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# *In-silico* Genome Editing Identification and Functional Protein Change of *Chlamydomonas reinhardtii* Acetyl-CoA Carboxylase (*CrACCase*)

Mentari Putri Pratami[1], Miftahul Huda Fendiyanto[1,2,*], Rizky Dwi Satrio[1,2] , Isna Arofatun Nikmah[1], Mo Awwanah[1], Nadya Farah[1], Nastiti Intan Permata Sari[1], Nurhadiyanta[1]

[1]*Study Program of Biology, Faculty of Military Mathematics and Natural Sciences, The Republic of Indonesia Defense University. Kampus Universitas Pertahanan RI, Sentul-Bogor, Kawasan IPSC, 16810, West Java, Indonesia;* [2]*Laboratory of Plant Physiology and Genetics, Department of Biology, Faculty of Mathematics and Natural Sciences, IPB University. Kampus IPB Darmaga Bogor 16680, West Java, Indonesia.*

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**Abstract.**

*The Acetyl-Coa Carboxylase* (*CrACCase*) in *Chlamydomonas reinhardtii* is a gene encoded triacylglyceride (TAG) and lipid (oil body) synthesis. The *CrACCase* gene was little studied and had not been genetically engineered either *in-silico* or *in-vivo*. In this study, we provide bioinformatic precision information for genome editing, especially in *CrACCase*. This study aimed to construct sgRNA and predict the functional region of the putative mutant protein of the *CrACCase*. Based on the results of molecular identification, the best *CrACCase* (GeneBank XM_001703135) can be genetically in-silico modified. The best potential sgRNA constructions in this study were GCGTCTGCTCAATCACACGGCGG, TTGAGGTCGGAACTCCAGCGG, and AGGCAATACCCTCAATTGGGTGG with efficiency values of 79.27, 68.25, and 65.17%, respectively. The best oligo sgRNA obtained has a protospacer adjacent motif (PAM) site with NGG especially in the form of CGG and TGG. The location of the engineered *CrACCase* gene mutation was found in the XM_001703135.1:1089 region in the *Chlamydomonas reinhardtii* genome, especially in the negative strand. CrACCase protein was predicted to have the structure of carboxyl transferase subunit of ACC, carboxyl transferase subunit of putative PCC, humanized carboxyltransferase domain of yeast Acetyl-CoA carboxylase, and Acetyl-CoA Carboxylase. Changes in frameshift mutations in the *CrACCase* gene influenced structural changes of the functional regions of the ligand-protein binding sites at residues D:C 92, 95, 111, and 114 where these sites are zinc ion binding sites. This structural change resulted in a change in the function of the CrACCase protein. This bioinformatics information is important to perform in-vivo genome editing on the *CrACCase* in the future so that mutants with the highest TAG production or the highest biodiesel (oil body) producer can be obtained. The manipulation of the *CrACCase* gene in *Chlamydomonas reinhardtii* can be applied to other microalgae organisms with the highest lipid percentages to increase future bioenergy production by molecular biologists and biotechnologists.

**Keywords:** Acetyl-CoA Carboxylase, *Chlamydomonas reinhardtii*, CRISPR/Cas 9, Genome Editing, Protospacer Adjacent Motif

## 1. Introduction

Microalgae can be used as an alternative energy source (renewable biofuel) to overcome the problem of energy difficulties in recent times (Alam *et al*., 2012; Jones and Mayfield, 2012; Suali and Sarbatly, 2012; Medipally *et al*., 2015). Microalgae are the best source of biofuels (Barqi, 2015; Eisentraut, 2010) when compared to palm oil, Jatropha, and lignocellulosic biomass (Milano *et al*., 2016) because the organisms have a very high lipid content, i.e in *Nannochloropsis oculata* it reaches 31-68%, while in *Chlorella* sp. reached 10-58% (Christi, 2007). However, the use of microalgae as a source of biofuel has not been carried out optimally and efficiently. This is due to the limited identification of species and molecular characterization.

Microalgae can produce 150-200 times more lipids (Demirbas, 2011; Peng *et al*., 2013; Bouabidi *et al*., 2018) than lipid-producing plants (oil palm, jatropha) at the best conditions of silver per year. Microalgae have the greatest potential as a producer of biodiesel feedstock (Godhe *et al*., 2008; Khan *et al*., 2009) compared to other plants. In contrast to other plants, microalgae are able to produce very high lipids for biodiesel production with fast harvest times (Bligh and Dyer, 1959; Lee, 1980; Brown *et al*., 1994; Chisti, 2007; Bringezu *et al*., 2007; Ratha and Prasanna, 2012). Recently, mass microalgae culture with bioreactors (Peng *et al*., 2016) can be used as an alternative to meet bioenergy needs, especially biodiesel (Brennan and Owende, 2010; Sharma *et al*., 2018).

* Corresponding author. e-mail: miftahul.fendiyanto@idu.ac.id.

However, the efficiency of lipid production through the triacylglyceride metabolic pathway (TAG) is vital to study to increase lipid production in microalgae (Cooksey *et al*., 1987; Duong *et al.*, 2015; Sharma *et al*., 2018). Especially for the species *Chlamydomonas reinhardtii*, genome editing of the ACCase gene has not been reported; therefore, this study is the first step in carrying out genetic manipulation to increase lipid production. In addition to increased lipid production with biocatalysts (Rodolfi *et al*., 2009; Yun *et al*., 2014), a genome editing approach becomes important. This genetic engineering can be done by looking for markers both at the gene level (Diaz *et al*., 2010; Fendiyanto *et al*., 2019a; Miftahudin *et al*., 2021), genome (Countway *et al*., 2005; Zhu *et al*., 2005; Fendiyanto *et al*., 2019b; Pratami *et al*., 2020), mRNA (Satrio *et al*., 2019), or metabolites (Chini-Zittelli *et al*., 2006; Ramasamy *et al*., 2015; Fendiyanto *et al*., 2020; Fendiyanto *et al*., 2021).

*Chlamydomonas reinhardtii* is a microalga with great potential as a source of biofuels, but this species has not been studied or molecular characterized completely. Therefore, the potential of microalgae particularly *Chlamydomonas reinhardtii* as a renewable bioenergy source is necessary to study. In addition, *C. reinhardtii* was found to have the *Acetyl-Coa Carboxylase (CrACCase)* gene, which encodes for triacylglyceride (TAG) and lipid (oil body) synthesis. TAG is the main form of energy storage in microalgae cells and is contained in 60-70% of its dry weight (Hu et al., 2008; Scott et al., 2010; Shuba and Kifleb, 2018). Research on the *CrACCase* gene in *Chlamydomonas reinhardtii* has not been carried out comprehensively and has not been characterized either in-silico or in-vivo, even though this gene plays an important role in producing TAG.

Lipid metabolism is the synthesis and degradation of lipids in cells, which involves the breakdown or storage of fat for energy (Spolaore *et al*., 2006). These fats are obtained from consuming food and absorbing them or are synthesized by plants or animals. Lipogenesis is the process of synthesizing these fats. The types of lipids found in the body are fatty acids and membrane lipids in microalgae (Weldy and Huesemann, 2007). Lipid metabolism often begins with hydrolysis, which occurs with the help of various important enzymes. Lipid metabolism also occurs in microalgae, although the process is different than in animals. In microalgae organisms, lipid biosynthesis is carried out by involving the ACC and GPAT enzymes (Ummalyma *et al.*, 2017) in the triacylglyceride (TAG) pathway starting from the acetyl-CoA precursor to oil bodies in the plastids and endoplasmic reticulum. One of the important enzymes analyzed in this study is ACCase, the protein encoded by *the Acetyl-Coa Carboxylase gene*. The gene can be found in *Chlamydomonas reinhardtii* and then we called *CrACCase*. We attempted to perform genome editing and search for mutation sites in silico in the *CrACCase* gene and predict changes in the functional structure of the protein. Therefore, this study aimed to construct oligo guide RNA and predict the functional region of the putative protein mutant of the *CrACCase* gene to engineer the increase in lipid content in *Chlamydomonas reinhardtii*.

## 2. Materials and Methods

### *2.1. CrACCase Gene Searching of Microalgae in GenBank Database*

*The Acetyl-Coa Carboxylase* (*CrACCase*) gene was done by performing an advance search ((Acetyl-Coa Carboxylase)(OR Microalgae) OR *Chlamydomonas reinhardtii*) using Nucleotide Query in the NCBI database (https://www.ncbi.nlm.nih.gov/). Gene selection was done by filtering only for mRNA sequences and coding sequences for the *CrACCase* gene in the species *Chlamydomonas reinhardtii*. From 1244 accessions obtained, we selected the *CrACCase* gene with accession XM_001703135, specifically the gene that has a high level of confidence in Microalgae. The XM_001703135 accession was then downloaded in FASTA and GENEBANK formats to determine the area of the open reading frame (ORF), promoter, and coding sequence (CDS). Accession Data visualization was performed using the UGENE program (Okonechnikov *et al.*, 2012). In addition, we also performed a BLASTn process to ensure the best access to the Acetyl-Coa Carboxylase gene in *Chlamydomonas reinhardtii* species. The selection of species of microalgae *Chlamydomonas reinhardtii* was carried out based on the reference of Sharma *et al*., (2018) which can be chosen as the main gene to regulate the synthesis of triacylglycerol (TAG) in producing oil bodies for biodiesel production in microalgae. Identification, characterization, and search for functional regions of genes were investigated with a precision tool using the UGENE program (Okonechnikov. *et al.*, 2012).

### *2.2. sgRNA Design of the CrACCase using in-silico CRISPR/Cas 9 approaches*

The design guide-RNA was carried out using a comparative approach for the initial study on genome editing CRISPR/Cas 9 (Sharma *et al.*, 2018). The design of guide-RNA in bioinformatics in the CRISPR/Cas 9 approach was carried out by searching for the protospacer adjacent motif (PAM) region in the genome of *Chlamydomonas reinhardtii*, especially the *CrACCase* gene. This search process is carried out with the help of the CHOP-CHOP application (https://chopchop.cbu.uib.no/) with the 'KNOCK OUT' format in the hope that the target gene undergoes a frameshift mutation. The primers obtained were then statistically tested and genetically verified through the value of GC nucleotide ratio and potential for self-complementarity. Self-complementarity was avoided to eliminate the potential for hairpin structure formation in the RNA oligo-guide of the generated *CrACCase* gene. sgRNA sequences obtained were also designed based on the choice of PAM, especially the NGG site on the *CrACCase* genome of the species *Chlamydomonas reinhardtii*. The obtained oligos are then used as a benchmark to see the potential for frameshift mutations in the *CrACCase* gene and its protein.

### *2.3. Identification Genes and Proteins of CrACCase*

Identification of the *CrACCase* gene was carried out using the BLASTn approach (Fendiyanto *et al.*, 2019a), while the search for CrACCase protein was carried out using the BLASTtn approach (Miftahudin *et al.*, 2021) on the NCBI database. We selected 50 genes with similar identity and query cover above 80% to identify the

*CrACCase* gene. The *CrACCase* gene with accession XM_001703135 was selected for further analysis because it is the only gene encoding the highest quality Acetyl-Coa Carboxylase in *Chlamydomonas reinhardtii* species. The gene with the accession was then searched for its protein sequence with the help of BLAST and characterization directly using the UGENE program. The protein obtained was then further analyzed for its 2-dimensional (2D), 3-dimensional (3D) structure, functional regions, protein sequence homology, and potential ligand binding to the active site of the CrACCase protein in *Chlamydomonas reinhardtii* species.

### *2.4. Confirmation of CrACCase Putative Protein and Prediction of Functional Region*

The putative protein sequences obtained from the UGENE and BLAST tools were then confirmed and further characterized. We constructed a 3D and crystal structure of the CrACCase protein using the SWISS-MODEL program (Bienert *et al.*, 2017; Waterhouse *et al.*, 2018) based on homology modeling and functional structure. The homology predictions obtained were four putative proteins, and we chose only one protein using the protein template based on the highest residue sequence composition (QMEANDisCo) (Waterhouse *et al.*, 2018). We also pay attention to the QMEAN Z-score value, local quality estimates, ligand interaction, amino acid composition, oligo state type, GMQE value, sequence identity, and structure assessment. Specifically for the study of ligand-protein interactions, we identified the ligands of the CrACCase protein in the chemical component summary description in the RCSB PDB database (https://www.rcsb.org/ligand/ZN) which includes identities, formulas, molecular weight, type, isomeric SMILES, InChl, InChlKey, formal charge, atom count, chiral atom count, bond count, and aromatic bond count. The quality of protein homology and identification of functional regions were also carried out with the ORION tool (Altschul *et al.*, 1997; de Brevern *et al.*, 2000; Söding, 2005; Ghouzam *et al.*, 2015). Identification of changes in protein function was carried out by looking at the structure of amino acid residues and ligands using the MODELLER, I-TASSER, and SWISS MODEL programs (Waterhouse *et al.*, 2018).

## 3. Results and Discussion

### *3.1. In silico Genome Editing of CrACCase in Chlamydomonas reinhardtii*

Based on the test for making oligo single-guide (sg) RNA, it was shown that the protospacer adjacent motif (PAM) region in the *CrACCase* gene, especially the *Chlamydomonas reinhardtii* genome, contained 188 regions spread from the 25th to 1300 bp nucleotides. The oligo sgRNA construction was carried out by randomly searching the PAM region of the *CrACCase* gene with nucleotide orientation either from the 5' to 3' end (positive thread) or vice versa from the 3' to 5' end (negative strand). There are three types of sgRNA in the *CrACCase* gene, namely green, red, and orange types. The different types of sgRNA regenerated in chop-chop tools depend on the type of PAM especially at the NGG site in the *CrACCase* genome. The most sgRNA was found in the red type, while the least in the green type. however, the green type SgRNA ranked 1st in statistical analysis, genome editing efficiency, and self-complementary. Green type SgRNA, rank 1, was found in the 1089 bp region with negative-strand (-).

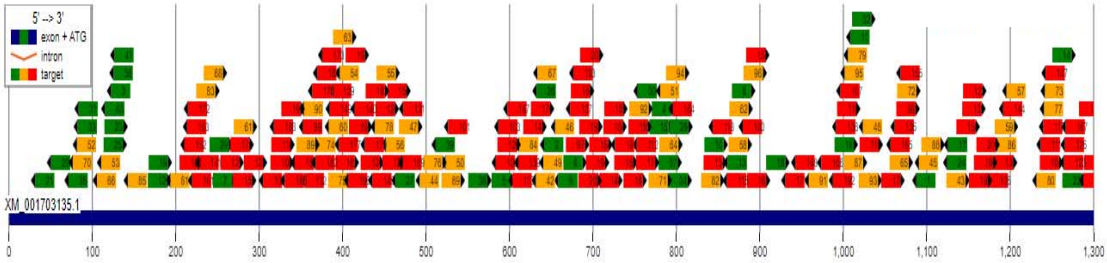


**Figure 1**. Oligo guide putative RNA was generated based on in silico genome editing studies of *CrACCase* genes in *Chlamydomonas reinhardtii*. There are three types of oligo-guide RNA based on genetic testing, namely type 1 (green), type 2 (orange), and type 3 (red). The black arrow indicates the NGG sequence at the protospacer adjacent motif (PAM) site in the *CrACCase* gene region. The accession number for the chop-chop program used in this study is XM_001703135.1. The size of the genome sequence was 1300 bp with an orientation from the 5' end to the 3' end.

A total of 50 selected top hits from a total of 188 sgRNAs were found in this study. The determination of the top 50 hits was mainly based on the efficiency of genome editing of the *CrACCase* gene, however, we also took into account the values of self complementarity, GC content, strand, and genomic location. Based on the parameters, the gene-editing efficiency value of fifty sgRNA Top hits showed a range of 65.98 to 79.27% and GC content of 35 to 65% (Table 1). In the preparation of primers for either sgRNA or ordinary PCR amplicon, it has high efficiency if the GC content value does not exceed 65%. The principle of targeting sgRNA sequences is basically similar to ordinary primer design (Galluzzi *et al.*, 2004; Galluzzi *et al.*, 2010), but this method pays attention to the PAM region in the genome/gene of interest.

Based on these parameters, the three best target sgRNA sequences are GCGTCTGCTCAATCACACGGCGG, TTGAGGTCGGAGAACTCCAGCGG, and AGGCAATACCCTCAATTGGGTGG. The sgRNAs formed was at 1089, 643, and 124 bp nucleotides. The NGG regions of the first and second rank sgRNAs are nucleotide type C, while the nucleotides of the third rank sgRNA are type T nucleotides. Regarding the second rank sgRNA, although it has a high potential for self-complementarity (as much as 2), the value of gene editing efficiency is relatively high at 68.25. % (Table 1). Based

on positive threads, oligo sgRNAs that have the highest efficiency value and have positive (+) threads, the best target sequences are ATGGATTTCACCTACATGGGTGG, ATCAACCACCTCATTGACGCCGG, and GTCTTACACCGACCGCATCAAGG. The three sgRNAs were in the fourth to sixth ranks, respectively, and were located at the 772, 580, and 666 bp nucleotides, respectively. However, we used sgRNA with the First Rank at the 1089 bp location because it has the highest gene editing efficiency value in the *CrACCase* gene from *Chlamydomonas reinhardtii*. Therefore, we further studied the analysis of sequences that cause nucleotide changes/mutations at the protein level, especially in functional areas.

**Table 1**. Fifty top hits guide RNA design for genome editing in *CrACCase* gene region

| Rank | Target sequence | Genomic location | Strand | GC content (%) | Self-complementarity | Efficiency |
|---|---|---|---|---|---|---|
| 1 | GCGTCTGCTCAATCACACGGCGG | XM_001703135.1:1089 | - | 60 | 0 | 79.27 |
| 2 | TTGAGGTCGGAGAACTCCAGCGG | XM_001703135.1:643 | - | 55 | 2 | 68.25 |
| 3 | AGGCAATACCCTCAATTGGGTGG | XM_001703135.1:124 | - | 50 | 0 | 65.17 |
| 4 | ATGGATTTCACCTACATGGGTGG | XM_001703135.1:772 | + | 45 | 1 | 64.36 |
| 5 | ATCAACCACCTCATTGACGCCGG | XM_001703135.1:580 | + | 50 | 0 | 62.68 |
| 6 | GTCTTACACCGACCGCATCAAGG | XM_001703135.1:666 | + | 55 | 0 | 62.17 |
| 7 | TGGTTGCAATGGAGTCAGGATGG | XM_001703135.1:246 | + | 50 | 1 | 62.59 |
| 8 | ATCATTGTGTGCACCTCGGGCGG | XM_001703135.1:868 | + | 55 | 0 | 60.75 |
| 9 | TGCGGTCGGTGTAAGACTTGAGG | XM_001703135.1:660 | - | 55 | 1 | 61.44 |
| 10 | ATCGAGTACGCCACGCAGGAGGG | XM_001703135.1:835 | + | 60 | 0 | 60.23 |
| 11 | GAACGAGGCCGTCACACCACCGG | XM_001703135.1:1010 | - | 65 | 0 | 60.2 |
| 12 | TAGATTACTGATCTAACGCTCGG | XM_001703135.1:168 | + | 35 | 0 | 60.1 |
| 13 | CGAGGTGCACACAATGATGACGG | XM_001703135.1:863 | - | 50 | 0 | 57.51 |
| 14 | AAGCGCGGCATGATCCCCTTCGG | XM_001703135.1:1252 | + | 60 | 0 | 56.56 |
| 15 | GTCATGGATTTCACCTACATGGG | XM_001703135.1:769 | + | 40 | 0 | 56.34 |
| 16 | ATCCTCACCTCGCCTACCACCGG | XM_001703135.1:991 | + | 60 | 0 | 54.12 |
| 17 | CTCCGCAGTCTGGAAGTCGTCGG | XM_001703135.1:1127 | - | 60 | 2 | 56.09 |
| 18 | CATCTTTTCGCTCATGCAGATGG | XM_001703135.1:909 | + | 45 | 0 | 53.93 |
| 19 | AGATTACTGATCTAACGCTCGGG | XM_001703135.1:169 | + | 40 | 0 | 53.55 |
| 20 | GGTAAAAGTGCTCGCTGACAAGG | XM_001703135.1:52 | - | 50 | 0 | 53.26 |
| 21 | AAGGTGTCAAAACTTGCGTTTGG | XM_001703135.1:33 | - | 40 | 0 | 53.18 |
| 22 | ATCCCCTTCGGCGTGCAGCACGG | XM_001703135.1:1264 | + | 65 | 2 | 54.71 |
| 23 | GCCAATTACCACCCAATTGAGGG | XM_001703135.1:116 | + | 45 | 0 | 52.43 |
| 24 | TCCGCAGTCTGGAAGTCGTCGGG | XM_001703135.1:1126 | - | 60 | 2 | 54.26 |
| 25 | CGCCAATTACCACCCAATTGAGG | XM_001703135.1:115 | + | 50 | 0 | 50.99 |
| 26 | GAGAACTCCAGCGGGTCCACGGG | XM_001703135.1:634 | - | 65 | 0 | 50.54 |
| 27 | ACTTGTCGCACCGGGTCCACAGG | XM_001703135.1:465 | - | 65 | 0 | 49.58 |
| 28 | GGATCCATGGGCAGTGTGGTGGG | XM_001703135.1:793 | + | 60 | 0 | 49.36 |
| 29 | AGCGTGGTTGCAATGGAGTCAGG | XM_001703135.1:242 | + | 55 | 1 | 50.04 |
| 30 | ATCCATGACGCCCAGCGCCACGG | XM_001703135.1:755 | - | 65 | 0 | 48.38 |
| 31 | GCACAAAAGCTGAGCTCGCGTGG | XM_001703135.1:85 | - | 60 | 1 | 48.95 |
| 32 | GGTGGTGTGACGGCCTCGTTCGG | XM_001703135.1:1012 | + | 65 | 0 | 47.47 |
| 33 | CACAAAAGCTGAGCTCGCGTGGG | XM_001703135.1:84 | - | 55 | 1 | 47.52 |
| 34 | TGGATCCATGGGCAGTGTGGTGG | XM_001703135.1:792 | + | 60 | 0 | 44.25 |
| 35 | AGCTCGCGTGGGGCAAGGATAGG | XM_001703135.1:73 | - | 65 | 0 | 44.23 |
| 36 | GCAAGGCAATACCCTCAATTGGG | XM_001703135.1:127 | - | 45 | 0 | 43.63 |
| 37 | CACCTACATGGGTGGATCCATGG | XM_001703135.1:780 | + | 55 | 2 | 43.78 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 38 | CCATCTCAAGATGAGCTCTATGG | XM_001703135.1:552 | + | 45 | 1 | 41.41 |
| 39 | AGATGTGGTGGTGCTCCTTCAGG | XM_001703135.1:513 | - | 55 | 0 | 37.42 |
| 40 | ACCCTCAATTGGGTGGTAATTGG | XM_001703135.1:117 | - | 45 | 1 | 36.58 |
| 41 | TGCAAGGCAATACCCTCAATTGG | XM_001703135.1:128 | - | 45 | 0 | 31.92 |
| 42 | AGAACTCCAGCGGGTCCACGGGG | XM_001703135.1:633 | - | 65 | 0 | 69.11 |
| 43 | GCCCGACGACTTCCAGACTGCGG | XM_001703135.1:1125 | + | 65 | 0 | 69.03 |
| 44 | CAGGTGCTTGATGTACAGGATGG | XM_001703135.1:494 | - | 50 | 0 | 67.77 |
| 45 | GCAGCGTCTGCTCAATCACACGG | XM_001703135.1:1092 | - | 55 | 1 | 67.63 |
| 46 | GTCGGTGTAAGACTTGAGGTCGG | XM_001703135.1:656 | - | 50 | 1 | 66.82 |
| 47 | TGGACCCGGTGCGACAAGTGCGG | XM_001703135.1:469 | + | 65 | 0 | 65.77 |
| 48 | GTCTCCCAGCATGCCGAACGAGG | XM_001703135.1:1025 | - | 65 | 0 | 65.4 |
| 49 | TGAGGTCGGAGAACTCCAGCGGG | XM_001703135.1:642 | - | 60 | 2 | 67.22 |
| 50 | TGCAGCCAAAGCAGATGTGGTGG | XM_001703135.1:525 | - | 55 | 1 | 65.98 |

Note: XM_ XM_001703135.1 is an accession of the *CrACCase* gene in chop-chop tools. The last nucleotide in the target sequence represents the NGG nucleotide in the protospacer adjacent motif (PAM) region of the *CrACCase* gene in *Chlamydomonas reinhardtii*. The + sign indicates the positive thread with the 5'-3' orientation, while the - sign indicates the negative thread with the 3'-5' orientation.

### *3.2. Crystal Structure Prediction of CrACCase Protein*

Based on the information on the sgRNA construction of the *CrACCase* gene at the -1089 bp nucleotide region with a negative orientation, we tried to find the functional region and crystal structure of the CrACCase protein. We used the ORION, SWISS-MODEL, Modeller, and I-TASSER programs to search for information on changes in amino acid residues of the sgRNA regions we designed. We found four crystal structures of CrACCase which have a similarity (seq identity) reaching 49.62 %. We found four crystal structure models of putative proteins similar to CrACCase, namely models 1, 2, 3, and 4. Model 1 is a protein similar to the acetyl-coenzyme A carboxylase carboxyl transferase beta subunit commonly found in *Staphylococcus aureus*. Model 2 is a protein similar to the hypothetical methyl malonyl-CoA decarboxylase alpha subunit commonly found in *Sulfolobus tokodaii*. Model 3 is a protein Acetyl-CoA carboxylase which is commonly found in Acetyl-CoA carboxylase in complex with compound 1, especially in yeast. Model 4 is the Acetyl-Coa Carboxylase protein ACC2 CT Domain in Complex with Inhibitor commonly found in Bovine (Figure 2). Based on the value of sequence identity, we chose the putative protein model 1 for further analysis and we made it a template for determining the functional regions of the CrACCase protein.

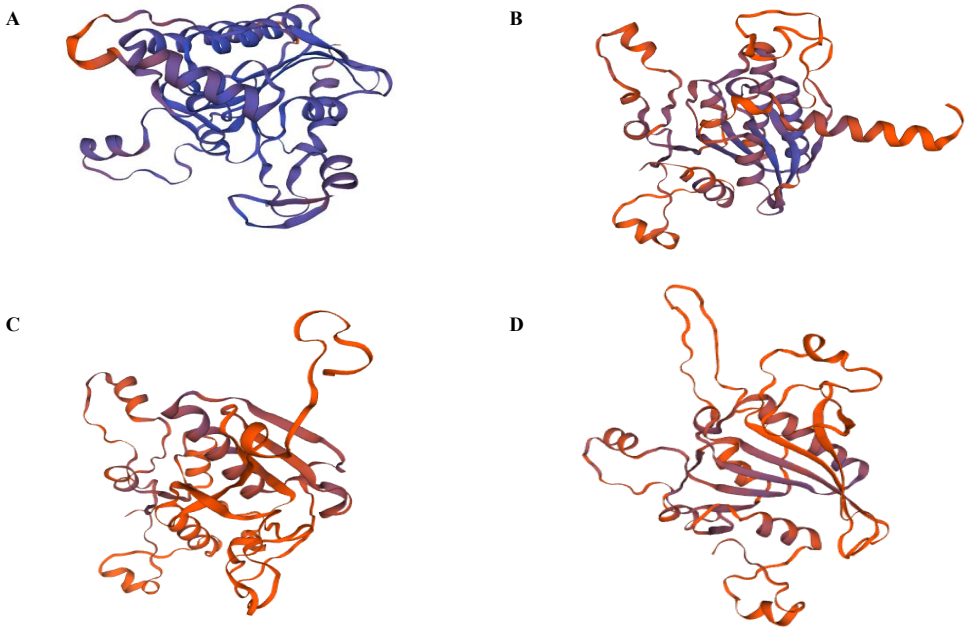


**Figure 2.** Prediction of the protein structure of CrACCase in *Chlamydomonas reinhardtii* using the SWISS-MODEL, I-TASSER, ORION, and MODELLER programs. The putative protein structures are acetyl-coenzyme A carboxylase carboxyl transferase subunit beta, Crystal Structure of the carboxyltransferase subunit of ACC (A). Hypothetical methyl malonyl-CoA decarboxylase alpha subunit, Crystal Structure of the carboxyl transferase subunit of putative PCC of *Sulfolobus tokodaii* (B). Acetyl-CoA carboxylase, Crystal Structure of the humanized carboxyltransferase domain of yeast Acetyl-CoA carboxylase in complex with compound 1 (C). Acetyl-Coa Carboxylase. Bovine ACC2 CT Domain in Complex with Inhibitor (D).

### *3.3. Functional Region Analysis of the CrACCase Protein*

Analysis of functional areas of CrACCase protein can be studied in several ways, one of which is by identifying important amino acid residues. The putative CrACCase protein was analyzed by general composition, glycine composition, proline composition, and pre-proline composition. Based on the Ramachandran Plots, the composition of amino acid residues in CrACCase protein is found at three main centers, while in the composition of Glycine there are six main points with 2 main centers that are opposite the residue values. The amino acid residue composition of proline shows two central values of Ramachandran Plots, whereas pre-proline is located at one main center point (Figure 3).

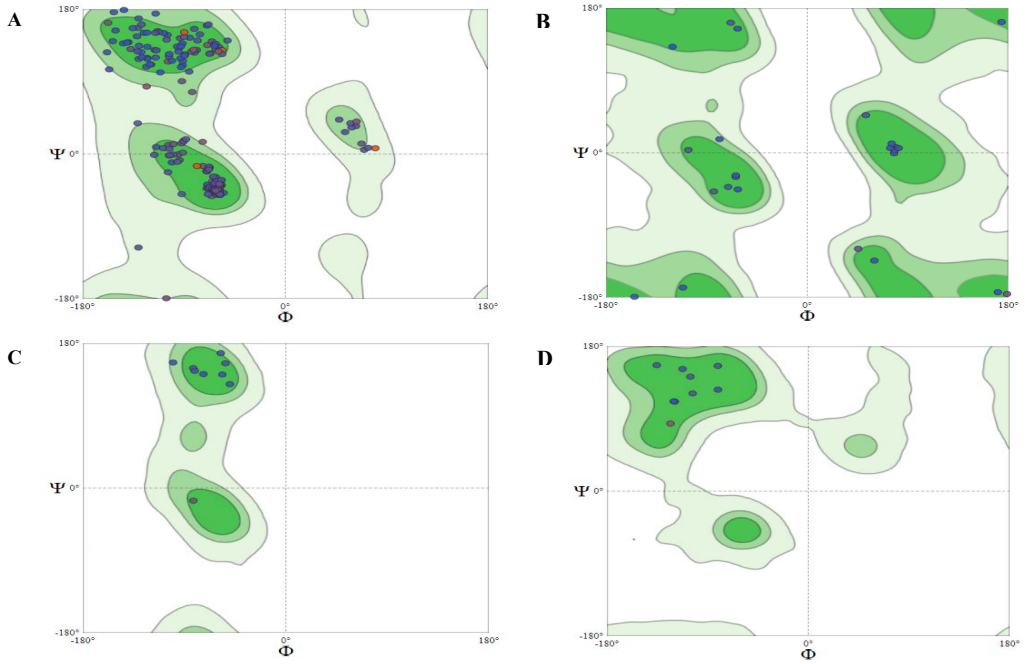


**Figure 3.** Ramachandran Plots of Amino Residue of putative protein CrACCase in *Chlamydomonas reinhardtii*. General (A), Glycine (B), Proline (C), Pre-Proline (D). Amino acid residues are generally concentrated at three main points. Amino acid residues were tested using the SWISS-MODEL program.

In addition to the composition of the amino acid residues, the functional regions of the CrACCase protein can be tested with local quality estimates, Z-scores of the non-redundant set of PDB structure, Global QMEANDisCo values, and QMEAN Z-scores (Figure 4). Based on local quality estimates, amino acid residues with values of 0.3-0.8 were found around the 150th and 260th residues. The majority of amino acid residues had predicted local similarity values of 0.7-0.9 at residues 90 to 340 (Figure 4A). The amino acid model constructed in this study showed a Z-score between 0.5 and 1.0, so the putative protein structure model tested in this study showed a high level of confidence (Figure 4B). Global QMEANDisCo value in this study also showed a value above 75 percent with details of QMEAN value of -0.76, Cβ of -0.88, All-atom of 0.41, solvation value of -0.34, and torsion of -0.54 (Figure 4C).

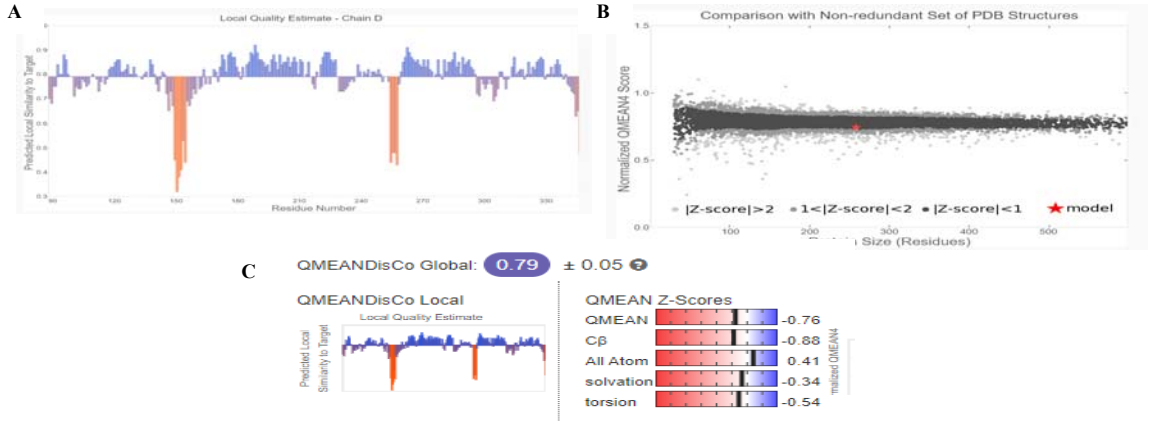


**Figure 4.** Quality estimates of the structure of the CrACCase protein in *Chlamydomonas reinhardtii*. Local quality estimates (A), Comparison PDB structure (B), and QMEANDisCo Global Value (C) of CrACCase protein structure.

### *3.4. Interaction between CrACCase Mutant Protein and Zinc Ligand*

The putative sequence of CrACCase showed a structural similarity with the template at the 90th to 346th amino acids. Based on protein sequence investigations, putative proteins had relatively similar QMEAN values for almost all amino acid residues (Figure 5A). Changes in frameshift mutations in the *CrACCase* gene are located at the 1086 bp nucleotide and the amino acids after that change and result in changes in protein structure. In addition to the structure, it also resulted in a change in the functional area of the ligand-protein binding site at residues D:C 92, 95, 111, and 114 where this site is a zinc ion binding site. The D or C residues are amino acid residues that code for cysteine and aspartic acid. These two amino acids play an important role in the binding of ligands in the form of zinc ions (Figure 5B, 5C). Four residues bind to zinc ions to form a D:C chain and four PLIP interactions form a metal complex structure. Based on the search of the ligand database on the RCSB PDB, we got information that the zinc ligand type in the putative protein CrACCase has a cation in the form of $zinc^{+2}$ cation, Zn formula, molecular weight 65.41, non-polymer type, isomeric smiles [Zn+2], InCHl = 1S /Zn/q+2, InChlKey PTFCDOFLOPIGGS-UHFFFAOYSA-N. In addition, the zinc ligand has a formal charge of 2, an atomic count of 1, and does not have a chiral atom count, bond count, and aromatic count. Thus, the bioinformatics construction of sgRNA can lead to changes in protein structure and changes in the binding of zinc ligands to the protein active site of CrACCase in *Chlamydomonas reinhardtii* species.

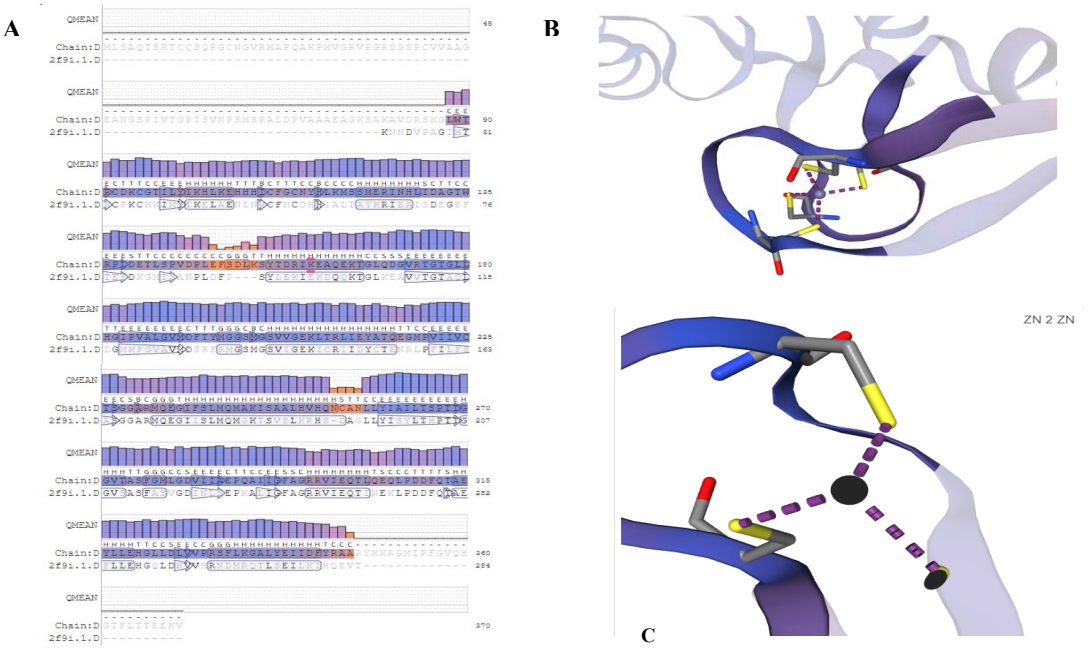


**Figure 5.** Amino acid residue analysis using SWISS-MODEL analysis. Alignment between CrACCase and protein template model (A), Ligand-amino acid residue interaction (PLIP/chain D interaction) in the active site of CrACCase protein (B), and Zinc-Zinc (ZN 2 ZN) binding (C).

## 4. Discussion

*Chlamydomonas reinhardtii* is a microalga belonging to the Division Chlorophyta, Classis Chlorophyceae, Ordo Volvocales, Family Chlamydomonadaceae, and Genus *Chlamydomonas* (Wilson and Loomis, 1962; Bold and Wynne, 1985; Barsanti and Gualtieri, 2006; Ghufran and Kordi, 2010). This organism is used very little as a source of biofuel even though Rengel *et al.*, (2018) reported that the content of triacylglyceride (TAG) is 2.4 times more than other microalgae species (Hariyanti, 1994; Yani, 2003; Harada *et al.*, 2007; Hubbard *et al.*, 2008; Bellinger and Sigee, 2010; Joo *et al.*, 2010). In recent studies, we developed the potential of the use of *C. reinhardtii,* especially as a resource of TAG content and Biofuel. We improved the in-silico method to find the potency of gene and protein of Acetyl-CoA Carboxylase (CrACCase) and predict their mutant. In this study, we provided Oligo guide putative RNA to engineer or create mutant genes of *CrACCase*. Based on our investigation, the best target sgRNA sequences were GCGTCTGCTCAATCACA CGGCGG, TTGAGGTCGGAACTCCAGCGG, and AGGCAATACCCTCAATTGGGGG (Figure 1, Table 1).

Changes in DNA structure in the functional area of a gene can replace the function and structure of a protein (Sharma *et al.*, 2018) including *CrACCase*. Based on information on changes in the mutant sequence in genome editing of sgRNA, we found that there were changes in the secondary, tertiary, and quaternary structure of the CrACCase protein, especially in the complex structure of the domain, β-sheet, and number of α-helix (Figure 2). Changes in the functional area of the CrACCase protein are thought to affect the function of CrACCase in acting as a key enzyme in the fatty acid biosynthesis pathway (Sharma *et al.*, 2018; Miftahudin *et al.*, 2021; Halim *et al.*, 2021). The enzymes CrACCase, Acetyl CoA synthase (ACS), and Acyl-ACP reductase which change their

structure can enhance fatty acid biosynthesis in microalgae, especially *C. reinhardtii* (Sharma *et al*., 2018). We suspect that genetically modified mutations in the *CrACCase* mutant in-silico could alter the structure and function of the enzyme and further increase TAG production and lipid biosynthesis. The production of mutants and transgenic over-expression of genetic engineering has been shown to increase the production of TAG in Microalgae, i.e., *C. cryptica*, *D. salina*, *Schizochytrium* sp., and *C. merolae* (Sharma *et al*., 2018). Genetic engineering of the *CrACCase* gene can be carried out further by considering the bioinformatics test data that we have done. Bioinformatics prediction with genome editing CRISPR/Cas9 was also commonly used in other organisms such as rice in studying *DLT* and *OsGERLP* genes (Miftahudin *et al*., 2021; Halim *et al*., 2021). In-silico information on both genes was proven after genetic engineering resulted in mutants and transgenics that were different from their wild type especially in the mRNA gene expressions (Siska *et al*., 2017; Fendiyanto *et al*., 2021; Satrio *et al.*, 2021; Miftahudin *et al*., 2021). Specifically for the microalgae *C. reinhardtii*, we examined the functional regions of the protein comprehensively and looked at the ligand-binding region, as well as changes in enzyme function.

Recently, mass microalgae culture with bioreactors (Peng *et al.*, 2016) can be used as an alternative to meet bioenergy needs, especially biodiesel (Brennan and Owende, 2010; Sharma *et al*., 2018). However, the efficiency of lipid production through the triacylglyceride metabolic pathway (TAG), is vital to study to increase lipid production in microalgae (Cooksey *et al*., 1987; Duong *et al*., 2015; Sharma *et al*., 2018). Especially for the species *Chlamydomonas reinhardtii*, genome editing of the ACCase gene has not been reported; therefore, this study is the first step in carrying out genetic manipulation to increase lipid production. In addition to increased lipid production with biocatalysts (Rodolfi *et al*., 2009; Yun *et al*., 2014), a genome editing approach becomes important. This genetic engineering can be done by looking for markers both at the gene level (Diaz *et al*., 2010; Fendiyanto *et al*., 2019a; Miftahudin *et al*., 2021), genome (Countway *et al*., 2005; Zhu *et al*., 2005; Fendiyanto *et al*., 2019b; Pratami *et al*., 2020), mRNA (Satrio *et al*., 2019), or metabolites (Chini-Zittelli *et al*., 2006; Ramasamy *et al*., 2015; Fendiyanto *et al*., 2020; Fendiyanto *et al*., 2021) in metabolic pathways certain.

Changes in the structure of amino acid residues in CrACCase seemed to change greatly based on the residue composition of Proline, Glycine, and Pre-Proline (Figures 3, 4). Changes in protein structure residues in Proline and Glycine are related to changes in the function of an enzyme according to Sharma *et al.*, (2018). The interaction of the CrACCase enzyme was also validated concerning the PLIP/chain D interaction in the active site of CrACCase protein and Zinc-Zinc (ZN 2 ZN) binding (Figure 5). Zinc ligands related to the role of CrACCase protein are thought to be related to regulatory functions or transcription factors that can work more actively in the presence of zinc ions. This structural and functional change resulted in the mode of action of the CrACCase protein. This bioinformatics information is important to perform in-vivo genome editing on the *CrACCase* in the future so that mutants with the highest TAG production or the highest biodiesel (oil body) producer can be obtained. The manipulation of the *CrACCase* gene in *Chlamydomonas reinhardtii* can be applied to other microalgae organisms with the highest lipid percentages to increase future bioenergy production by molecular biologists and biotechnologists.

## 5. Conclusion

The location of the mutation of the *CrACCase* gene, the result of genome editing using chop-chop tools, was found in the XM_001703135.1:1089 region, especially in the negative thread. The putative protein of CrACCase was predicted to have the structure of carboxyl transferase subunit of ACC, carboxyl transferase subunit of putative PCC, humanized carboxyltransferase domain of yeast Acetyl-CoA carboxylase, and Acetyl-CoA Carboxylase. Changes in frameshift mutations in the *CrACCase* gene result in structural changes of the functional regions of the ligand-protein binding sites at residues D:C 92, 95, 111, and 114 where these sites are zinc ion binding sites. This structural change results in a change in the function of the CrACCase protein. Manipulation of the *CrACCase* gene in *Chlamydomonas reinhardtii* can also be used as a source of information to biotechnologists and biologists to increase the production of lipids or TAG as an alternative to biodiesel or renewable bioenergy in the future.

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