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Optimization of methanol-induced expression and His-tag purification of *Saccharomycopsis fibuligera* R64 mutant α-amylase in *Pichia pastoris*

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ABSTRACT The Sfamy R64 α-amylase mutant from *Saccharomycopsis fibuligera* was expressed in *Pichia pastoris* to explore its industrial potential. The gene encoding the mutant enzyme was cloned into the pPICZαA vector and transformed into *P. pastoris* SMD1168. Optimal expression was achieved at 1.5% methanol concentration, with the highest enzyme activity observed at 48 h, reaching 24.06 U/mL. The recombinant protein was purified using Ni-Sepharose affinity chromatography in native and denaturing conditions. The native conditions retained higher protein integrity and activity, while the denaturing process resulted in partial degradation. Molecular dynamics (MD) simulations conducted to assess the structural stability of the His-tagged Sfamy R64 α-amylase mutant and its interaction with the maltose substrate. The simulation confirmed the stable binding of maltose in the active site and the solvent accessibility of the His-tag, supporting its effectiveness in affinity chromatography. The RMSD, RMSF, and time-evolution snapshots demonstrated that the protein remained structurally stable over 100 ns at an optimum temperature of 50 °C. The findings suggest that the Sfamy R64 mutant α-amylase is a promising candidate for industrial applications, combining high expression yields, efficient purification, and stable enzyme-substrate interactions. The results offer a strong basis for further optimization and large-scale enzyme production.

KEYWORDS Affinity chromatography; MD simulation; Methanol induction; *Pichia pastoris*; Sfamy R64 mutant

1. Introduction

α-Amylase is a crucial enzyme with extensive applications, particularly in the starch industry and biomedical fields. It catalyzes the hydrolysis of starch at α-1,4-glycosidic linkages, producing maltodextrins, glucose, and other oligosaccharides, which are essential intermediates in the production of syrups, sweeteners, and modified starches. In the pharmaceutical industry, α-amylase is used as a digestive aid and for its anti-inflammatory properties. Its versatility extends to food processing, textiles, and detergents (Gopinath et al. 2017; Abdel-Mageed et al. 2019).

In Indonesia, *Saccharomycopsis fibuligera* R64 strain has been identified as a yeast-producing α-amylase (Sfamy R64) with superior enzymatic activity compared to other strains. Recent advances have focused on developing mutant variants of α-amylase, engineered to enhance specific properties such as substrate binding and catalytic efficiency. One such mutant, Sfamy R64 mutant, has been modified in silico to improve its binding capacity to starch, making it an ideal candidate for further study (Baroroh et al. 2019).

Recombinant protein expression systems are fundamental in biotechnology, with *P. pastoris* emerging as a popular choice due to its eukaryotic protein folding mechanisms, rapid growth, and cost-effectiveness. The gene encoding this Sfamy R64 mutant has been constructed in pPICZαA vector to express the recombinant protein in *P. pastoris* SMD1168 (Baroroh et al. 2022). This yeast is particularly suited for producing recombinant proteins guided by the highly inducible AOX1 promoter, which methanol activates. Methanol not only serves as a carbon source but also acts as an inducer for protein production (Love et al. 2016; Kielkopf et al. 2021).

P. pastoris expression system offers numerous advantages (Sembiring et al. 2024). These include the ease with which its genes can be manipulated, the availability of efficient vectors such as the tightly regulated AOX1 (alcohol oxidase) promoter, and its ability to produce correctly folded proteins through the construction of disulfide bonds and post-translational modifications (Byrne 2015;

Mohanty et al. 2023). Therefore, the *P. pastoris* expression system is an optimal tool for laboratory-based studies and potential industrial applications (Ahmad et al. 2014). The Sfamy R64 mutant, which contains three disulfide bonds and undergoes post-translational modifications (Hasan et al. 2008), is highly compatible with this system, making it a strong candidate for producing the recombinant enzyme.

During *P. pastoris* fermentation, methanol serves multiple roles as an inducer, energy source, and carbon source for protein expression (Kielkopf et al. 2021; Unver and Dagci 2024). AOX initiates the methanol utilization pathway by converting methanol into formaldehyde and hydrogen peroxide. Catalase then decomposes the hydrogen peroxide into water and oxygen, while formaldehyde is further oxidized into formate and carbon dioxide, supplying energy for cell growth. Among the two AOX genes in *P. pastoris*, AOX1 plays the primary role, being tightly regulated and strongly induced by methanol. Using the correct methanol concentration is crucial to optimizing recombinant protein expression (Gasser et al. 2015; Abed 2024).

In this study, we explore various methanol concentrations to determine the optimal conditions for maximizing Sfamy R64 mutant α-amylase expression in *P. pastoris*. Since this new mutant has not been previously optimized, the optimization process is a crucial step to ensure its successful production. In addition, recombinant proteins often include a His-tag to ease purification through immobilized metal affinity chromatography (IMAC). The His-tag's accessibility to solvents can significantly influence the efficiency of purification. This study also employed molecular dynamics (MD) simulations to assess the solvent exposure of the His-tag. Further, we compared the purification of Sfamy R64 under both native and denaturing conditions to determine which purification method is more effective for the characteristics of the Sfamy R64 mutant protein.

2. Materials and Methods

2.1. Strain, plasmid, and gene construction

The *E. coli* TOP10F' host cell was sourced from Merck (Darmstadt, Germany); the *P. pastoris* SMD1168 host cell was purchased from Thermo Scientific (Vilnius, Lithuania). At the same time, the plasmid pPICZαA-sfamy-mutant was synthesized from Genscript (Nanjing, China). Cloning and cell growth in *E. coli* were conducted using Low Salt Luria-Bertani (LSLB) medium supplemented with 10 µg/mL tetracycline from Merck (Darmstadt, Germany), while cloning and cell growth in *P. pastoris* were conducted using YPD medium supplemented with 100 µg/mL zeocin from Thermo Scientific (Vilnius, Lithuania).

The Sfamy R64 mutant α-amylase gene was cloned into the pPICZαA vector under the control of the AOX1 promoter for expression in *P. pastoris*. The gene was designed to include a His-tag to facilitate subsequent purification. The construct was first propagated in *E. coli* TOP10 cells following the $CaCl_2$-heat shock method (Green and Sambrook 2012; Tantray et al. 2023). After confirming the plasmid by PmeI restriction digestion, the pPICZαA-Sfamy R64 mutant was ready for transformation into *P. pastoris* SMD1168.

2.2. Transformation of pPICZαA-Sfamy-mutant into P. pastoris

Transformation into *P. pastoris* SMD1168 was performed by electroporation, following the protocol from the Eppendorf transformation protocol. The pPICZαA-Sfamy R64 mutant plasmid was linearized using PmeI to enhance its integration efficiency into the yeast genome. Linearized plasmid DNA with approximately 5–10 µg was introduced into electrocompetent *P. pastoris* cells. After electroporation (Eppendorf), the cells were incubated in YPDS medium and plated on YPDS plates containing 100 µg/mL Zeocin™ to select transformants. Transformants were confirmed using agarose gel electrophoresis and restriction digestion.

2.3. Methanol induction for Sfamy R64 mutant expression

Positive *P. pastoris* transformants were grown in BMGY medium (2% peptone, 1% yeast extract, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base (YNB), 4×10^{-5}% biotin, 1% glycerol) until the OD_{600} reached 12–15. Cells were collected by centrifugation at 3000 × g for 5 min at room temperature and then resuspended to 10 mL in BMMY medium (similar to BMGY but containing a carbon source, methanol). Recombinant protein expression was induced by adding methanol at varying concentrations: 0.75%, 1%, and 1.5%. Every 24 h, methanol was added to sustain induction for up to 144 h. Culture samples were taken at specific times (24, 48, 72, 96, 120, and 144 h) to measure α-amylase activity and protein production. The protein expression was characterized by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

2.4. Sfamy R64 mutant activity assay

The starch-iodine method assessed the α-amylase activity before protein purification (Fuwa 1954). In this assay, the enzyme was obtained as the total expressed protein present in the culture medium. The enzyme's ability to hydrolyze starch was controlled by the decrease in the intensity of the blue starch-iodine complex. The absorbance was measured at 600 nm, and one unit of α-amylase activity was labeled as the amount of enzyme that caused a 10% decrease in absorbance under the assay conditions. The enzyme activity was evaluated at various methanol concentrations and time points to determine the optimal induction conditions.

2.5. His-tag protein purification

The soluble Sfamy R64 mutant α-amylase was purified using Ni-Sepharose affinity chromatography in both native and denaturing conditions. For purification under native conditions, the culture supernatant from methanol-induced *P. pastoris* was processed using Buffer A, consisting of 0.02 M sodium phosphate and 0.5 M NaCl at pH 7.0, to equilibrate the column. After applying the sample, unbound proteins were washed off with Buffer A, and the target protein was eluted using Buffer B, which contained 0.02 M sodium phosphate, 0.5 M NaCl, and 0.5 M imidazole at pH 7.0. For denaturing conditions, the process was modified to include 8 M urea in both Buffer A and Buffer B to solubilize inclusion bodies and improve His-tag accessibility. The eluted fractions were collected for analysis via silver-stained SDS-PAGE to determine the purity of the protein target.

2.6. MD simulation

MD simulations were accomplished using AMBER 22 and the AMBER force field to assess the structural stability and behavior of the His-tagged Sfamy R64 mutant α-amylase, including its interaction with the maltose substrate. The protein was modeled using MODELLER 9.19 (Webb and Sali 2016) with PDB id 2GUY as a template. Both sequences shared high similarity, with 73% and 57% identity, respectively. The structure of 2GUY consists of a complex with maltose, thus the coordinates of this complex were superimposed as the initial model coordinates. The model's quality was evaluated using the Ramachandran plot (Sobolev et al. 2020). The model was then prepared in a solvated system with periodic boundary conditions, TIP3PBOX model, and Na^+ ions were added to neutralize the charge. The simulation was run for 100 nanoseconds at an active temperature of 50 °C, reflecting the enzyme's operational conditions in industrial applications.

Throughout the simulation, critical structural and dynamic metrics were measured, including the root mean square deviation (RMSD) to assess the overall stability of the protein, the RMSD of the substrate (maltose) to evaluate the enzyme-substrate interaction, and the root mean square fluctuation (RMSF) to determine the flexibility of individual amino acid residues. A time evolution snapshot of the protein-substrate complex was captured at several intervals during the simulation (Case et al. 2005).

3. Results and Discussion

3.1. Cloning of Sfamy R64 mutant

The pPICZαA-Sfamy R64 mutant plasmid was successfully constructed and propagated in *E. coli* TOP10 cells. After transformation, the transformants were selected on Zeocin-resistant plates, and colonies were detectable after 24–48 h of incubation at 37 °C. The presence of the recombinant plasmid in *E. coli* transformants was confirmed by colony growth on the selective medium. In contrast, no growth was observed in the negative control (Figure S1), which was *E. coli* TOP10 cells without the plasmid.

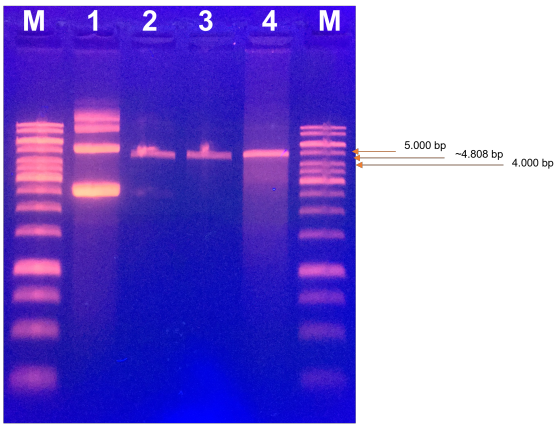
To verify the precise insertion of the Sfamy R64 mutant gene (1,470 bp), the plasmid (4,808 bp) containing the Sfamy R64 mutant gene and the pPICZaA vector was digested using the PmeI restriction enzyme. The agarose gel electrophoresis showed two distinct bands: the undigested plasmid (lane 1) and the linearized plasmid (lane 2) after digestion, with the expected fragment size of approximately 4808 bp, confirming successful cloning (Figure 1).

The successful cloning of the Sfamy R64 mutant α-amylase gene into the pPICZαA vector is a critical step in expressing recombinant proteins in *P. pastoris*. The Zeocin resistance of the transformed *E. coli* colonies confirmed the uptake of the pPICZαA-Sfamy mutant plasmid, as expected for clones carrying the Zeocin-resistance marker gene (Green and Sambrook 2012). The absence of growth in the negative control confirmed the selectivity of the antibiotic, further validating the success of the transformation process.

3.2. Transformation of P. pastoris

The transformation of *P. pastoris* was successful, as evidenced by the growth patterns observed in the selective media. On the competent cell positive control plate (YPD agar without zeocin), *P. pastoris* cells grew as expected. In contrast, no colony growth was observed on the negative control plate (YPD agar containing 100 µg/mL zeocin), confirming the antibiotic's effectiveness in inhibiting non-transformed cells. Several colonies were observed on the transformant plate (YPD agar supplemented with 100 µg/mL zeocin), confirming the uptake and stable integration of the plasmid into the *P. pastoris* genome, conferring resistance to zeocin. The colonies appeared large and distinct, indicating healthy cell growth and a successful transformation (Figure S2).

P. pastoris is a methylotrophic yeast that can me-



M: Marker DNA 1kb
1: pPICZaA-Sfamy-mutant **Uncut**
2: pPICZaA-Sfamy-mutant **Cut** (*PmeI*)
3: pPICZaA-Sfamy-mutant **Purification**
4: pPICZaA-Sfamy-mutant **Concentrated**

FIGURE 1 Agarose electrophoresis of pPICZαA-Sfamy-mutant digested by PmeI.

tabolize methanol as the sole carbon source through the methanol utilization (Mut) pathway. AOX enzymes are involved in the first step of methanol metabolism. AOX1 and AOX2 genes in *P. pastoris* are responsible for encoding them (Juturu and Wu 2018). AOX1 gene expression is controlled by AOX1 strong promoter, which was used in this study to guide the heterologous expression of Sfamy R64 mutant protein in *P. pastoris*. The linearization of the plasmid increases the likelihood of successful integration at the AOX1 locus, allowing for high-level expression under methanol induction (Love et al. 2016; Anggiani et al. 2018).

3.3. Expression of Sfamy R64 mutant

Recombinant expression of the Sfamy R64 mutant α-amylase in *P. pastoris* was successfully induced using varying methanol concentrations of 0.75%, 1%, and 1.5%. Protein samples from the culture supernatant were analyzed by SDS-PAGE after 144 h of induction, revealing distinct protein bands at approximately 54 kDa, corresponding to the predicted molecular weight of the expressed α-amylase (Figure 2). Among the methanol concentrations tested, 1.5% methanol resulted in the most intense protein band, indicating the highest expression levels. However, prolonged exposure to high methanol concentrations can lead to methanol toxicity or cellular stress, which may reduce protein expression over extended periods (Ahmad et al. 2014; Wang et al. 2024).

Methanol acts as both an inducer and carbon source for *P. pastoris* via the AOX1 promoter, but higher concentrations, such as 1.5%, may eventually inhibit protein production due to metabolic stress. This was reflected in the lower expression levels observed at 0.75% and 1% methanol, which were insufficient to sustain high expression levels. While 1.5% methanol initially led to high recombinant protein yields, the continued use of this concentration caused declining protein production, likely due to the accumulation of toxic by products (Yasokawa et al. 2010; Love et al. 2016; Wang et al. 2024).

Enzyme activity assays further supported these findings, with the highest α-amylase activity recorded at 48 h post-induction with 1.5% methanol, reaching 24.06 U/mL (Figure 3). After 72 h, enzyme activity began to decline, likely caused by methanol toxicity and the accumulation of metabolic byproducts that negatively impacted cell viability and overall protein production (Tsuda and Nonaka 2024). These results demonstrate that 48 h of induction at 1.5% methanol is optimal for maximizing both α-amylase

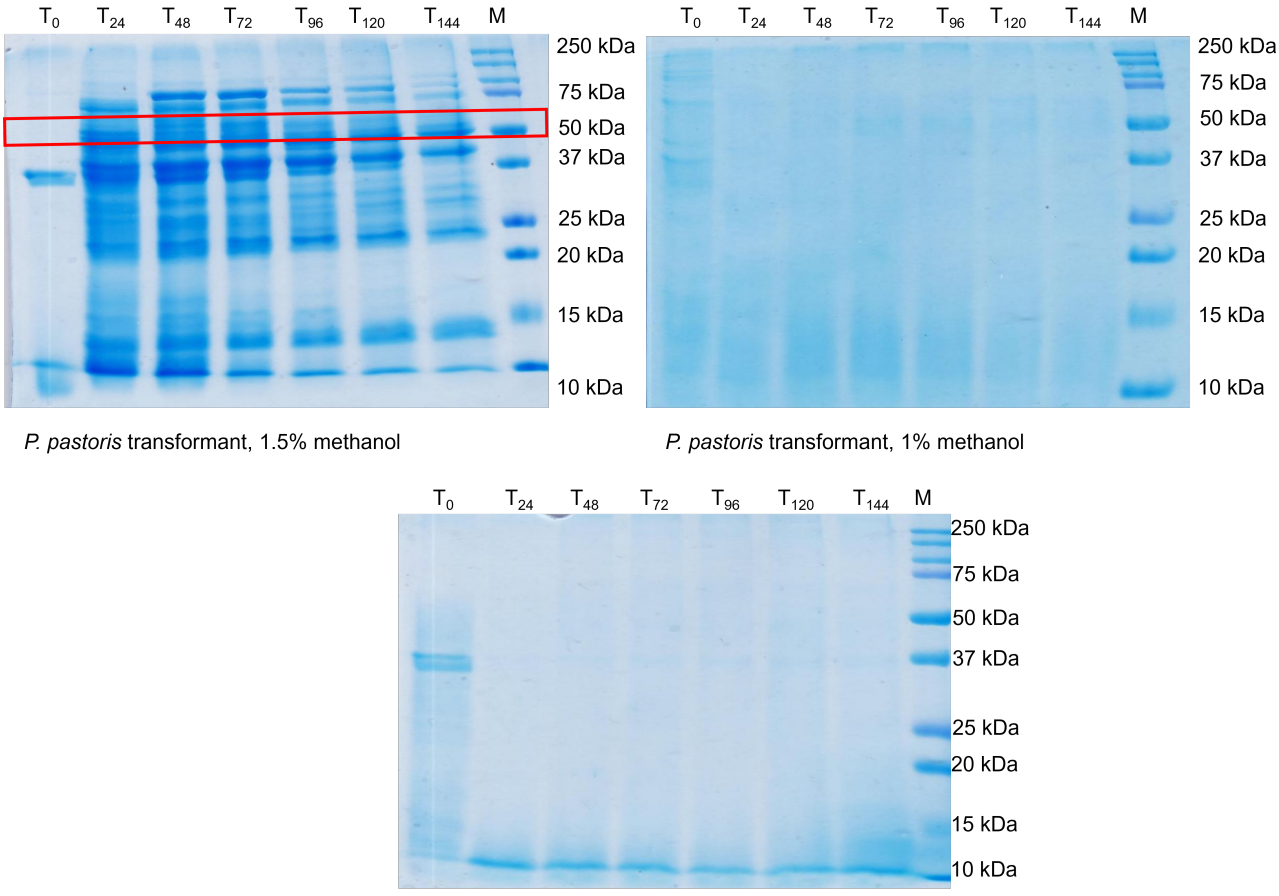


FIGURE 2 Electropherogram SDS-PAGE 12% of P. pastoris after induction with (a) 1.5%, (b) 1%, and (c) 0.75% methanol. T indicates the induction time (T_0, T_4, T_8, T_{12}, T_{20}, T_{24}), while M represents the protein marker.

expression and activity.

In *P. pastoris*, the secretion of recombinant proteins depends on a signal sequence that guides the protein to the endoplasmic reticulum (ER) for correct folding. The *S. cerevisiae* α-factor signal sequence has been successfully used for this purpose, both in its full-length and truncated forms (Gaffar et al. 2015). Protein secretion often becomes a rate-limiting step in the production process, as proper folding is essential for efficient secretion from the ER. Optimization of signal peptide through site-directed mutagenesis can improve secretion efficiency. A study related to this finding has successfully enhanced the secretion 1.4 fold of mutant SPKM19 (Alias et al. 2022).

Expression analysis of the Sfamy R64 mutant was performed using one of the Mut+ *P. pastoris* recombinant strains. Cultures of the recombinant were grown in BMMY medium, with methanol added every 24 h at concentrations of 0.75%, 1%, and 1.5% to maintain induction over 144 h. Analysis of the culture supernatant via SDS-PAGE using Commassie Blue dye, revealed a protein band at approximately 54 kDa after 144 h of 1.5% methanol induction, indicating the successful secretion of the Sfamy R64 mutant α-amylase. In contrast, 0.75% and 1% methanol concentrations did not result in detectable secreted protein in the *P. pastoris* transformants, further highlighting that 1.5% methanol is required for optimal expression and secretion.

The enzyme activity was assessed for all variants containing a His-tag. The maximum activity of Sfamy R64 mutant on *P. pastoris* was obtained at 48 h in 1.5% induction time which was 24.06 U/mL (Figure 3). The low activity observed in the other variant was likely due to the limited amount of secreted protein, as indicated by the thin band in SDS-PAGE electrophoresis. However, this activity is still lower than previous results, where the highest Sfamy activity in the culture supernatant was recorded at 32.29 U/mL after 72 h of methanol induction (Gaffar et al. 2015), suggesting that further optimization of recombinant protein expression is necessary. The enzyme activity decreased at 72 h of induction, possibly due to the presence of native proteases expressed by *P. pastoris*. This may have led to a reduction in expression levels (Goyal et al. 2005; Gaffar et al. 2015).

3.4. Purification of Sfamy R64 mutant

Soluble Sfamy R64 mutant α-amylase was purified using Ni-Sepharose (IMAC, Immobilized metal affinity chromatography) under native and denaturing conditions. Those two conditions showed a clear band around 48 kDa. However, in the native condition, Sfamy R64 mutant α-amylase showed a greater concentration over the denaturing condition, as visualized by Coomassie Blue staining. In contrast, due to the lower eluate concentration in purification-denaturing condition, Silver staining was used for visualization with a limit detection between 0.1–1 ng of protein (Jin et al. 2004; Arnau et al. 2006).

Additionally, in native condition, Sfamy R64 mutant α-amylase was eluted at 20 mM imidazole while in denaturing condition was eluted starting at 100 mM imidazole. Elution of his-tagged proteins under 50 mM imidazole is considered as low binding strength, and between 50–150 mM imidazole is considered as moderate binding strength. Notably, the theoretical Molecular weight of Sfamy R64 mutant α-amylase is 54 kDa, however it was characterized around 48 kDa (Figure 4). It was probably due to intracellular proteolytic degradation during expression (Zahrl et al. 2018). Therefore, Sfamy R64 mutant α-amylase purification under native conditions was more effective in capturing a higher amount of protein fusion with his-tag (Sinha et al. 2005).

The purpose of purification under native conditions was to purify protein while maintaining its natural conformation, avoiding protein denaturation. It's suitable for soluble protein. Whereas, purified under denaturing conditions involves 8M urea as chaotropic agents to completely unfold the protein, increasing the His-tag accessi-

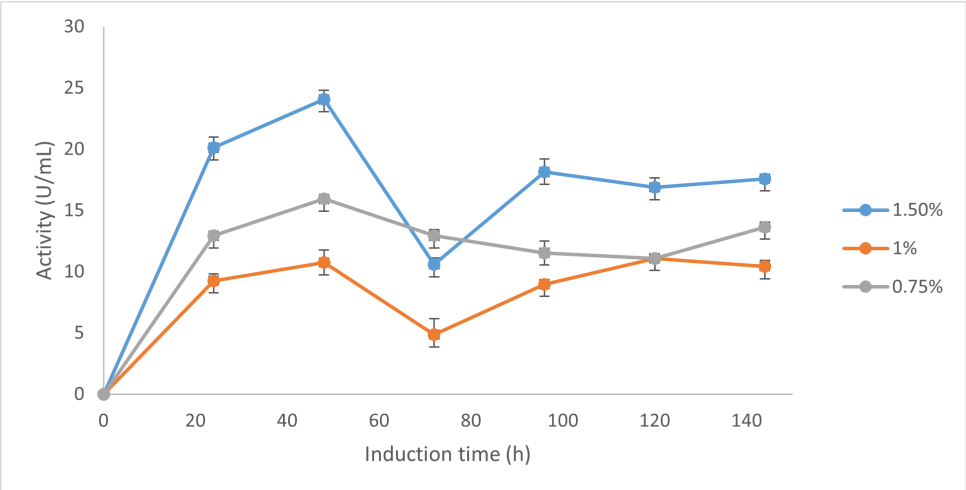


FIGURE 3 Sfamy R64 mutant activity every 24 h induction time with 1.5% methanol. Culture was harvested every 24 h and centrifuged to separate the supernatant from the cell pellet. Enzyme activity was then assessed using Fuwa method.

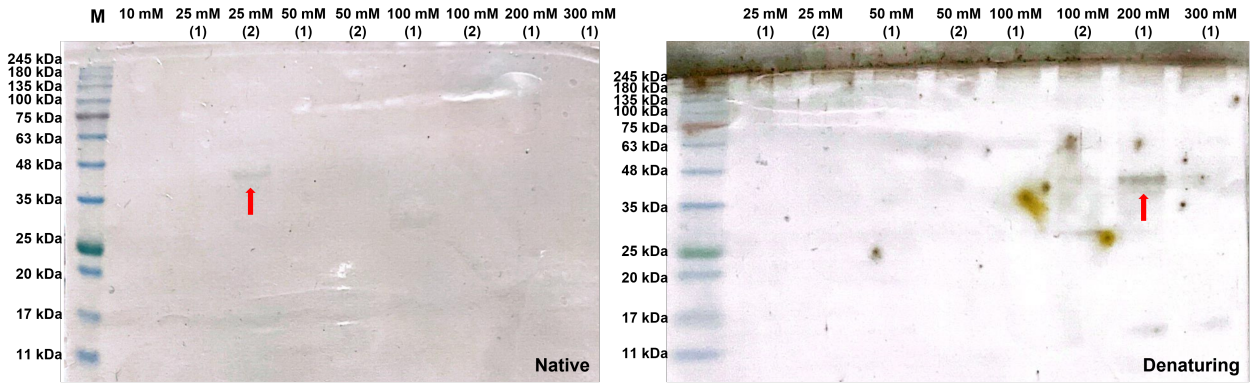


FIGURE 4 Electrophoregram SDS-PAGE 12% of Sfamy R64 purification under native and denaturing conditions. M represent for protein market and the imidazole is indicated at the top. The red arrow shows the band of purified protein.

bility toward the nickel column. As a result, in the denaturing condition, the Sfamy R64 mutant α-amylase eluated at a higher imidazole concentration due to increased His-tag accessibility in unfolded conformation (Hernández-Moreno et al. 2015; Wingfield 2015).

The use of His-tagged proteins and Ni-Sepharose ensured high specificity during the purification process, making it an efficient method for isolating the recombinant α-amylase. However, the results indicate that purification under native conditions is preferable for applications requiring enzymatic activity. If denaturing conditions are necessary, additional steps, such as protein refolding, may be needed to restore functionality (Yan et al. 2023).

3.5. MD simulation of His-Tag exposure

The structure of the Sfamy R64 mutant was modeled using MODELLER and evaluated with a Ramachandran plot. The model showed that more than 90% residues located in the most favored regions, 9.1% residues in additional allowed region, and 0.5% residues in generously allowed regions. The model structure was acceptable and could be used for further analysis (Laskowski et al. 1993; Sobolev et al. 2020) The analysis revealed that Sfamy R64 consists of three domains: Domain A/B, which contains the active site, and Domain C, described as the surface binding site (SBS), facilitating substrate binding before the hydrolysis reaction (Baroroh et al. 2019). Based on its sequences, the protein is also predicted to contain three disulfide bonds and two potential N-glycosylation sites (Figure 5).

The MD simulation demonstrated that the Sfamy R64 mutant maintained structural stability throughout the 100 ns run, with RMSD values stabilizing after approximately 20 ns, indicating overall structural integrity. However, the RMSD of the Sfamy R64 mutant without the His-tag was more stable from the beginning, suggesting that the core enzyme structure remained stable throughout the simula-

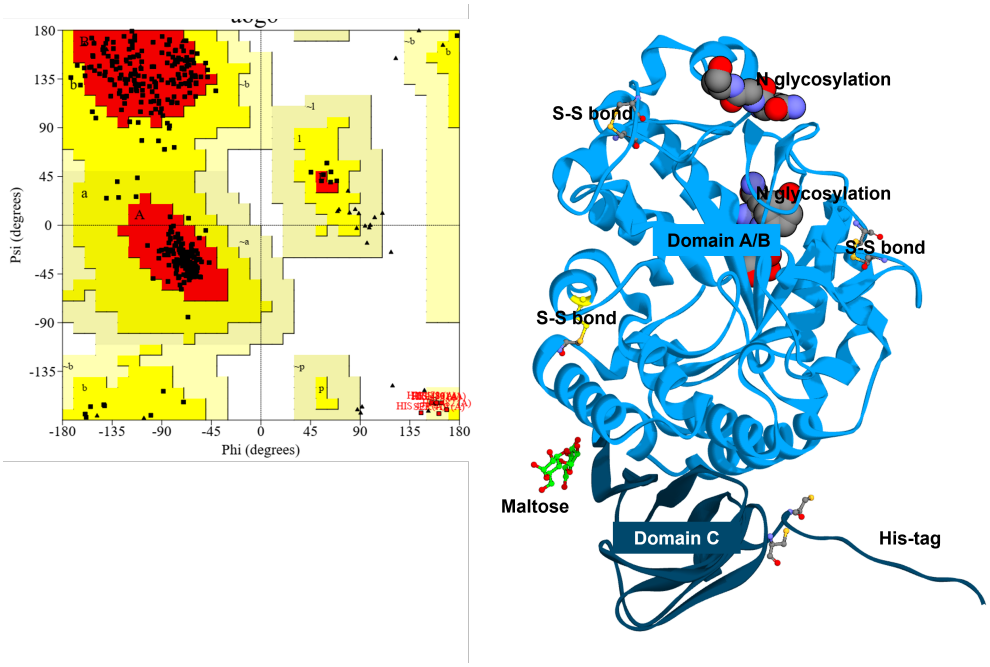


FIGURE 5 The model of Sfamy R64 mutant and its Ramachandran plot evaluation.

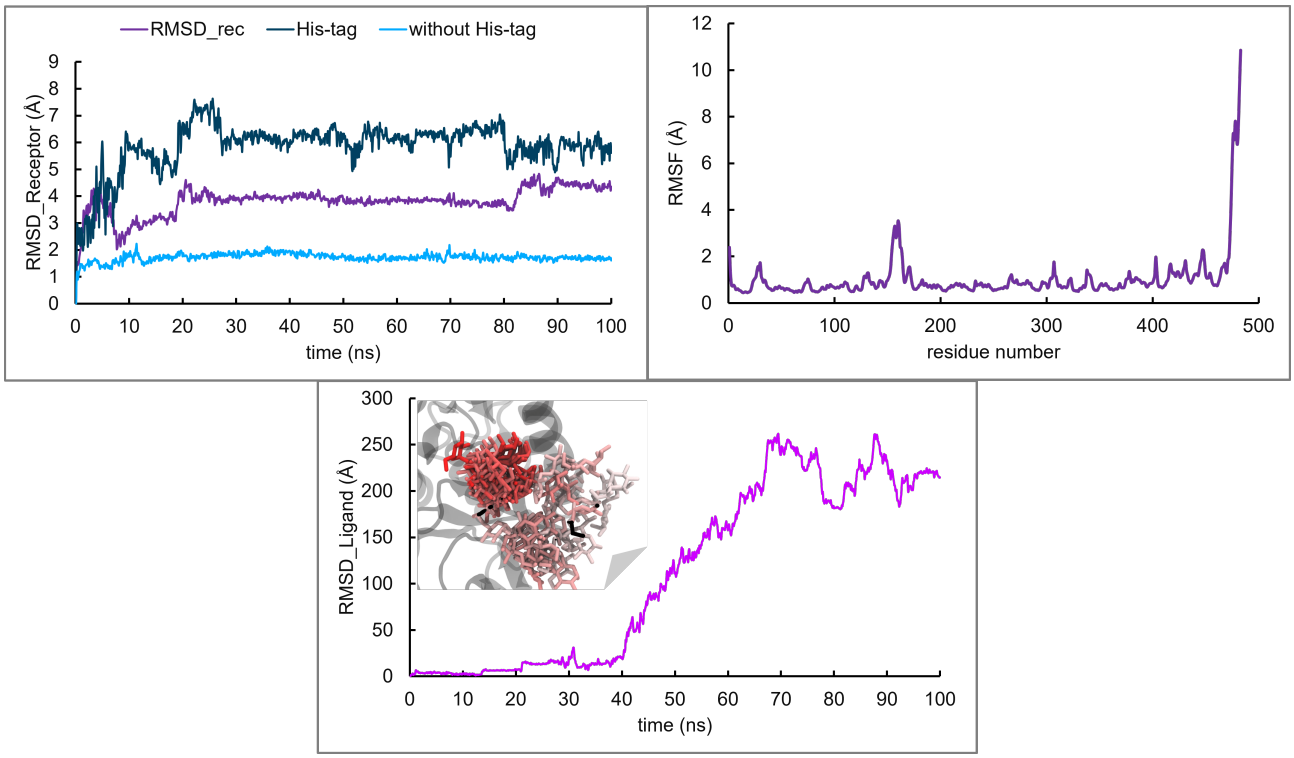


FIGURE 6 RMSD, RMSD substrate, and RMSF of Sfamy R64 complex throughout 100 ns MD simulation.

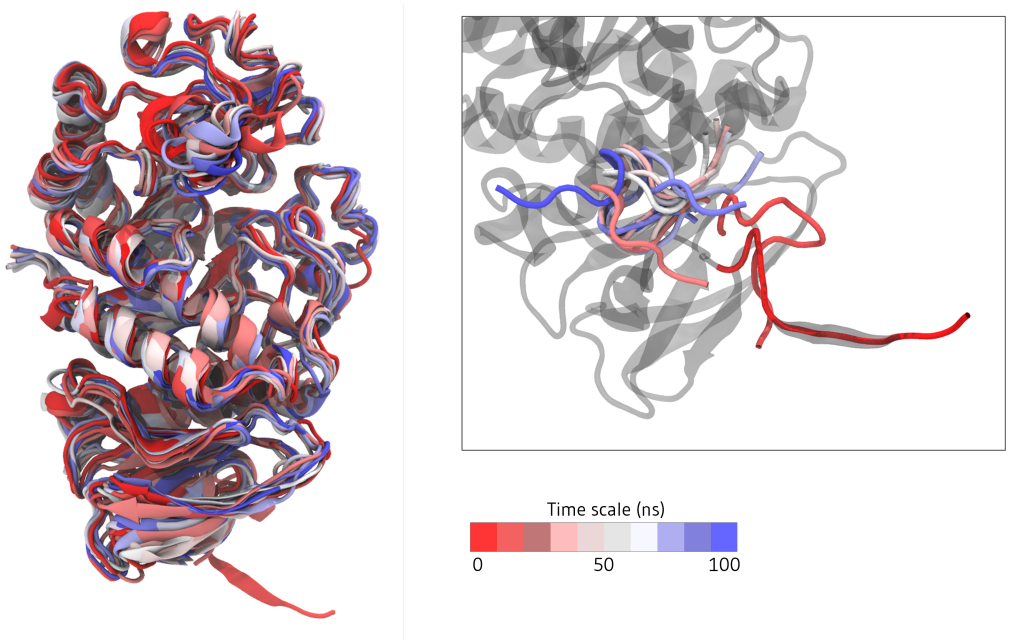


FIGURE 7 Time evolution snapshot of Sfamy R64 mutant and His-tag throughout 100 ns MD simulation.

tion. The RMSD of the His-tag showed significant deviations, especially during the first 30 ns, as it sought a more stable conformation. This was unsurprising due to its position at the C-terminus. However, after 30 ns, the His-tag stabilized, suggesting that it had adopted a stable configuration, which could facilitate efficient binding during affinity chromatography.

The RMSF analysis showed protein regions that exhibited higher flexibility, particularly in the loop regions neighboring the active site, which are critical for substrate binding and catalysis. The time evolution snapshots of the enzyme-maltose complex revealed that the substrate remained bound to the surface binding site (SBS) until 40 ns, after which it transitioned to the active site, indicating a stable binding process and proper substrate movement towards the catalytic region (Figure 6). This suggests that

the enzyme retains its integrity over time, even at the optimal temperature of 50 °C, which is critical for its use in industrial applications (Hernández-Moreno et al. 2015; Schartner et al. 2018).

The time evolution snapshots further support the conclusion that the enzyme-maltose complex remained stable throughout the simulation, with no significant structural changes that could impact the enzyme's function (Figure 7). These results align with the experimental data from the purification process, especially in native conditions where the His-tag was explored.

4. Conclusions

In this study, we successfully expressed, purified, and characterized the Sfamy R64 mutant α-amylase in *P. pastoris*. The recombinant protein was expressed under various methanol concentrations, with 1.5% methanol yielding the highest expression levels and enzyme activity after 48 h of induction. The purification of the Sfamy R64 mutant using Ni-Sepharose affinity chromatography was effective in both native and denaturing conditions. However, the native purification method provided better structural integrity and higher activity retention. MD simulations confirmed the stability of the Sfamy R64 mutant and its interaction with the maltose substrate. The His-tag remained solvent-accessible throughout the simulation, supporting its efficiency in the purification process. The combination of experimental and computational approaches provided valuable insights into the stability, functionality, and purification of the Sfamy R64 mutant α-amylase. These results provide a foundation for the enzyme's further optimization and large-scale production.

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Authors' contributions

UB, MTN, SK, MY designed the study. CC, ED, RA, MTN, TRT carried out the laboratory work. UB, CC, ED, MTN, DA, SK analyzed the data. UB, CC, ED, RA, MTN wrote the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

We declare that there is no competing interest.

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