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In-silico Development of Novel Plasmid Construct for Production of Recombinant Chitinase in *Saccharomyces cerevisiae*

Rima Akter[1], Sk Amir Hossain[1]*

[1] *Biotechnology and Genetic Engineering Discipline, Khulna University, Khulna, 9208, BANGLADESH*

*Corresponding Author: skamir@bge.ku.ac.bd
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Abstract

This study presents the design and potential application of novel plasmid constructs for chitinase enzyme production in Saccharomyces cerevisiae. Using advanced molecular techniques in recombinant DNA technology, we constructed two unique plasmids—pESC-TRPGAL1, 10Chitinase2 and pESC-TRPPHO5chitinase2—specifically engineered to facilitate the insertion of the Candida albicans chitinase-encoding gene (CHT3) into yeast. The plasmid pESC-TRPGAL1, 10Chitinase2 incorporates a genetic cassette with GAL1 and GAL10 strong bidirectional promoters, a signal sequence, and a 6xHis tag. Conversely, pESC-TRPPHO5chitinase2 features the GAL1 promoter in one direction and the PHO5 promoter in the other, along with a signal sequence and 6xHis tag. The plasmid design enables the targeted expression of recombinant chitinase when transformed into S. cerevisiae, with functionality and activity potentially assessable via selective culture with 1–2% chitin media. This work emphasizes the innovation in plasmid construction for recombinant chitinase expression and establishes the groundwork for experimental validation, which will focus on assessing the activity of enzyme and thermostability relative to wild-type C. albicans chitinase.

1. Introduction

Chitin, a β-(1→4)-linked N-acetylglucosamine polymer, represents the second most abundant natural polymer after cellulose, playing crucial structural roles in fungal cell walls, insect exoskeletons, and crustacean shells [1]. The abundance and renewable nature of chitin make it an attractive target for industrial bioconversion applications, particularly in sustainable biotechnology [2]. Despite its structural stability, chitin does not accumulate extensively in natural environments due to the action of chitinolytic enzymes, primarily chitinases, which catalyze its degradation into bioavailable components as in Fig.1 [3]. These enzymes are classified into two main categories: endochitinases, which cleave internal glycosidic bonds, and exochitinases, which process the polymer from its termini [4]. The diverse applications of chitinases span multiple industries, from agricultural pest control to biomedical applications and environmental waste management [5].

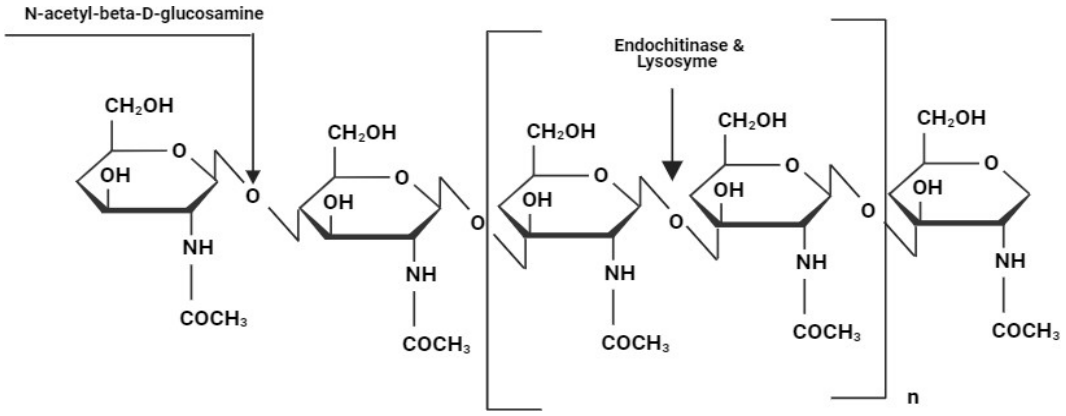

Fig. 1 *Action mechanism of chitinase*

Saccharomyces cerevisiae has emerged as a valuable platform for heterologous protein production, driven by its extensive genetic characterization, ease of genetic manipulation, and rapid growth rates, which facilitate large-scale applications [6]. Its GRAS (Generally Recognized as Safe) status further promotes its use, particularly for generating enzymes with applications in food, pharmaceutical, and environmental industries. Importantly, *S. cerevisiae* also performs eukaryotic post-translational modifications, a property that makes it advantageous over prokaryotic systems when producing complex eukaryotic proteins that require glycosylation, phosphorylation, or other modifications [7].

This research focuses on the expression of recombinant chitinase in *S. cerevisiae* via innovative plasmid designs aimed at maximizing gene expression efficiency and protein yield [8]. Utilizing two specific promoter systems—the *GAL1/10* and *PHO5* promoters—this approach allows precise control over the expression process under varying conditions [9]. The *GAL1/10* promoter, known for its strong and tightly regulated expression in the presence of galactose, enables high expression levels upon induction, making it ideal for large-scale, time-controlled production [10]. On the other hand, the *PHO5* promoter, which is sensitive to phosphate availability, offers an alternative regulation mode, potentially reducing production costs when phosphate levels can be modulated [11,12]. By leveraging both promoters, this dual strategy provides flexibility in controlling expression rates and adapting production to different industrial requirements.

The chitinase gene, *CHT3*, originating from *Candida albicans*, was selected due to its high catalytic efficiency on crystalline chitin, a challenging substrate with significant applications in agriculture and waste management [13]. The choice of *CHT3* aligns with prior research indicating the compatibility of *C. albicans* chitinase with protein folding and secretion pathways of *S. cerevisiae*, thus supporting efficient downstream purification and reducing processing complexity [14]. In our study, the recombinant plasmids were engineered to include tailored signal peptides that facilitate chitinase secretion into the culture medium, along with polyhistidine (His) tags, which enable simplified protein purification via affinity chromatography and facilitate activity assays [15].

This novel plasmid design represents a notable advancement, incorporating components optimized for stability and activity of the expressed chitinase in industrial settings. Signal sequences enhance protein transit through the secretory pathway, facilitating extracellular release, while His-tags offer robust tools for tracking enzyme functionality during production stages [16]. This approach addresses enzymatic efficiency and aligns with sustainable production goals, supporting broader applications of *S. cerevisiae* as an enzyme-producing platform. Furthermore, the engineered yeast strains present a sustainable approach to chitinase production, meeting objectives to reduce environmental impact and reliance on traditional extraction sources [17]. By optimizing genetic and bioprocess parameters, this study enhances utility of *S. cerevisiae* as a bio-manufacturing platform for chitinase and other bioproducts, advancing renewable biotechnology solutions [17].

2. Materials and Methods

2.1 Construction of pESC-TRPGAl1,10 Chitinase2 and pESC-TRPPHO5chitinase2

Two distinct plasmid vectors were engineered using the pESC-TRP backbone, chosen for its stability and high copy number in *S. cerevisiae*. The first construct, pESC-TRPGAl1,10 Chitinase2, incorporated both *GAL1* and *GAL10* promoters for coordinated expression control. The second construct, pESC-TRPPHO5chitinase2, featured a combination of *GAL1* and *PHO5* promoters, offering dual regulation through galactose induction and phosphate repression mechanisms. The plasmid designs were initially created and analyzed using SnapGene software, which enabled precise mapping of restriction sites and regulatory elements [18].

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The construction process began with the preparation of the pESC-TRP backbone through restriction digestion using appropriate enzymes for each promoter insertion. The *GAL1* promoter region was retained in both constructs, while the *GAL10* promoter was either maintained or replaced with the *PHO5* promoter sequence. Each construct was engineered to include a 6xHis tag for protein purification and appropriate signal sequences for efficient protein secretion [19]. The plasmid details and primers for each are summarized in Table 1 and Table 2, respectively.

Table 1 *List of primers used in this work*

Primer	Sequence	Length	GC Content (%)
CHT3_FOR1	GCGCGAATTCCATCATCACCATCACCACATAAACCATATTGCTGCTATTCACCAAGT	57	44
CHT3_REV1	GAGCTCCATCATCACCATCACCACCGAAACAAGACGAAACAAAACAACGAAC	52	46
CHT3_FOR2	CCCGGGCATCATCACCATCACCACATAAACCATATTGCTGCTATTCACCAAGT	53	47
CHT3_REV2	CCGCGGCATCATCACCATCACCACCGAAACAAGACGAAACAAAACAACGAAC	52	50

Table 2 *List of primers used in this work*

Primer	Sequence	Length	GC Content (%)
CHT3_FOR1	GCGCGAATTCCATCATCACCATCACCACATAAACCATATTGCTGCTATTCACCAAGT	57	44
CHT3_REV1	GAGCTCCATCATCACCATCACCACCGAAACAAGACGAAACAAAACAACGAAC	52	46
CHT3_FOR2	CCCGGGCATCATCACCATCACCACATAAACCATATTGCTGCTATTCACCAAGT	53	47
CHT3_REV2	CCGCGGCATCATCACCATCACCACCGAAACAAGACGAAACAAAACAACGAAC	52	50
PHO5_FOR	GCGCCACCGGTATGTTTAAATCTGTTGTTTATTCAATTTTAGCCGCTT	48	40
PHO5_REV	GCTTCGAACTATTGTCTCAATAGACTGGCGTTGTAATGA	39	41

2.2 Materials

The materials used in this study include the pESC-TRP vector, known for its dual-promoter functionality, employing *GAL1* and *GAL10* promoters to facilitate gene expression under galactose-inducing conditions [10]. Additionally, the *PHO5* promoter was incorporated, which enables regulated expression in response to varying phosphate levels, enhancing control over gene expression [12]. A 6x His tag was included to streamline protein purification and tracking throughout the production stages [15]. Custom primers were meticulously designed for PCR amplification, ensuring specificity for the target genes and compatibility with restriction enzyme sites, enabling seamless cloning [20]. Gel electrophoresis was employed to verify the size and purity of PCR products, allowing for quality control before further experimental steps and ensuring accuracy in gene insertion and cloning processes [20].

2.3 Primer Design

Primers were tailored for the *CHT3* gene to enable amplification and insertion at both ends of the plasmid for each configuration. For pESC-TRPGAl1,10 Chitinase2, primers were designed with SacI and EcoRI sites for CHT3_ FOR1 and CHT3_REV1 (for *GAL1*) and with XmaI and SacII sites for CHT3_FOR2 and CHT3_REV2 (for *GAL10*). For pESC-TRPPHO5chitinase2, *PHO5*-specific primers were synthesized with AgeI and BstBI restriction sites (PHO5_FOR and PHO5_REV). These restriction sites were used to replace *GAL10* with *PHO5* upstream of the chitinase gene. The primers exhibited GC content ranging from 40% to 50% and lengths of 38–57 nucleotides, ensuring reliable PCR amplification.

2.4 PCR Amplification

PCR was conducted to amplify the *CHT3* gene, using primers specific to each promoter insertion site. The reaction utilized an initial denaturation at 95°C, followed by amplification cycles optimized for both primer specificity and yield, with annealing temperatures varying between 59°C and 61°C. Cycle conditions were as follows:

Cycle 1: 5 minutes at 95°C; 29 seconds at 59°C; 20 seconds at 72°C
Cycle 2: 5 cycles of 45 seconds at 95°C; 29 seconds at 59°C; 20 seconds at 72°C
Cycle 3: 30 cycles of 45 seconds at 95°C; 29 seconds at 61°C; 30 seconds at 72°C.

Gel electrophoresis confirmed successful amplification of PCR products, producing expected fragment sizes.

2.5 Insertion of Amplified Fragments

Following PCR, the amplified *CHT3* fragments were purified and digested with respective restriction enzymes. For pESC-TRPGAl1,10 Chitinase2, the *CHT3* insert was ligated into the backbone using SacI and EcoRI for *GAL1* and XmaI and SacII for *GAL10*. In pESC-TRPPHO5chitinase2, the *PHO5* promoter replaced *GAL10*, inserted upstream of the chitinase gene using AgeI and BstBI restriction sites.

Gel electrophoresis was employed to verify the correct insertion of the *PHO5* promoter and chitinase fragment. SnapGene analysis confirmed the sequence orientation and complete assembly of both plasmids, enabling a reliable dual-promoter system with *GAL1* and *GAL10* (or *PHO5*) for controlled chitinase expression.

Table 3 *List of plasmids used in constructed plasmid*

Plasmid	Description	Origin/marker	Reference
pESC-TRP	Plasmid encoding gene under control of *GAL1,10* promoter	PpTRP2, AmpR	[5]
pESC-TRPGAl1,10 Chitinase2	Plasmid encoding gene under control *of GAL1,10* promoter	PpTRP2, AmpR	This work
pESC-TRPPHO5chitinase2	Plasmid encoding gene under control of *GAL1* and *PHO5* promoter	PpTRP2, AmpR	This work

3. Results and Discussion

The primary objective of this study was to construct a functional chitinase-expressing plasmid for transformation into *S. cerevisiae* through in silico methods, followed by predictive assessments of recombinant chitinase activity and stability. The recombinant plasmid design, informed by the activity of the *CHT3* gene from *C. albicans*, was chosen for its demonstrated efficiency in chitin degradation and secretion, which simplifies downstream enzyme purification processes [13]. Comparative analyses were performed between wild-type chitinase from *C. albicans* and the recombinant chitinase, focusing on conditions favorable to chitinase activity: incubation at 37°C, a slightly acidic pH of 5.5, and nutrient-rich media such as yeast extract or peptone [13].

To achieve optimal chitinase production, the *GAL1/10* promoter system was utilized to drive gene expression upon galactose induction, while the *PHO5* promoter was integrated to enable gene regulation based on inorganic phosphate levels, offering flexibility in controlling expression dynamics [10]. Two recombinant plasmid constructs, pESC-TRPGAL1,10-CHT3 and pESC-TRPPHO5-CHT3, were created, with pESC-TRPPHO5chitinase2 in particular providing a platform for fine-tuned chitinase expression in response to environmental factors like galactose and phosphate availability [12].

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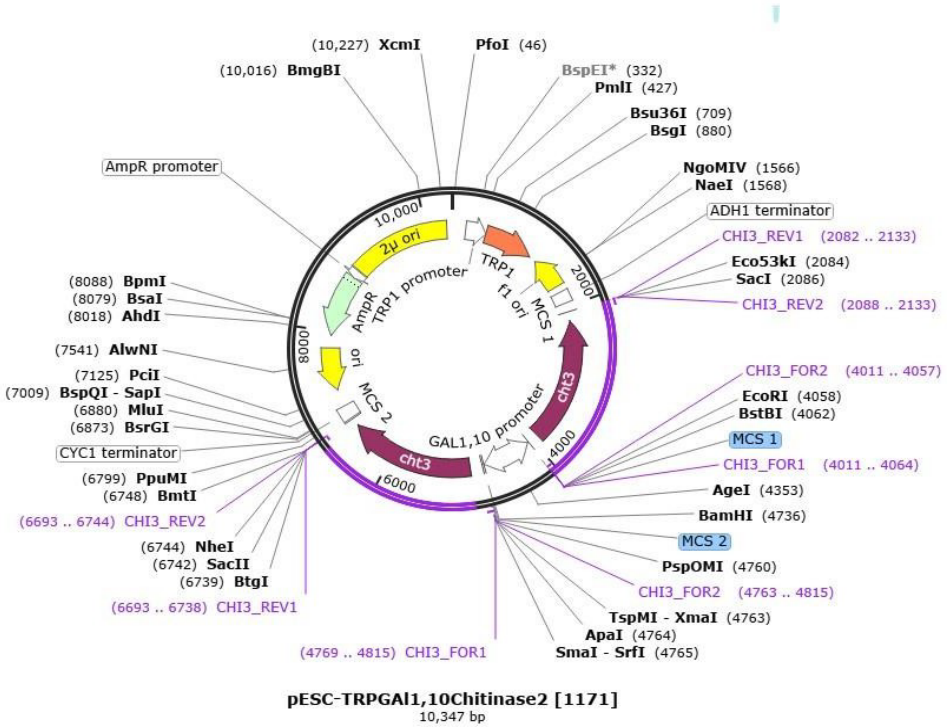

Fig. 2 *Genetic map of pESC-TRPGAl1,10 Chitinase2*

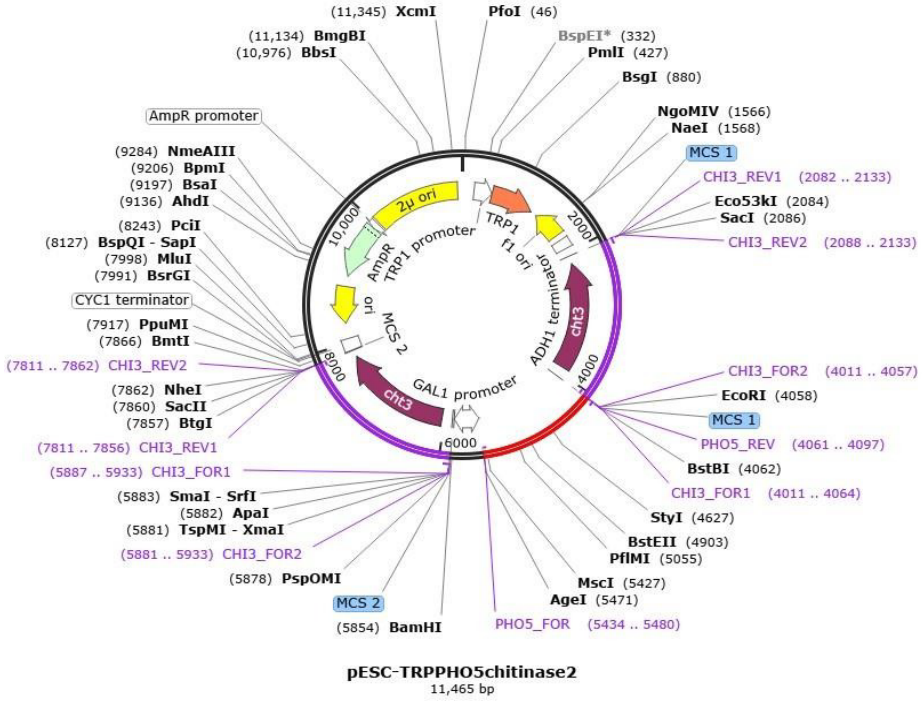

Fig. 3 *Genetic map of pESC-TRPPHO5chitinase2*

Fig. 2 and 3 display the genetic maps of these plasmids, with clear annotation of functional elements: the 2μ origin (yellow), *TRP1* (brown), *GAL1/10* (white), and the *CHT3* gene (purple) in pESC-TRPGAL1,10-CHT3, and an added *PHO5* promoter region (red) in pESC-TRPPHO5chitinase2. Each plasmid was designed in SnapGene and exported in compatible formats, including FASTA and GenBank, to facilitate further computational analyses and bioinformatic applications [11].

Simulated gel electrophoresis of restriction-digested DNA fragments (Fig. 4) was conducted using SnapGene's in silico modeling. This simulation predicts the fragment patterns expected in experimental conditions and provides preliminary validation of enzyme digestion and PCR fragment sizing. For example, the *CHT3* gene fragment (1982 bp) was digested using EcoRI and SacI, while the *PHO5* fragment (1423 bp) was digested with AgeI and BstBI, resulting in specific banding patterns that will guide future in-lab validations [12]. The SnapGene simulation offers a reliable, high-clarity visualization of DNA fragment sizes and electrophoresis patterns in a controlled digital environment. Nonetheless, physical gel electrophoresis in laboratory settings is advised for final confirmation [18].

In the in silico model, simulated environmental conditions demonstrated that chitinase expression in *S. cerevisiae* can be optimized through *GAL1/10* activation by galactose, producing a noticeable increase in recombinant chitinase [11]. This result aligns with previous findings where galactose induction substantially increased protein expression through the *GAL1/10* promoter system. Furthermore, protein yield could be modulated by adjusting inorganic phosphate (Pi) levels, with elevated Pi levels repressing *GAL1/10* expression and reducing overall chitinase output [14]. Optimal protein expression was achieved at pH 5.5, supporting both yeast growth and chitinase production [21]. Temperature settings of 30°C were maintained across simulations to enhance enzyme activity and support yeast cell proliferation [22].

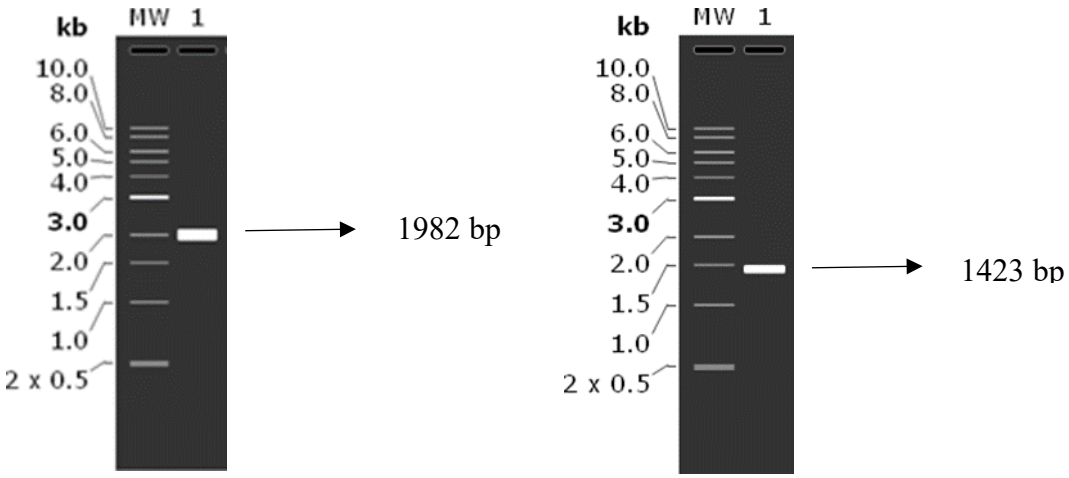

Fig. 4 *Simulated gel electrophoresis using SnapGene: (a) CHT3 gene fragment (1982 bp) digested with EcoRI and SacI; and (b) PHO5 gene fragment (1423 bp) digested with AgeI and BstBI*

Fig. 4(a) and 4(b) present the gel electrophoresis simulations of key fragments. Fig. 4(a) demonstrates the *CHT3* fragment digested with EcoRI and SacI, showing a clear 1982 bp band, while Fig. 4(b) displays the *PHO5* fragment digested with AgeI and BstBI, with a distinct 1423 bp band. The in silico gel simulations were carefully positioned and arranged to enhance layout clarity and provide an orderly visual reference within the manuscript. This tidy and clear presentation ensures that the simulated gel results effectively support the narrative structure of the paper.

The SnapGene platform streamlined the design, construction, and preliminary validation of the plasmids, offering a comprehensive in silico approach that reduces reliance on experimental trial and error and supports data reproducibility [18]. The plasmid designs, activity predictions, and environmental response simulations highlight *S. cerevisiae* as an efficient bio-manufacturing host for chitinase, supporting potential applications in eco-friendly industries such as textile manufacturing [23]. This study demonstrates the potential of combining genetic modifications and computational modeling to establish *S. cerevisiae* as a robust platform for sustainable chitinase production, providing a valuable foundation for future experimental and practical applications in biotechnology and industry [24].

4. Conclusion

This study has successfully demonstrated the use of *S. cerevisiae* as a robust and efficient host for the production of chitinase through the construction of two targeted plasmid systems: pESC-TRP/GAL1,10Chitinase2 and pESC-TRPPHO5Chitinase2. The application of inducible promoters—*GAL1/10*, which initiates chitinase expression upon galactose addition, and *PHO5*, which responds to inorganic phosphate (Pi) levels—enabled a controlled and scalable enzyme production system, as previously reported in the literature [12, 16]. By regulating enzyme yield in response to specific environmental conditions, such as galactose and Pi availability, these plasmids offer a versatile approach for optimized enzyme expression in yeast.

Through in silico simulation, the study confirmed optimal conditions for maintaining stable protein expression and activity in yeast, specifically at a neutral to slightly acidic pH (~7.0) and a temperature range of 25–37°C [7]. The SnapGene software allowed for a detailed simulation of enzyme restriction, digestion, and electrophoresis patterns, which provided a preliminary validation of the recombinant plasmid constructs, including the specific banding patterns expected from each gene fragment under restriction enzyme digestion [18]. These simulations not only streamlined the initial validation process but also provided a high-clarity digital visualization of expected outcomes, laying the foundation for subsequent laboratory validation.

The findings underscore the potential of yeast as a viable and adaptable host for large-scale chitinase production, meeting industrial needs for eco-friendly enzyme production with minimal cost implications [16]. Future work will include experimental validation of in silico predictions through laboratory-based gel electrophoresis, activity assays, and yield optimization studies. This approach could enhance practical applications, such as sustainable biotechnology, agriculture, and waste management, by providing an efficient enzyme production method using recombinant yeast systems. The outcomes of this research provide a valuable framework for developing enzyme production processes tailored to environmental and industrial requirements, and further improvements could expand the applications of recombinant chitinase across various sectors [17].

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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, data collection and draft manuscript preparation:*** *Rima Akter;* ***analysis and interpretation of results:*** *Sk Amir Hossain; Rima Akter; All authors reviewed the results and approved the final version of the manuscript.*

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