 (Figure 2). The designed primers only amplified 1238 bp of the ORF that contains the Der p 2/1S hypoallergen gene.

Several studies have also used an auxotrophic strain of *S. boulardii* for genome integration and heterologous expression of recombinant proteins, hence confirming the effectiveness and feasibility of the host strain [17, 22, 23]. The utilization of the *S. boulardii* auxotrophic mutant strain, Sb-M2, made it possible to transform and integrate the plasmid into the genome through homologous recombination [24]. Homologous recombination is a well-established mechanism in yeast that enables precise genetic modifications by allowing exogenous DNA, like plasmid, to replace or insert into a target locus based on sequence homology [25].

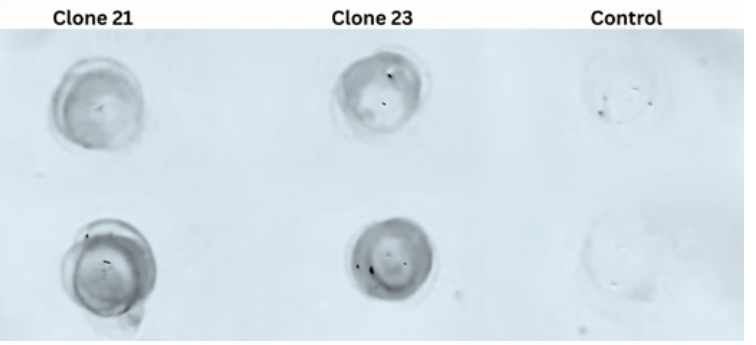

Figure 3. Dot blot assay identifying His-tag in the expressed protein of Sb-Der p 2/1S clone 21 (A) and clone 23 (B); and Sb-expressing Rab7 (C) as control.

Henceforth, the plasmid linearization through the Stu1 restriction enzyme produces sticky ends between the URA3 gene, allowing the plasmid DNA to bind into the genome of the auxotroph uracil-depleted gene strain, Sb-M2. This allows the positive transformant to grow in the uracil-deficient media, SD-URA. Moreover, the presence of hypoallergen Der p 2/1S in the genome of the positive transformant further verifies its successful integration.

Expression of Der p 2/1S Hypoallergen. The Der p 2/1S hypoallergen was expressed as a recombinant protein with 6-histidine-taig in the positive transformant, Sb-Derp2/1S (Figure 3). Dot blot immunoassay using anti-His antibodies showed positive expression of the His-tagged recombinant Der p 2/1S in two clones of Sb-Derp2/1S. The specificity of the presence of expressed protein was confirmed with the negative signal of Sb-expressing Rab 7 without a Histidine tag.

Dot blot assay is a highly specific immunoassay used for detection and verification of expressed proteins using antibodies [26]. The confirmation of the expressed protein is determined by using an antibody against the 6-histidine tag.

The Kozak consensus sequence optimized the activity of the TEF1 promoter which further enhances the initiation of protein translation, meanwhile, the presence of the α-mating factor enables the yeast to secrete recombinant proteins outside the cell [22]. The α-factor is a secretional signal that guides recombinant proteins through the yeast secretory pathway which involves processing and maturation of proteins then transported outside the cells via vesicles [27]. Moreover, codon optimization improved yields of protein expression [27].

Several studies have used *S. boulardii* to express recombinant proteins, such as GFP [24], ovalbumin [17], and leucocin C [22], regardless of limited genetic studies on genetic manipulation of *S. boulardii* [27] yet still confirms its possibility to produce recombinant proteins. The Sb-M2 strain has the potential to deliver and express recombinant proteins amidst selective pressure, which suggests the stability of the protein. [17].

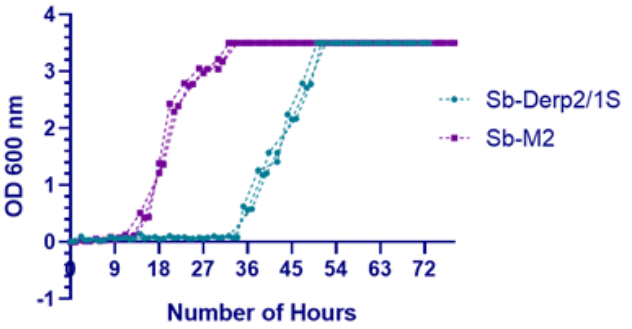

Figure 4. The growth curve of Sb-Derp2/1S and Sb-M2 was observed for 72 hours, measured in OD 600 nm, and cultured in synthetic defined broth media.

Growth Kinetics of Sb-transformant. The growth kinetics were performed for 72 hours to monitor and compare the growth of the positive transformant, Sb-Derp2/1S, to Sb-M2, as control. The growth curve indicates the delay of growth of Sb-Derp2/1S, showing a lagged onset of the exponential growth phase for the 36th hour compared to the Sb-M2 at the 15th hour (Figure 4). However, Sb-Derp2/1S met the maximum cell concentration, which read at an optical density of 3.5, in 18 hours, compared to Sb-M2 which is 21 hours. Both strains reached and remained at the maximum optical density until the 72nd hour, yet their growth curves are significantly different ($p = 0.02$).

The extended lag phase of Sb-Derp2/1S may suggest introducing metabolic stress, potentially affecting cellular adaptation to the growth environment. Previous studies have also shown that recombinant S. boulardii strains expressing heterologous proteins often experience altered growth rates due to variations in metabolic flux and secretion-associated stress [28, 22]. A similar observation of prolonged lag phase in transformant Sb-LecC yet yielded a lower cell density [22] unlike in Sb-Derp2/1S. Despite the delayed growth, Sb-Derp2/1S and Sb-M2 reached a comparable maximum optical density (OD) of 3.5 by the 72nd, indicating that the transformation did not impair the overall biomass yield.

Furthermore, the recombinant plasmid DNA was constructed incorporating key regulatory elements to effectively express the hypoallergen Der p 2/1S protein. Hence, a strong transcriptional promoter (TEF1) and a terminator (CYC1) were included to ensure efficient transcription, stable mRNA production, and to prevent degradation. Additionally, a Kozak sequence was introduced to enhance translation initiation, and an α-factor signal sequence to facilitate secretion of the expressed protein outside the cell, contributing to robust protein expression. *Saccharomyces boulardii* was selected as a host organism due to its capacity for post-translational modifications, which help stabilize the expressed protein [16]. Furthermore, *S. boulardii* expressing the hypoallergenic Der p 2/1S protein holds potential for in vivo applications, offering a promising avenue for investigating therapeutic effects on immune modulation and the alleviation of allergic symptoms.

Conclusion

The successful expression of the recombinant Der p 2/1S hypoallergen protein in *S. boulardii* was made possible through the yeast integrating expression system. The plasmid design further enhances the production and secretion of recombinant protein, likewise, the integration of the construct into the yeast genome aids in efficiently maintaining recombinant protein for long-term use. Meanwhile, growth kinetics analysis indicated a transient delay in the exponential phase of Sb-Derp2/1S, but ultimately, it reached a comparable biomass yield to the control strain, suggesting that transformation did not impair overall growth capacity. Dot blot immunoassay confirmed the presence of the His-tagged recombinant protein in positive transformants, verifying successful protein expression. These findings confirm that the auxotrophic mutant *S. boulardii* is a viable host for recombinant protein production, demonstrating stable genome integration and protein expression of Der p 2/1S hypoallergen. Future studies can test the efficacy and functional activity of the combination of probiotic *S. boulardii* and hypoallergen Der p 2/1S for therapeutic applications.

Acknowledgments

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization, Ramos JDA, and Austriaco NPG; Methodology, Ramos JDA, Austriaco, NPG, and Mendoza GR; Plasmid design, Ramos, JDA; Yeast transformation, Mendoza, GR.; Other experiment and data collection, Manalo AJI, Interpretation of data, Manalo AJI and Ramos, JDA; Manuscript writing, Manalo AJI and Ramos, JDA.

Institutional Review Board Statement

Not applicable.

Informed Consent Statement

Not applicable.

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