International Journal of Technology

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Research Article

Halothermophilic Cas9 and dCas9 Interaction with Variety sgRNA In-silico

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Abstract: Industrial bioprocesses often require microorganisms that can adapt to specific conditions, such as halothermophilic bacteria capable of producing biosurfactants with various applications. Enhancing production yield is important, and one approach genetic modification. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) is a highly effective method for genetic editing; however, its use in halothermophilic bacteria remains limited. So far, optimal genetic editing with Cas9 has only been achieved in thermophilic bacteria unsuitable for high-salt environments. For determining optimal CRISPR involves analyzing the binding interaction between sgRNA (single guide RNA) and Cas9 using Molecular Docking in halothermophilic bacteria. This includes preparing the structures of Cas9 and sgRNA, simulating them with HDOCK software, and data analysis. Molecular Docking has advantages such as cost-effectiveness and time efficiency in designing the optimal sgRNA length. Optimization of sgRNA is achieved by varying the lengths of repeat, spacer, and tracrRNA, aiming for the lowest binding energy values and visually feasible designs. For Cas9 from the selected bacterium, Klebsiella pneumoniae, the optimal sgRNA design for Cas9 involves a spacer of 10 nt. In contrast, dCas9 involves a spacer of 10 nt, a repeat of 36 nt, and tracrRNA of 63 nt. The native temperature of the bacteria did not significantly affect the optimal sgRNA length.

Keywords: Biosurfactant; CRISPR-Cas9 Optimization; Halothermophilic; Molecular docking; sgRNA Design

1. Introduction

Nearly half of the global energy demand increase stems from rapidly developing countries, leading to a continuous rise in energy consumption each year. There are over 1.6 trillion barrels of

This work was supported by a fundamental research grant by Kemendibukristek, Indonesia Number: NKB-945/UN2.RST/HKP.05.00/2024

https://doi.org/10.14716/ijtech.v16i1.7367
Received October 2024; Revised October 2024; Accepted November 2024; Published January 2025

oil reserves worldwide, but 67% of this total consists of residual crude oil in reservoirs that are difficult to process (Shibulal et al., 2014). One alternative for processing this residual oil involves the use of biosurfactants produced by bacteria, either in-situ or ex-situ, through the use of bioreactors (Geetha, Banat, and Joshi, 2018). Biosurfactants are also utilized in the cosmetic, pharmaceutical, food, and agricultural industries, serving as emulsifying agents, foaming agents, and solvents (Akbari et al., 2018). Owing to their diverse applications and environmentally friendly benefits, the biosurfactant market is projected to grow at a CAGR of 5.5% from 2023 to 2028 (Mordor Intelligence, n.d.).

Halothermophilic bacteria, resistant to high temperatures and salinity, can optimally produce biosurfactants for commercial-scale production at temperatures above 40°C and are commonly found in hot springs, deep-sea hydrothermal vents, or high-salinity lakes, such as those in the Arabian Gulf (Lischer et al., 2020; Roda and Bassam, 2017;Elazzazy, Abdelmoneim and Almaghrabi, 2015). Halothermophilic bacteria have a wide range of potential applications in various industries, including dye decolorization, as well as in pharmaceuticals, cosmetics, and food. They are used in the production of extremophile lipase enzymes (Memarpoor-Yazdi et al., 2017) and biocatalyst production (Rodrigues et al., 2017). The optimization of biosurfactant production can be achieved through genetic engineering, such as inserting the rh1AB or rh1A genes to produce more biosurfactants or using mutagenesis to modify amino acid sequences (Jimoh et al., 2021). However, genetic engineering in halothermophilic bacteria remains limited due to the difficulties in gene editing and the lack of related research. The technology to do genetic engineering still needs to be developed. Current technology, such as TALEN, ZINC Finger, and current Cas9, cannot withstand the harsh environment where halothermopphilic bacteria grow. One genetic modification method is CRISPR/Cas9, which can cut DNA at specific locations and alter its sequence. CRISPR-Cas9 is a highly effective, efficient, and accurate genome-editing tool used across various scientific disciplines. Cas9 encodes a multidomain protein that targets and cuts DNA, while dCas9 binds to specific DNA segments without cutting, typically through modifications in the RuvC and HNH nuclease domains (Mengstie and Wondimu, 2021; Makarova et al., 2015). However, CRISPR/Cas9 is currently optimal at 37°C, which is less suitable for thermophilic bacteria (Mougiakos et al., 2017). Studies on CRISPR/Cas9 interactions in thermophilic bacteria have been conducted, identifying optimal spacer, repeat, and tracrRNA lengths for genetic modification (Pramayuditya, 2023; Angela, 2022). Cas9 application has been studied in halophilic bacteria in *Halomonas spp.* (Qin et al., 2018). However, research on halothermophilic bacteria is limited, with initial exploration using Single Amplified Genomes (SAGs) by (Grötzinger et al., 2018)

To address the challenges of operating at high temperatures and salinity, further studies are needed on the potential of Cas9 and dCas9 in halothermophilic bacteria. This includes optimizing the lengths of spacers, repeats, and tracrRNA using Molecular Docking simulations to predict interactions and binding affinities (Karlson et al., 2021; Uppada et al., 2018). The findings from these analyses can inform the design of optimal sgRNAs for halothermophilic bacteria, followed by in vitro and in vivo studies to validate their accuracy and enhance biosurfactant production post-genetic modification. Most docking studies are conducted to observe the interactions between drugs and their targets (Sahlan et al., 2023; Husnawati et al., 2023; Sahlan, et al., 2020). However, this research differs in that it focuses on docking to analyze the interactions between gRNA and Cas9 as well as dCas9.

2. Methods

In general, the experiment is divided into three major tasks, with two of them able to be conducted in parallel: Protein Sequence Identification and 3D Visualization of Cas9/dCas9 and sgRNA, then Molecular Docking Simulation for each variant with respective variations.

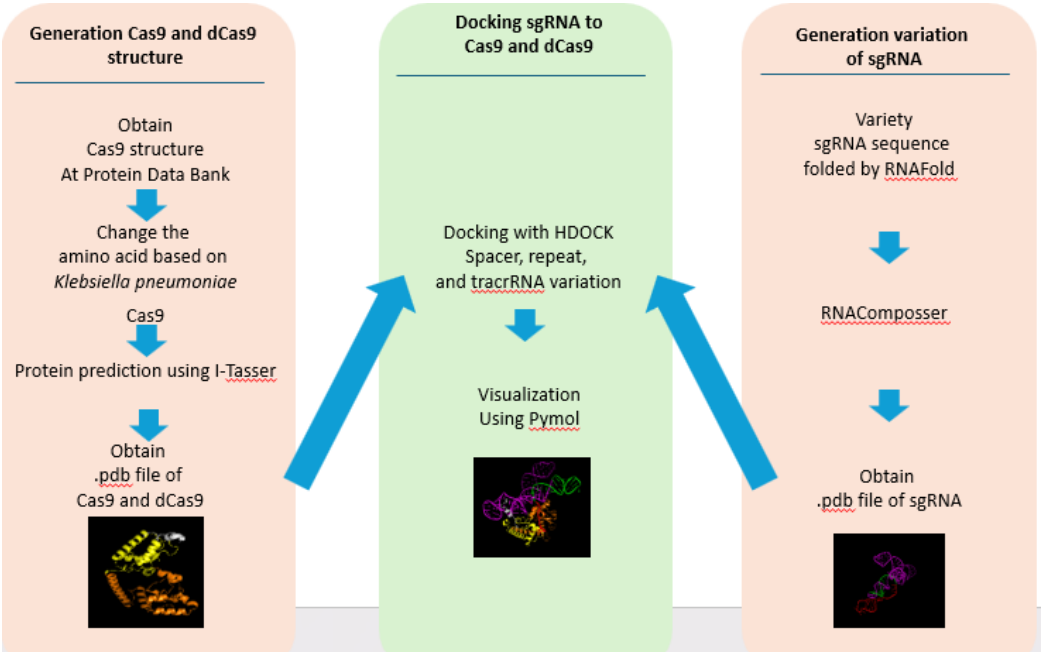


Figure 1 Scheme of the docking process

2.1. Preparation of Sequences and 3D Visualization of Cas9 and dCas9 Proteins from Halothermophilic Bacteria

The 3D structure visualization of Cas9 and dCas9 proteins can be performed after obtaining their respective protein sequences. In this study, Cas9 from the bacterium *Klebsiella pneumoniae* was used, sourced from the Protein Data Bank with the accession code MCI3999334.1. The protein sequence was obtained by accessing the NCBI website and searching for "type II CRISPR RNA-guided endonuclease Cas9 [target bacterium name]," in this study, we are using *Klebsiella pneumoniae*. The "Identical Protein Groups" database was selected, and the target protein sequence was accessed and downloaded in FASTA format.

Before visualizing, dCas9 was derived based on the study by Mougiakos et al. (2017) on ThermodCas9, which involves mutating the Cas9 sequence at amino acids 8 and 582. The 3D visualization was then performed using the cloud-based server I-TASSER, where the protein sequence file in FASTA format was uploaded, an email and password were entered to receive the results, an optional ID name was provided to distinguish the visualization results, and the I-TASSER tool was run (Zhou et al., 2022; Zheng et al., 2021; Yang and Zhang, 2015). The results were sent via email upon completion of the protein visualization.

2.2. Preparation of Sequences and 3D Visualization of sgRNA Variants

The sgRNA sequences used in this study are based on sgRNA previously utilized for thermophilic bacteria by Harrington et al. (2017), as detailed in supplementary data 3 of their journal. Each variant was derived as follows: the base spacer of 20 nt was directly obtained from the reference journal data. The 10 nt spacer was created by deleting 9 nt from the right side of the 19 nt spacer sequence: UGUAAGCGGAUGCCAUAUG. The 30 nt spacer was created by adding 9 nt to the left side of the 21 nt spacer sequence: GUCUGUAAGUCUGUAAGCGGAUGCCAUAUG. The repeat and tracrRNA sequences were directly taken from the journal's existing data. The folding of repeat and tracrRNA was performed using RNAfold website, and 3D visualization of sgRNA was carried out using RNAComposer (Sarzynska et al., 2023; Zuker, 2023).

2.3. Molecular Docking Simulation of sgRNA-Cas9/dCas9 Complex

The molecular docking process was performed using HDOCK(Yan et al., 2020), following these steps: The 3D structure pdb data of Cas9 from halothermophilic bacteria submitted as the receptor

in "Input Receptor Molecule". The 3D structure pdb data of sgRNA also submitted as the ligand in "Input Ligand Molecule". The interaction site residues of sgRNA within Cas9 were manually entered through the "Advanced Options (Optionals)" > Receptor Binding Site Residue(s) section. The residues were entered in the format M: A, where "M" represents the residue number of the protein interaction site and "A" is the protein chain. The interaction sites are based on a study done by Sun et al. (2019). A name was given to the file, and a personal email address was provided so that the docking results could be sent directly to the specified address upon completion. The process was initiated by clicking submit to start the molecular docking. The results will include the top 10 models in 3D, along with binding affinity values (docking score), confidence score, ligand RMSD, and interface residues. These docking results could be downloaded by clicking "All the results in a package."

2.4. Visualization of sgRNA-Cas9/dCas9 Interaction in Halothermophilic Bacteria

The sgRNA-Cas9 complex formed from the HDOCK simulation can be visualized using PyMOL or directly viewed on the website. Using PyMOL, the docking results must be in chain B format before visualization can be performed. The steps for visualization are as follows: Open the PyMOL application and select File > Open > the pdb file from the simulation. Type the command "alter lig_(x).pdb, chain='B'" and save the chain B file by selecting File > Export Molecule > Save. For analysis with LigPlot+, open the application and select Open > PDB Files > Browse... Choose DIMPLOT and fill in domain 1 with *A and domain 2 with *B, then click Run to view the visualization.

3. Results and Discussion

3.1. Cas9 Protein Alignment Result

The Cas9 protein from *Klebsiella pneumoniae* was aligned with the well-characterized Cas9 protein from *Geobacillus kaustophilus*, as described by (Harrington et al., 2017). This alignment aimed to map the target domains and assist in determining the mutation points necessary for forming dCas9. The alignment shown in Figure 1 revealed that the data obtained for *Klebsiella pneumoniae* only covered partial domains of REC, RuvC II and III, and HNH. Consequently, mutations were only performed within the HNH domain (Figure 2).

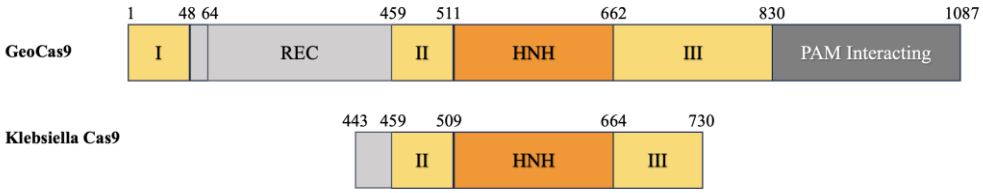


Figure 2 Visualization of Cas9 Domain.

Cas9 from halothermopilic bacteria is significantly shorter (287 Amino Acids)) compared to geobacillus (1087 Amino Acid). The visualization results indicate a significant difference in the coverage of target domains between the two species, highlighting the necessity for specific mutations in the HNH domain to achieve the desired functionality of dCas9 in *Klebsiella pneumoniae*. This finding is crucial for understanding the structural and functional adaptations required for effective gene editing in halothermophilic bacteria.

3.2. Visualization of 3 Dimension Structure of Cas9/dCas9 and sgRNA

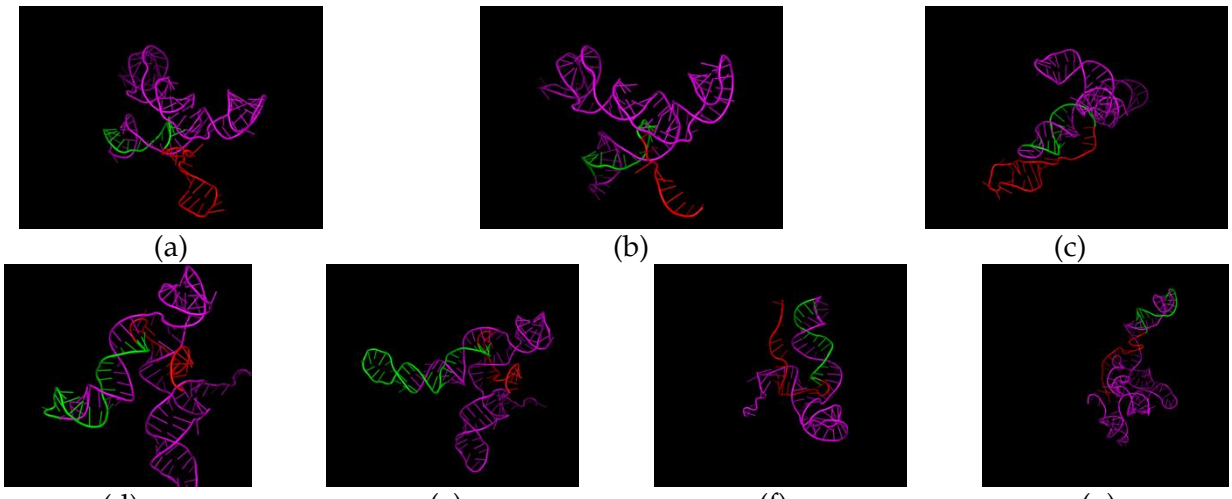
The second sequence visualized was sourced from the same locations: Air Panas Jono, Bleduku, and Grobogan, Bleduku in Semarang, East Java. The sequence contains 289 amino acid pairs, as shown in Figure 3.

(a) (b)

Figure 3 3-Dimension Structures (a) Cas9 and (b) dCas9 of *Klebsiella pneumoniae*.

The colors in the structure represent specific areas: yellow for RuvC, orange for HNH, and light gray for REC. Mutations were introduced exclusively at positions A582F/A145F, which correspond to the HNH domain, due to the limited Cas9 sequence data. The results show that the dCas9 fold is simpler, with fewer alpha helices compared to Cas9. However, the structural similarity is high, with an RMSD value of 0.542, indicating minor differences. This suggests that the mutation affects functionality rather than structure.

For sgRNA, the spacer, repeat, and tracrRNA were folded separately since the spacer is expected to be linear. Initially, the construction of the 2D structure served as a bridge between the primary and tertiary structures, providing insights into how the sequence and base pairs form a functional 3D structure. It also confirmed that each sgRNA component forms the expected structure. Figure 4 shows the visualization of each sgRNA, with a red spacer, a repeat in green, and a tracrRNA in magenta. The visualization indicates that shorter nucleotide spacers form linear, unstructured regions. In contrast, longer nucleotides result in folds, which are undesirable as the spacer's function is to complement and bind the target DNA sequence for subsequent cutting.



(a) (b) (c)

(d) (e) (f) (g)

Figure 4 3-Dimensional Structures of sgRNA with Variations: (a) Control, (b) Spacer 10 nt, (c) Spacer 30 nt, (d) Repeat 25 nt, (e) Repeat 36 nt, (f) tracrRNA 63 nt, (g) tracrRNA 140 nt. (spacer in red, repeat in green, and tracrRNA in magenta)

Furthermore, changes in spacer length did not affect the structure or folding of the repeat and tracrRNA regions. Notable differences were observed in the tracrRNA variants, where different lengths altered the overall structure's orientation, indirectly affecting the potential binding interactions.

3.3. Molecular Docking Result of sgRNA-Cas9/dCas9 in Halothermophilic

After performing simulations between sgRNA and Cas9, data consisting of the top 10 structures of the sgRNA-Cas9/dCas9 complex were obtained. The structure selected was the top one that most closely approached ideal conditions, where the spacer was positioned closest to the RuvC and HNH domains. Docking was performed on Cas9/dCas9 from Klebsiella pneumoniae, paired with sgRNA variants that incorporated changes in spacer, repeat, and tracrRNA lengths. The goal was to observe their impact on the formed complex and assess whether any variant formed a more stable complex compared to the control. After identifying each variant of the spacer, repeat, and tracrRNA for both Cas9 and dCas9, the best length of each variant was selected by evaluating the lowest binding affinity values, highest confidence scores, and the position where the spacer was linear and closest to RuvC and HNH. For Cas9, the optimal sgRNA length was found to be a spacer of 10 nt, a repeat of 16 nt, and a tracrRNA of 98 nt, with a binding affinity value of -358.99 and a confidence score of 0.9849. Figure 5 (a) shows the linear spacer (red) positioned between HNH (orange) and RuvC (yellow).

(a) (b)

Figure 5 3-Dimension Structures of (a) Cas9 and (b) dCas9.

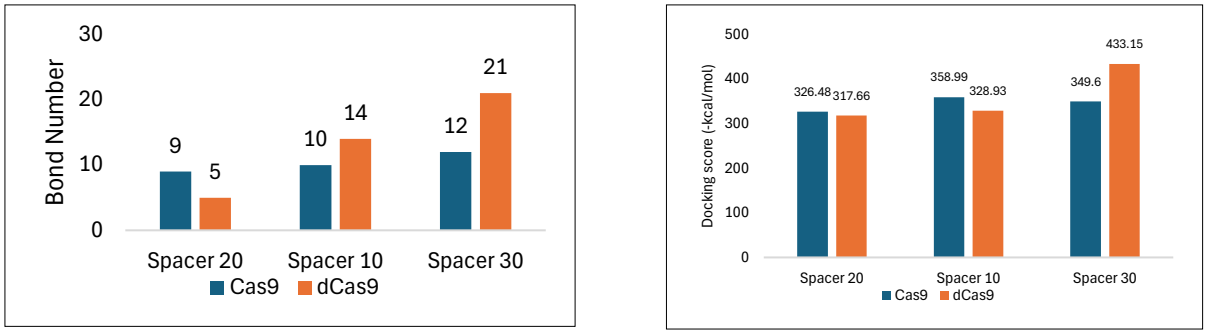
The optimal sgRNA design for dCas9 was a spacer of 10 nt, a repeat of 36 nt, and tracrRNA of 63 nt, with a binding affinity value of -354.28 and a confidence score of 0.9835. Figure 4 (b) shows the 3D structure of the complex with the linear spacer (red) positioned between HNH (orange) and RuvC (yellow) at a sufficiently close distance. These results indicate that the specific lengths of spacer, repeat, and tracrRNA significantly impact the binding affinity and overall structure of the sgRNA-Cas9/dCas9 complex, providing insights into the optimal design for effective gene editing in halothermophilic bacteria.

3.4. Influence of sgRNA Length on Binding Affinity in Molecular Interaction

The visualization of intermolecular interactions with varying spacer lengths showed that increasing the length of the spacer in Cas9 does not significantly affect hydrogen bonding, whereas, for dCas9, the opposite is true. This suggests that longer spacers can optimize the positioning or flexibility of sgRNA, leading to better interactions with the target DNA. This result is reflected in the binding affinity values/docking scores in the two graphs in Figure 6, where the pattern tends to be linear with the number of hydrogen bonds. From both graphs, it can be concluded that there is a correlation between hydrogen bonds in the complex and binding affinity values. An increase in the number of hydrogen bonds formed indicates enhanced interaction and stability.

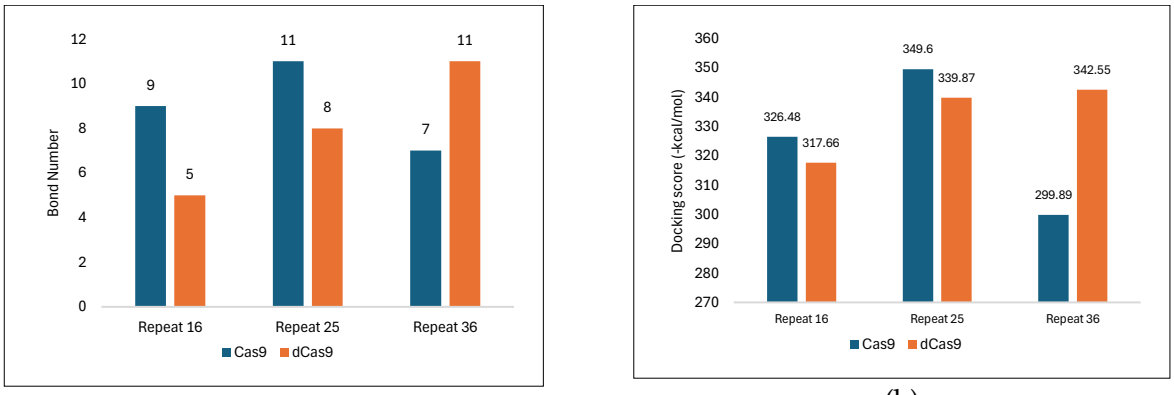
For the repeat region, there is a correlation between nucleotide length and hydrogen bonding, especially in dCas9. In Cas9, the most hydrogen bonds are found with a 25 nt repeat, while a 36 nt repeat shows a decrease due to the 3D structure folding away from Cas9. This indicates that the length of the repeat affects the folding and flexibility of sgRNA, thereby influencing its interaction with Cas9. The binding affinity values/docking scores in Figure 7 show a similar pattern, with a peak at 25 nt for Cas9 and a decrease at 36 nt. In the case of dCas9, the variation in binding affinity values between 25 nt and 36 nt does not correspond directly to the number of hydrogen bonds, possibly due to the flexibility of both molecules and the arrangement of atoms within the folds. Comparing these two parameters shows that repeat length significantly affects the number of hydrogen bonds and docking scores, impacting the structural stability and functional efficacy of

the complex. The 25 nt repeat is most optimal for Cas9, whereas the longest repeat is best for dCas9. However, the selection of sgRNA length must consider the qualitative structure of the complex.



(a) (b)

Figure 6 (a) Hydrogen Bond and (b) Intermolecular Binding Affinity on Spacer Variations

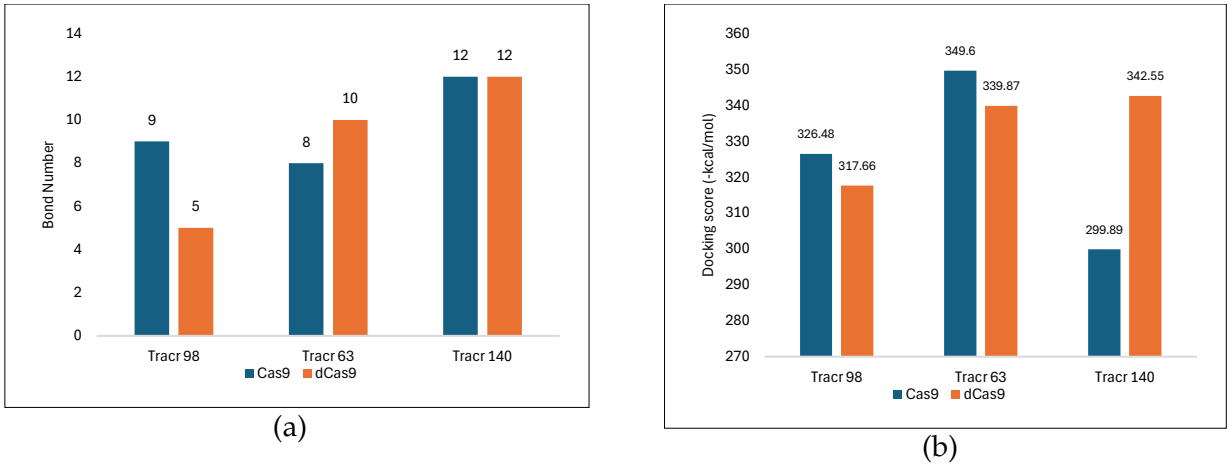


(a) (b)

Figure 7 (a) Hydrogen Bond and (b) Intermolecular Binding Affinity on Repeat Variations

Regarding tracrRNA variations, the pattern in Figure 8 shows that in Cas9, longer tracrRNA results in more hydrogen bonds. However, in dCas9, the 98 nucleotide variation results in fewer bonds. Overall, this indicates that longer tracrRNA increases the likelihood of forming bonds between Cas9 and sgRNA. However, the binding affinity values for Cas9 decrease with longer nucleotides, making the complex less effective in interactions. For dCas9, the binding affinity values increase with longer tracrRNA, although this does not always correlate with the number of hydrogen bonds formed. The results in Figure 8 demonstrate that optimal binding is not solely dependent on the number of hydrogen bonds but also on the configuration of sgRNA, particularly the tracrRNA domain, which has the largest proportion in the complex.

Hydrogen bonds are interactions between a hydrogen atom and an electronegative atom such as F, N, or O. These bonds are stronger than Van der Waals forces but not as strong as covalent bonds, making them ideal for biological interactions due to their stability and flexibility (Nelson and Cox, 2001). Hydrogen bonds are crucial in forming protein structures like alpha helices and beta sheets (Baker and Htjabardt, 1984).



(a) (b)

Figure 8 (a) Hydrogen Bond and (b) Intermolecular Binding Affinity on tracrRNA Variations

In molecular docking, hydrogen bonds are calculated to predict the most stable and selective binding positions (Kitchen et al., 2004). The docking results can be compared with previous studies, such as (Pramayuditya, 2023), which explored Cas9 and dCas9 in *Geobacillus kaustophilus* and *Klebsiella pneumoniae*, demonstrating that optimal spacer lengths vary depending on the bacteria and environmental conditions. Matson et al. (2019) found that shorter spacers increase the specificity of sgRNA-Cas9, particularly under high temperature and salt concentration conditions. Although hydrophobic interactions contribute to ligand-protein interactions, they are not the primary focus here due to their nonspecific nature and the challenges in quantitatively interpreting them (Sarkar and Kellogg, 2010).

The selected structures from the molecular docking simulation were based on binding affinity values and visual validation to ensure correct orientation and significant interactions (Meng et al., 2011). This preliminary study supports biosurfactant production by providing insights into the optimal design of sgRNA for Cas9/dCas9 systems. The next steps involve in vitro and in vivo testing to evaluate the effectiveness of sgRNA on Cas9/dCas9 in biosurfactant production conditions. In addition, future research should explore the production of cas9 and dcas9 and its activities in vitro and in vivo (Arumsari et al., 2024).

4. Conclusions

The appropriate design for sgRNA for Cas9 includes a spacer of 10 nt, a repeat of 16 nt, and tracrRNA of 98 nt. In contrast, for dCas9, the arrangement involves a spacer of 10 nt, a repeat of 36 nt, and a tracrRNA of 63 nt. Variations in spacer length do not significantly affect binding affinity values, whereas an increase in repeat length impacts binding affinity values. However, the binding affinity value decreases with increasing tracrRNA length in Cas9, which is opposite to what occurs in dCas9. The native temperature of the bacteria does not significantly affect the optimal sgRNA length for bacteria from that group. The results from in silico studies can be applied both in vivo and in vitro, especially in halothermophilic bacteria.

Acknowledgements

The author would like to extend sincere gratitude to the individuals and institutions for their invaluable assistance and support throughout this research: my supervisors for guiding me throughout the writing process, thesis examiners and the Department of Chemical Engineering, Faculty of Engineering, Universitas Indonesia, that participate in the process. This paper is supported by a fundamental research grant by Kemendibukristek, Indonesia Number: NKB-945/UN2.RST/HKP.05.00/2024.

Author Contributions

KL, FA, YN, do conceptual, analysis, supervise, and finalization draft of paper. HM draft first paper and analysis of the result. ACK, RWN do conceptual design and analysis. MSMA works on finalization of paper.

Conflict of Interest

The authors declare that there are no conflicts of interest

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