Markerless Genome Editing in *C.glutamicum*

Using CRISPR-Cas9

by

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A Thesis Presented in Partial Fulfillment
of the Requirements for the Degree
Master of Science

Approved April 2024 by the
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ARIZONA STATE UNIVERSITY

May 2024

ABSTRACT

Metabolic engineering has emerged as a highly effective approach to optimizing industrial fermentation processes by introducing purposeful genetic alterations using recombinant DNA technology. Successful metabolic engineering begins with a careful investigation of cellular function, and based on the outcomes of this analysis, an improved strain is created and then constructed using genetic engineering. By modifying the genetic makeup of cells, can increase the production of important chemicals, biofuels, medications, and agricultural products. The most often used genetic engineering tool is plasmid-based gene editing. In plasmid-based gene editing, the desired gene sequence is flanked by similar genome sequences, which encourages the foreign gene's integration into the genome. The main flaw of plasmid-based editing is the presence of selectable markers in the integrated DNA, which impacts cell stability as well as downstream applications that are critical to industries. Recently, with the growth of science, the new gene-editing technology CRISPR (clustered regularly interspaced short palindromic repeat) has revolutionized the field of gene editing. It has been used to incorporate the foreign genes into the genome of the microbial host without any mark and has more efficiency than the plasmid-based gene editing technique. CRISPR is utilized to achieve markerless integration of genes in genomes of microbes, which promotes cell stability and is also especially beneficial for applications in industries. In this experiment successfully integrated two genes into the genome of *C.glutamicum* employing markerless integration via homologous recombination, allowing cells to metabolize acetate into acetyl-CoA and improve the conversion of pyruvate into lactate. Further, this strain of *C.glutamicum* can be utilized as a platform for producing ethyl lactate, a green solvent using a microbial host.

ACKNOWLEDGMENTS

There are many people I would love to thank for their support in my project and throughout my time at Arizona State. First and foremost, thank you Dr. Arul Varman for your advice, kindness, and investment in my growth – I have learned so much from you and am so glad to have been a member of the lab. Thank you Nima Hajinajaf, Arren Liu, Apurv Mhatre, and Dr. Aditya Sarnaik for your encouragement and enthusiastic mentorship. Thank you Jackson Comes for helping with experiments and being my first research partner. Thank you to my lab mates Nandini Kannoju, Haley McKeown, Rachel Rick, Pedro Ochoa, Tegun Young, Sarai Ramani, Tyler O'Kane, Zulema Guzman, Linda Toma, and Reem Alamer for making our lab feel like a home and for inspiring me to always do and be my best. Thank you to Dr. David Nielsen and Dr. Jong Seto for your support on my thesis committee.

Outside of this lab, I extend my heartfelt gratitude to my mother and sister for affording me this opportunity, as well as to you, my family, for your unwavering support. Additionally, I am deeply thankful to Vanshika Kamble, Jayesh Katade, Dev Shroff, Kartike Dhote, Ketan Patil, Manthan Kotpalliwar, Nayan Zope, Vivek Chaware, Akash Padval, Harsh Sant, Pragati Daryanani, Shresth Vyas, Nachiket Ayare, and Jeet Badki, for your support and encouragement. Your support and belief in me have been invaluable throughout this journey, and for that, I am forever grateful.

Finally, thank you to Arizona State University, the Fulton Schools of Engineering, and the Master's Opportunity for Research in Engineering (MORE) for funding this project.

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CHAPTER 1

INTRODUCTION

Recently microbial communities have been researched to overcome many energy, fuel, and food demands. Because of their inherent complexity and versatility, these communities provide an appealing approach in a variety of sectors. Unlike single-species systems, microbial communities are made up of various populations of microorganisms that interact synergistically in their environment. This variety allows them to effectively break down complex organic substances, create useful metabolites, and adapt to changing environmental circumstances. Furthermore, microbial communities have a remarkable capability for self-regulation and resilience, allowing them to remain stable in turbulent environments. Leveraging microbial communities gives a comprehensive view of biological processes, including insights into ecosystem dynamics, biogeochemical cycles, and ecological interactions. Harnessing the power of microbial communities is a possible route for long-term solutions to a variety of socioeconomic and environmental issues.

1.1 Development of gene editing tools:

Advances in molecular biology, genetics, and biotechnology have resulted in substantial evolution of gene editing approaches. Early gene editing approaches relied on simple procedures like restriction digestion, which helps to cut the DNA at a certain spot and allows the researcher to insert a gene using DNA ligation enzymes. This technology established the groundwork for genetic engineering by giving a tool for inserting, deleting, or modifying the genome's unique DNA sequence. However, due to the enzymes' capacity

to detect short DNA sequences, this technique has limited flexibility. Furthermore, ligating the other DNA fragment may cause mutations and mistakes, altering the desired outcome. Due to these reasons, Restriction Digestion is not preferred as a gene editing tool.

Genome engineering approaches have advanced genome research, allowing for applications in a variety of industries such as medicine, agriculture, food, and energy. The development of transcription-activator-like effector nucleases (TALEN) and Zinc Finger nucleases (ZFNs) gene editing tools has enabled more efficient gene editing than previous methods. Zinc finger nucleases (ZFNs) and Transcription activator-like effector nucleases (TALEN) are artificial restriction enzymes that precisely edit or cleave target DNA. They achieve this by utilizing zinc finger DNA binding domains or TAL effector DNA binding domains, respectively. This approach generates a double-stranded break (DSB) in the target genome, which is then repaired using the error-prone non-homologous end joining (NHEJ) pathway or homology-directed repair (HDR). Although the discoveries of ZFNs and TALEN improved genome editing efficacy, targeting various sites in the genome necessitated the redesign or re-engineering of new sets of proteins. Because of these difficulties, ZFNs and TALENs have not been widely used by researchers as gene editing tools.

1.2 Emerging CRISPR tool:

The CRISPR-based methods are gaining popularity among scientists due to their strong compatibility with bacterial systems. The mechanism of editing involves targeting using a self-designed guide sequence which is complementary to the unique sequence of a site of interest, promoting break at a site which is then repaired at high rates with the help

of homologous recombination. CRISPR-Cas9 is no longer merely a gene-editing tool; the applications of catalytically hindered inactive Cas9, such as gene control, epigenetic editing, chromatin engineering, and imaging, now outnumber the gene-editing capabilities of WT-Cas9. These developments have enabled researchers to take advantage of the CRISPR tools in a variety of scientific advances. CRISPR, with its extraordinary adaptability and precision, is being extensively researched and applied, particularly in the field of human genome editing. This bears the promise of a transformative breakthrough, poised to overhaul the landscape of genetic medicine, and maybe redefine the boundaries of human health and well-being.

CRISPR-Cas9 is an RNA-guided DNA endonuclease that targets a specific gene of interest. CRISPR (clustered regularly interspaced short palindromic repeats) is a set of 30-40 bp short, direct repeat sequences separated by a spacer sequence that matches the foreign DNA sequence. It consists of nuclease Cas9, the crRNA (tracrRNA) that facilitates the processing of the crRNA array into discrete units. The CRISPR-cas9 is marked by the specificity which is owned by guide RNA (gRNA), that permits specific binding to the desired DNA site. This initiates the double-stranded break into the target site and then the gene of interest can be inserted or deleted from the system using homology-directed repair (HDR).

The microbial fermentation of renewable resources into green fuels and chemicals is helping to drive the bio-based economy forward. Production costs for these ecologically friendly methods must be reduced before they can compete with traditional fossil fuel-based industries. To this purpose, bacteria other than the commonly used model organisms, such as Escherichia coli and Saccharomyces cerevisiae, are being assessed for their

potential as production hosts(5). In prokaryotes, most scarless genome engineering approaches rely on selection-counterselection processes. The substances (antibiotics) required for counterselection are sometimes toxic, and there is often a lack of sufficient counterselection markers. Additionally, it decreases the growth rate of cells and increases instability within the cells.

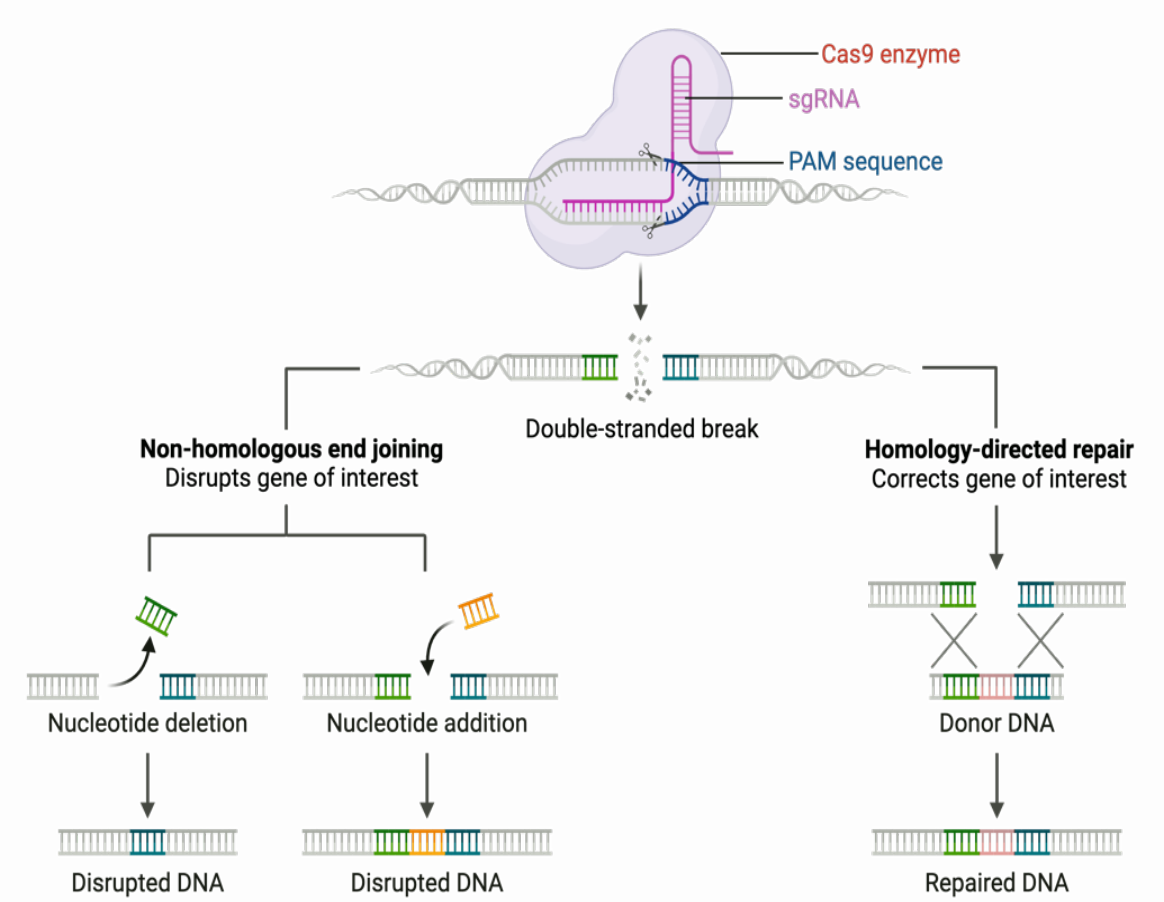

Figure 1: Description of the CRISPR_Cas9 gene editing tool performing double-stranded break and two DNA repair techniques used by the bacteria to repair the broken DNA.

CRISPR-based markerless genome engineering has an advantage over plasmid-based markerless genome engineering. CRISPR does not require a selection-counterselection method to select the engineered strain. CRISPR-Cas9 system can be inserted in a plasmid with the homology arms to initiate the repair after the double-stranded break. In the previous work done by Liang et. al, they successfully engineered *E.coli* using the CRISPR-Cas9 toolbox to produce Isopropanol with 0.75 mol/mol. In the work done by Nagaraju et. al they have successfully engineered *Clostridium autoethanogenum*, using the CRISPR/Cas9 system to make the strain capable of fermenting CO, CO_2, H_2 into biofuel ethanol and 2,3-butanediol. The table below lists the development of certain microorganisms using CRISPR-based tools to produce industrial compounds.

Product	**Bacterial species**	**Modifications**	**Reference**
Isopropanol	*Escherichia coli*	CRISPR-Cas9 (knock-in and RBS replacement of *thl*, *atoDA*, *adc*, *adh*)	16
n-Butanol	*Escherichia coli*	CRISPR knockout of *adhE* gene	17
1,4 BDO	*Escherichia coli*	CRISPR-Cas9 knockout of *lpdA and sad*	18
Glutarate	*Escherichia coli*	CRISPR-Cas9 elimination of *arcA, ldhA, atoB and pflB*	19
Succinate	*Synechococcus elongatus*	CRISPR-Cas9 knockout *glgC*	20
g-Amniobutyr	*Corynebacterium glutamicum*	CRISPR/Cas9 deleted of *Ncgl1221, gabT and gabP genes*	21

ic acid (GABA)			
Free Fatty acid	*Synechococcus elongatus UTEX 2973*	CRISPR-Cas9 *recJ* gene knock-out	22
L-homoserine	*Corynebacterium glutamicum*	CRISPR-Cas9 *ldh, glyr, serA* and *sdaA* genes knock-out	23
Free Fatty acid	*S. cerevisiae*	CRSIPR-Cas9 *pox1,faa1,faa2,pah1,lpp1,dpp1,are1,are2* genes knock-out	24

Table 1: Previous work done by implementing the CRISPR-Cas9 gene editing tool for editing the genome of microbial hosts by knocking out certain genes to enhance the production of the desired chemical.

1.3 Green alternative for petroleum-based chemicals:

At present petroleum-derived chemicals are widely used in many industries as their primary solvent. As of 2022, the demand for petroleum-derived solvents has experienced a significant upsurge, reflecting a robust market appetite for these versatile chemical products. The market for these hydrocarbon solvents is estimated to reach $9.87 billion by 2030. However, this solvent possesses many environmental and health concerns as it contains volatile organic compounds (VOCs). VOCs contribute to air pollution, which has negative impacts on both the environment and human health. Their presence in the atmosphere can produce ground-level ozone and smog, aggravating respiratory problems

and contributing to environmental deterioration. Thus, there is an urgent need to find eco-friendly solvents that can be produced using sustainable processes.

Ethyl lactate, a biodegradable and renewable solvent, has gained significant attention in recent years due to its potential as a green alternative to conventional solvents. Green organic solvents such as ethyl lactate have broad industrial applications and favorable environmental benefits. The use of ethyl lactate can be seen in various industries such as the paint industry, pharmaceutical industry, and skincare industry. Solvents are widely used as a primary component in paints, coating, adhesives, printing inks, and cleaning agents due to their ability to dissolve a wide range of substances. Currently, petroleum-based solvents are extensively used by many industries as their preferred chemical to use as a solvent. Derived from crude oil refining, these solvents encompass a wide range of compounds, including hydrocarbons such as aliphatic, aromatics, and cycloaliphatic. However, the extensive usage of petroleum-based solvents has raised many environmental and health concerns around the world. These solvents often contain volatile organic compounds (VOCs) that contribute to air pollution, and ozone layer depletion and pose a high risk to human as well as animal health due to inhalation or dermal exposure. Extracting and refining these solvents from crude oil requires high energy usage and stands as a leading contributor to air pollution and environmental degradation. There is an urgent need to find a greener and environmentally friendly alternative to these petroleum-based solvents. Due to this there any much research going around the world to find alternatives to produce these solvents by using renewable stocks or producing through sustainable processes to satisfy the demand for eco-friendly solvents.

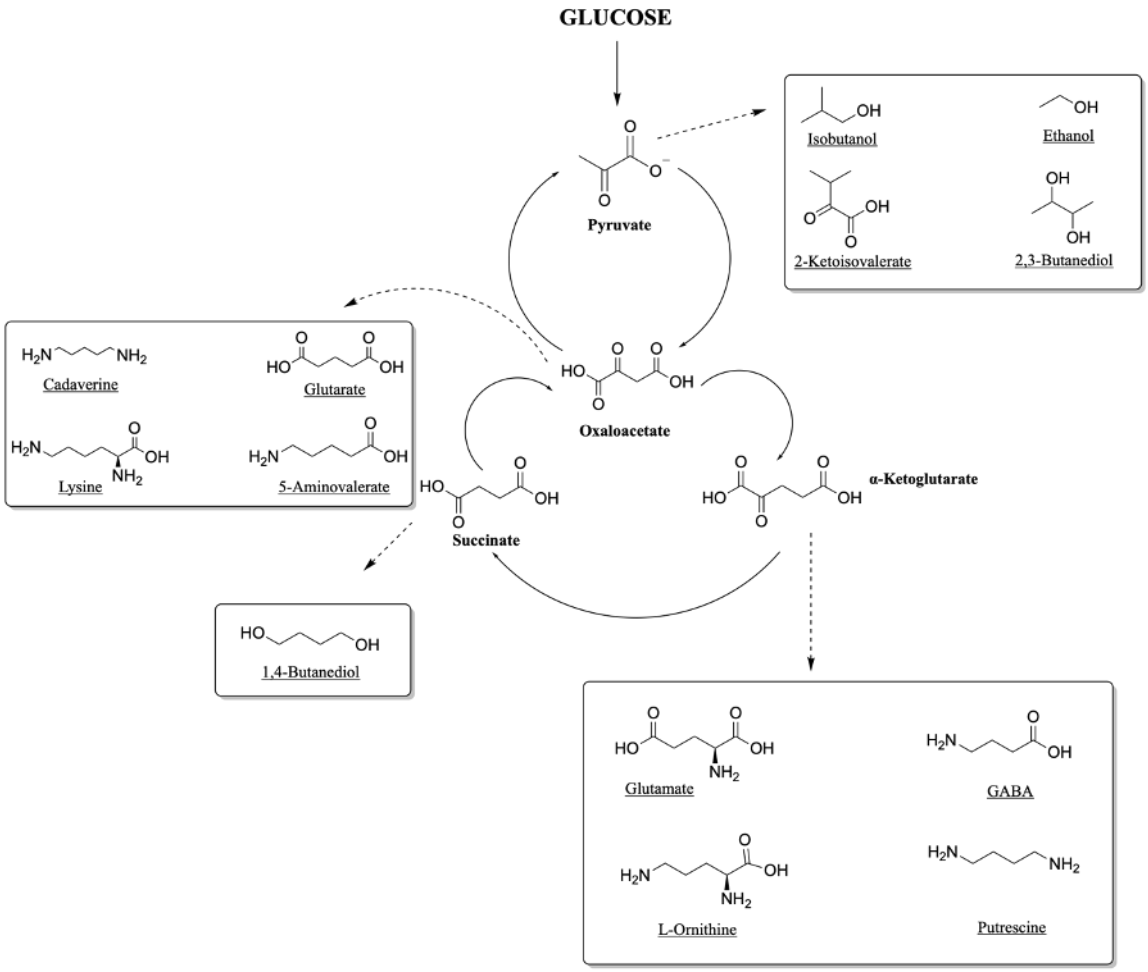

Figure 2: Pathway overview of *Corynebacterium glutamicum* for amino acids and C2–C5 platform chemical production. The pathways are based on enhanced production of key precursors, pyruvate, oxaloacetate, succinate, and α-ketoglutarate. C2 chemicals, ethanol, ethylene glycol, and glycolate. C3 hydroxy acid chemical, 3-hydroxypropionic acid. C3 diols, 1,2-propanediol, and 1,3-propanediol, are enclosed. C4 alcohols, isobutanol, and 2,3-butanediol.

Corynebacterium glutamicum is a fast-growing, aerobic, and non-pathogenic Gram-positive soil bacterium exhibiting physiological traits that could make it an interesting alternative for producing various industrial chemicals. *C. glutamicum* can naturally uptake glucose and further metabolize it to produce specific chemicals. *C. glutamicum* is known to secrete L-lactate, succinate, and amino acids at higher concentrations as fermentation end products from glucose due to its robust glycolytic pathway(27). Owing to the industrial importance of *C. glutamicum*, the genomes of several of its strains have been sequenced(26). The availability of genetic data for *C. glutamicum* led to the discovery of genetic clusters encoding aromatic degradation pathways with the aid of genomic bioinformatics(11). Improved genetic tools and strategies have enabled the successful implementation of heterologous synthetic pathways for the utilization of alternative carbon sources and the production of natural products(28,29). For example, heterologous pathway expression using optimized expression vectors and promoters to produce recombinant proteins, such as endoxylanase, α-amylase, and camelid antibody fragment (VHH), and non-natural metabolites, such as gamma-aminobutyrate, cadaverine, and 5-amino valerate, by *C. glutamicum* has been successfully demonstrated(30).

Engineering *C. glutamicum* for sustainable production of chemicals is potentially profitable because of its several unique advantages (1) flexible cellular metabolism (2) high-stress tolerance to carbon source and target products (3) maintenance of metabolic activity by growth-arrested cells and resistance to fermentation inhibitors (4) genetic stability because lack of recombination repair system and limited restriction-modification system(28).

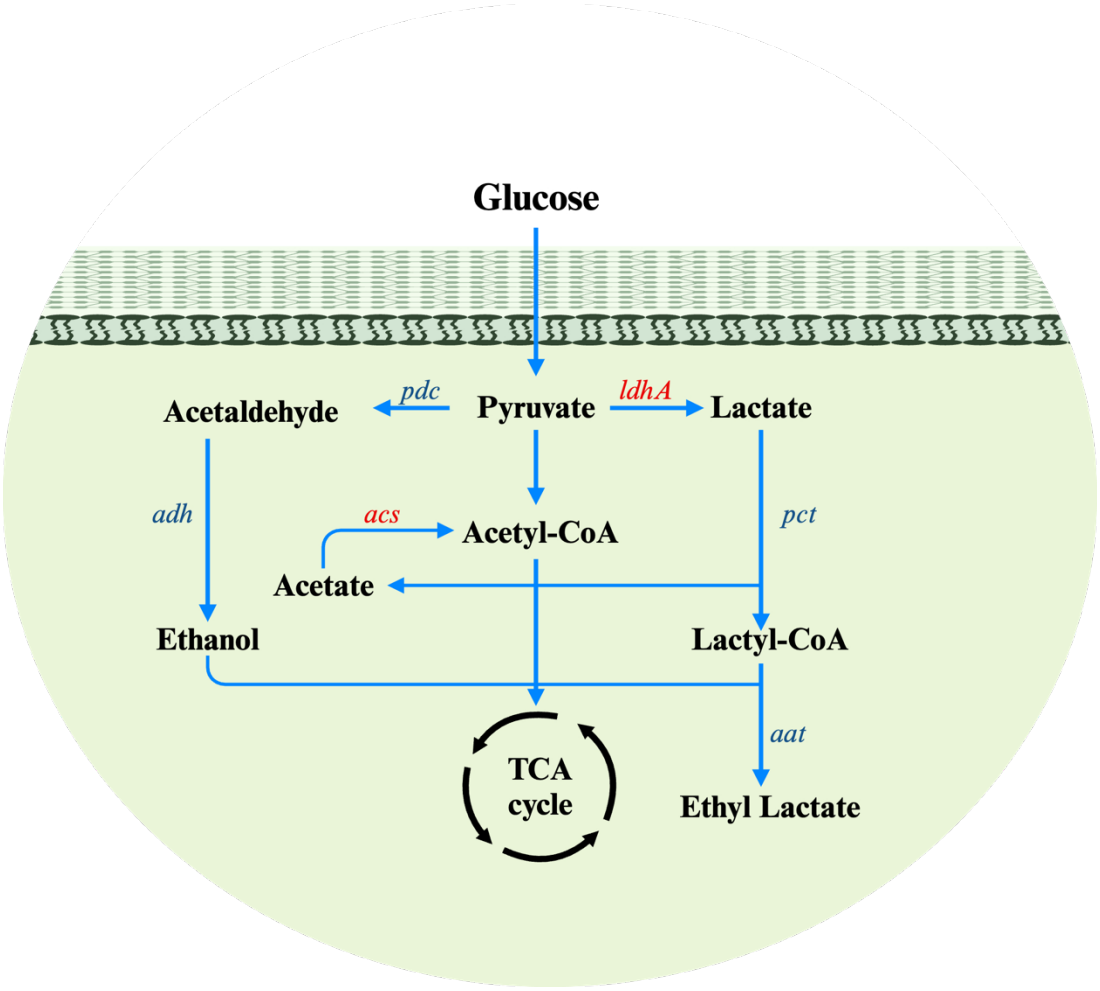

Figure 3: Metabolic Pathway for ethyl lactate production. Genes encoded *pdc*: Pyruvate decarboxylase, *adh*: Alcohol dehydrogenase, *ldhA*: Lactate dehydrogenase, *pct*: Propionyl coenzyme A transferase, *acs*: Acetyl-CoA synthetase. Red-texted genes are incorporated into the genome of the *C. glutamicum.* Blue texted genes are expressed via a plasmid.

1.4 Study focus:

In this study, the *Corynebacterium glutamicum* ATCC 13032 strain is engineered to enhance the production of lactate from pyruvate by knocking out the native *ldh* gene and replacing it with the heterologous *ldhA* gene. Further, incorporating of *acs* gene to uptake acetate and boost the production of acetyl-CoA. The engineering done in this study is carried out by using the CRISPR-Cas9 gene editing tool and the repair after the DSB is performed by homologous recombination. During the homologous recombination, heterologous *ldhA gene* and *acs* genes are integrated into the genome of the *Corynebacterium glutamicum* ATCC 13032 strain. This study emphasizes the idea of markerless integration of the gene into the genome of the microbial host for enhancing metabolic pathways. In the future, this strain can be used as the platform and can be engineered further for the production of green solvents like ethyl lactate.

CHAPTER 2

MATERIALS AND METHOD

2.1 Bacterial Strain and Plasmid

The strain *C. glutamicum* ATCC 13032 was used as the microbial host in this work. *E.coli* DH5α was used as a host for quick replication of engineered plasmids. *E.coli* GM 272 was used as a host for the demethylation of the engineered plasmids before transforming in ATCC 13031. The plasmid pDD02 harboring *ldhA* and *acs* genes was derived from the plasmid pCRB204 as the backbone, pDA04Cr1 and pDA04Cr2 containing CRISPR-Cas9 gene were constructed using pVWEx1araBAD as the backbone. The plasmids after demethylation were then transformed into *C.glutamicum* ATCC 13032 for the metabolic engineering of the microbial host for the uptake of pyruvate and acetate. All the plasmid and bacterial strains are listed below in Table 2.

Strain	Purpose
E. coli DH5α	General Cloning Host
E.coli GM 272	General Cloning Host
C. glutamicum ATCC 13032	Wild-Type ATCC 13032
SB01	*C. glutamicum* strain containing pDD02 *(ldhA and acs)*
SB02	*C. glutamicum* strain containing plasmid pDD02 *(ldhA and acs) and* pDA04Cr1
SB03	*C. glutamicum* strain containing plasmids pDD02 *(ldhA and acs) and* pDA04Cr2
SB04	*C. glutamicum* strain containing *ldhA* and *acs* genes
Plasmid	
pDD02	pCRB204 based recombinant plasmid harboring *ldhA* and *acs* gene with homology arms
pDA04Cr1	pVWEx1araBAD based recombinant plasmid containing CRISPR-Cas9 gene with gRNA target 1
pDA04Cr2	pVWEx1araBAD based recombinant plasmid containing CRISPR-Cas9 gene with gRNA target 2

Table 2: Bacterial strains and plasmid used in this study

2.2 Transformation

The SB01 strain was generated by transforming *C. glutamicum*, as previously described (11). Overnight *C. glutamicum* seed cultures were established in a 50 ml culturing tube with 5 ml of BHIS (brain-heart infusion with sorbitol) media. The overnight cultures were resuspended and transferred to a 250 mL conical flask containing 100 mL BHIS while maintaining an OD of 0.3. The cultures were incubated and cultivated at 30°C and 230 RPM until the optical density at 600 nm [OD600] reached 0.9–1.0. The cells were rinsed twice with ice-cold 10% glycerol (v/v). Finally, the cells were resuspended in 1 mL of 10% glycerol. Each transformation involved mixing 100 µL of competent cells with at least 500ng of DNA. Electroporation was carried out in a 2.0 cm cuvette using a gene pulser system (Bio-Rad Laboratories, Richmond, CA) set to 25 uF, 200 Ω, 2.5 kV, and 5ms. The electroporated cells were immediately transferred to 1 mL of BHIS medium and incubated at 30°C for 2 hours. The cultures were then plated on the appropriate BHI agar plate with the necessary antibiotics and IPTG. Recombinant colonies often formed within 24 hours after plating. Single colonies were chosen to confirm the recombination of the desired gene.

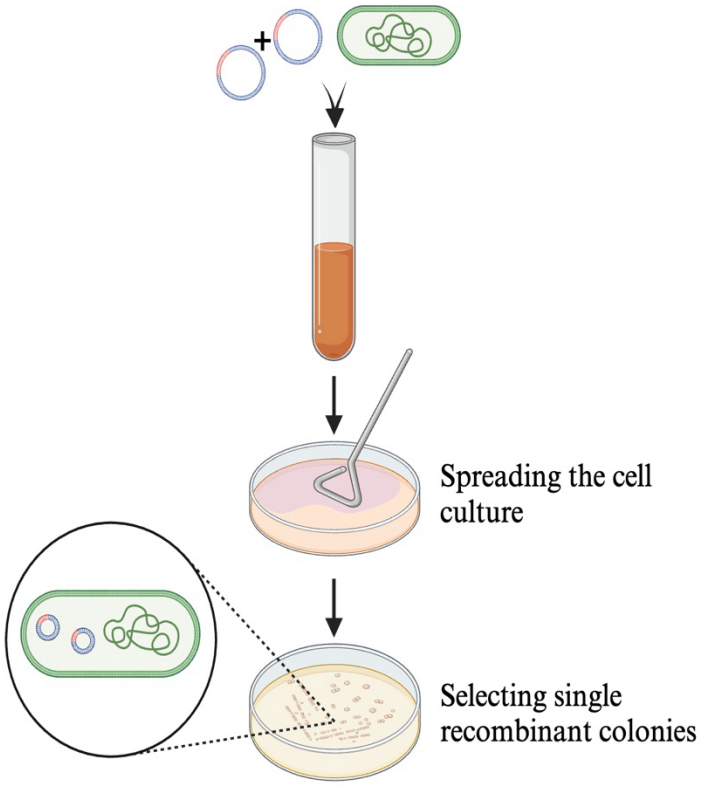

Figure 5: Schematic Representation of Plasmid Transformation into microbial host cells. After recovering the cells patching them on appropriate antibiotic selection to achieve recombinant colonies.

2.3 Media and Culture Conditions

E. coli strains (DH5α and GM 272) were cultured in LB medium supplemented with 25 µg/ml chloramphenicol for pDD02 plasmid and 50 µg/ml kanamycin for pDA04Cr1 and pDA04Cr2 plasmids, at 37°C in a shaker at 250 rpm or on LB agar plates. The *C. glutamicum* strains were cultured in BHI medium or BTM2 medium as required. BTM2 medium was made by adding the following ingredients (per liter): 7 g (NH4)2SO4, 0.5 g KH2PO4, 0.5 g K2HPO4, 0.5 g MgSO4.7H2O, 2 g urea, 8.4 g NaHCO3, 1 × vitamin

solution, and 1 × trace metal solution. The 1,000x trace metal solution (per liter) comprised FeSO4.7H2O 6 g, MnSO4.7H2O 10 g, ZnSO4.7H2O 0.56 g, and CuSO4.5H2O 0.2 g, whereas the 1,000x vitamin solution (per liter) contained biotin and thiamine. Furthermore, the BTM2 medium was added with 2% MOPS to give extra buffering. BHI agar plates were supplemented with chloramphenicol(5µg/ml) and kanamycin(25µg/ml) as required with 1mM IPTG to obtain colonies of the engineered strain at 30°C. Single colonies were inoculated into 5 ml of BHI medium and cultured overnight in 50 ml culture tubes at 30°C and 250 rpm.

2.4 In-vitro Gene Insertion Culture Conditions

To increase the chances of gene insertion into the microbial host different conditions were performed. The single colonies were selected from the plates with chloramphenicol and kanamycin with 1 mM IPTG and cultured in 50 mL culture tubes with 5 mL of BHI media with appropriate antibiotics and 1 mM IPTG overnight till the OD 0.5. Two inoculations of 5µL and 50µL cell culture were transferred into BTM2 media with the same composition as before. Three different concentrations of sodium acetate (10g/L, 14g/L, and 20g/L) were used for the invitro gene insertion analysis. The pH of the BTM2 medium was maintained at 7.0 to provide the appropriate for cell growth. All the liquid cultures were incubated at 30°C at 250 rpm overnight. The cells were grown till the OD reached 1-1.5 and then the culture was diluted accordingly to achieve single colonies. The diluted culture was spread on the BHI agar plates with preferred antibiotics and plates without antibiotics. The gene insertion was confirmed by performing colony-PCR with the

proper set of primers on the recombinant colonies achieved on the plates after incubating at 30°C overnight.

Primer Name	**Sequence**	**Purpose**
ldh2_F	GAAAGGAGAGGATTGATGACTAAAATTTTTGC	Amplification and Confirmation of *ldhA* Gene
ldh_R	ACTAGTTTAGCCAACCTTAACTGGAGTTTCAGC	Amplification and Confirmation of *ldhA* Gene
Cas9_PO_F	CAATTTCACACAGGAAACAGAATT	Amplification and Confirmation of CRISPR-Cas9 Gene
Cas9_Term_R	TATAAACGCAGAAAGGCCC	Amplification and Confirmation of CRISPR-Cas9 Gene
G5' FP	TTTCGCCGGTGCGTACTTTT	CPCR verification of *ldhA* gene
G5' RP	ATCGGCATGGGTCTTGC	CPCR verification of

		native *ldhA* gene
G3' FP	CGTTTTTGTCCACCAGCTG	CPCR verification of *acs* gene
G3' RP	AACCTCTCAATTAGGAGCTTGAC	CPCR verification of native *ldhA* gene
5' ldhA FP	ATGACTAAAATTTTTGCTTACGCAATTC	CPCR verification of *ldhA* gene
5' ldhA RP	TTAGCCAACCTTAACTGGAGTTTC	CPCR verification of *ldhA* gene
3' ACS FP	ATGAGCCAAATTCACAAACACACC	CPCR verification of *acs* gene
3' ACS RP	TTACGATGGCATCGCGATAG	CPCR verification of *acs* gene

Table 3: List of primers used in this study. Specific primers were selected for required amplification.

CHAPTER 3

RESULTS

3.1 Verification of genes

To verify the genes inside the plasmids PCR was performed to determine the proper length of the genes as well as the orientation of the homology arms. First, the genes from the pDD02 plasmid were verified using PCR and gel electrophoresis. The primers used to verify the genes are listed in Table 2. For the verification of the *ldhA* gene inside the plasmid primer set ldh2_F and ldh_R were used while running a PCR reaction, the expected size was around 1000 bp. For the verification of the 3' flanking region primer set 3'FR_F and 3'FR_R were used for the PCR reaction, the expected size of the band was 400 bp. To rectify the entire cargo that is heterologous *ldhA* gene and *acs* gene primer set ldh2_F and 3'FR_R were used to amplify the targeted region, the size expected was 3700 bp. All the regions were correctly amplified and checked with gel electrophoresis. The figure for the gel electrophoresis is shown below with each region labeled. Further, the CRISPR-Cas9 gene was amplified to verify the pDA04CR1 and pDA02CR2 plasmid construction. To amplify the selected region the primer set used was Cas9_PO_F and Cas9_Term_R, the expected size of the band was 4 Kb. The gel image is attached below, showing the correct amplification of the CRISPR-Cas9 gene.

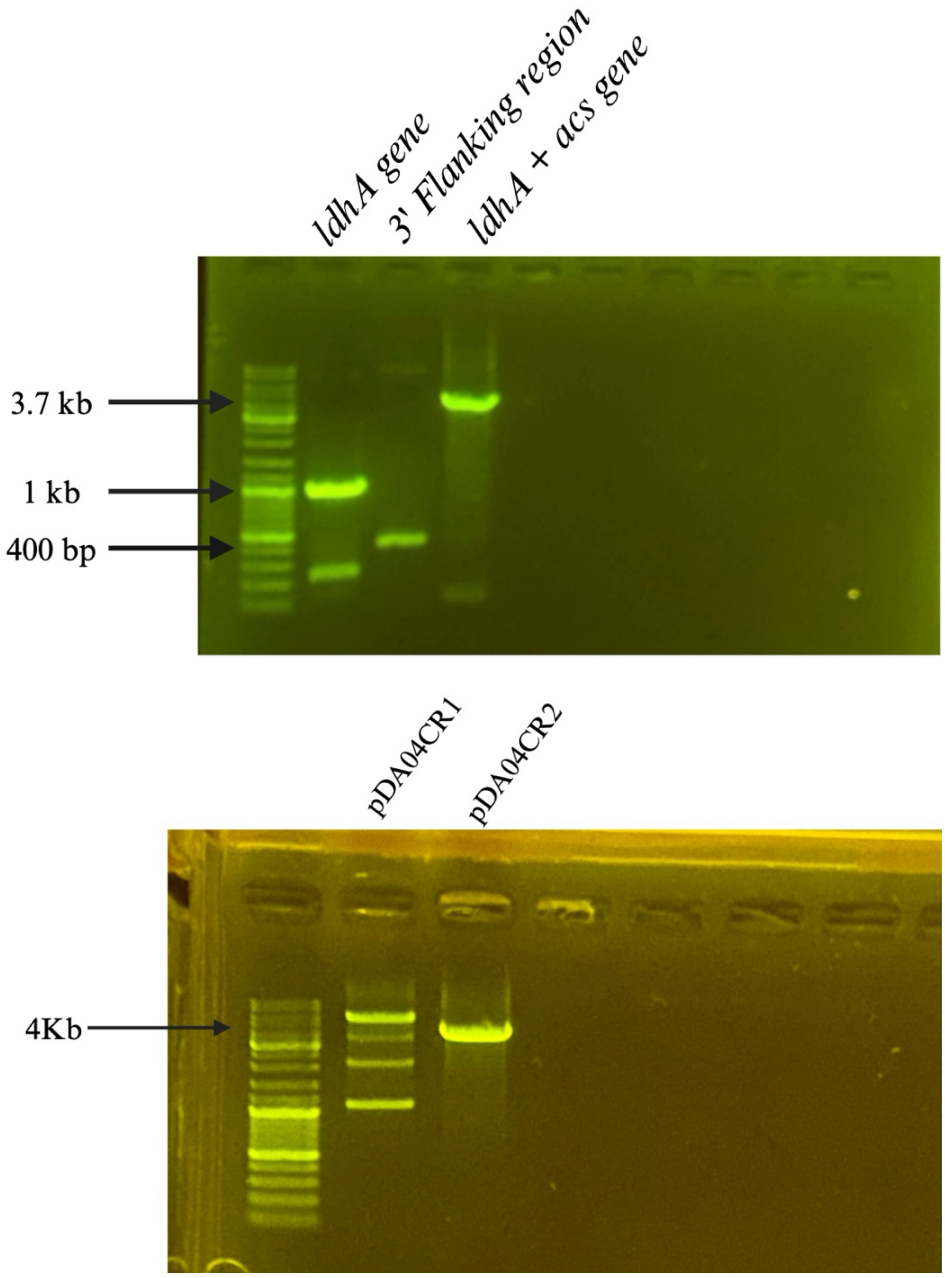

Figure 6: Gel Electrophoresis image for the verification of the selected amplified genes *ldhA, acs* and CRISPR-Cas9 inside the plasmids pDD02, pDA04Cr1 and pDA04CR2.

3.2 Verification of the plasmid inside the cell

After, successfully determining and confirming the plasmids and also the genes inside the plasmid, the mentioned plasmids pDD02, pDA04CR1, and pDA04CR2 were transformed in sequence. First, the pDD02 plasmid harboring the homology arms and foreign gene *ldhA* and *acs* was transformed into the *C. glutamicum* ATCC 13032 by following the transformation protocol mentioned above. The recombinant colonies were achieved after plating the recovered cells on BHI agar plates containing the appropriate selection marker. Colony PCR was carried out to screen the positive colonies for further necessary steps. The gel image for screening of the positive colonies is attached below Figure . The primers listed in Table 2 were used accordingly to amplify the desired region. Selecting the positive colonies, freezer stocks were made to preserve the recombinant cells and for further experimental usage. The selected colonies were made competent for the next step of the transformation.

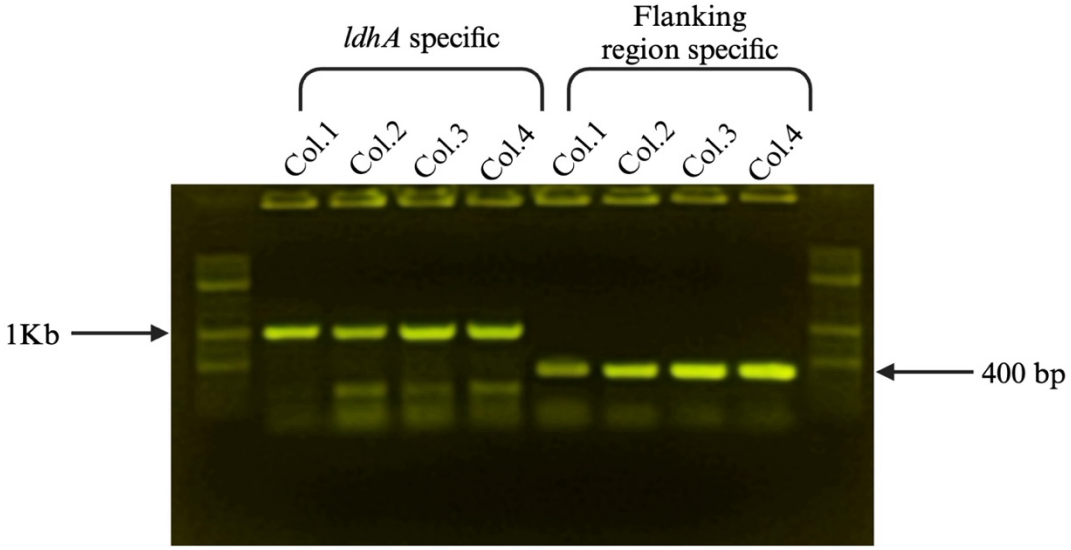

Figure 7: Sample gel electrophoresis from colony PCR confirming plasmids in *C. glutamicum* transformants. Bands obtained from colony PCR show the expected length of DNA.

After this step, the plasmids pDA04CR1 (MOQR) and pDA04CR2 (MPNR) carrying CRISPR-Cas9 and two different guide RNA targets were transformed into the cells already containing the pDD02 plasmid which has the repair for the DSB done my cas9. As the CRISPR-Cas9 performs the double-stranded break (DSB) on the targeted DNA site having the plasmid carrying the homology arm for the DNA repair before is considered optimal as after DSB the cells die due to no proper repair is available. Non-homologous end joining is performed by the cells but due to this, there is an unnecessary mutation that happens during the repair process, due to this selected cargo cannot be introduced into the genome of the *C. glutamicum.* Therefore, the CRISPR plasmids were transformed later for the necessary gene disruption and insertion. Further, the cells were electroporated to achieve high transformation efficiency. The cells were recovered for 2 hrs at 30°C before plating them on the BHI agar plates with necessary antibiotics for positive results.

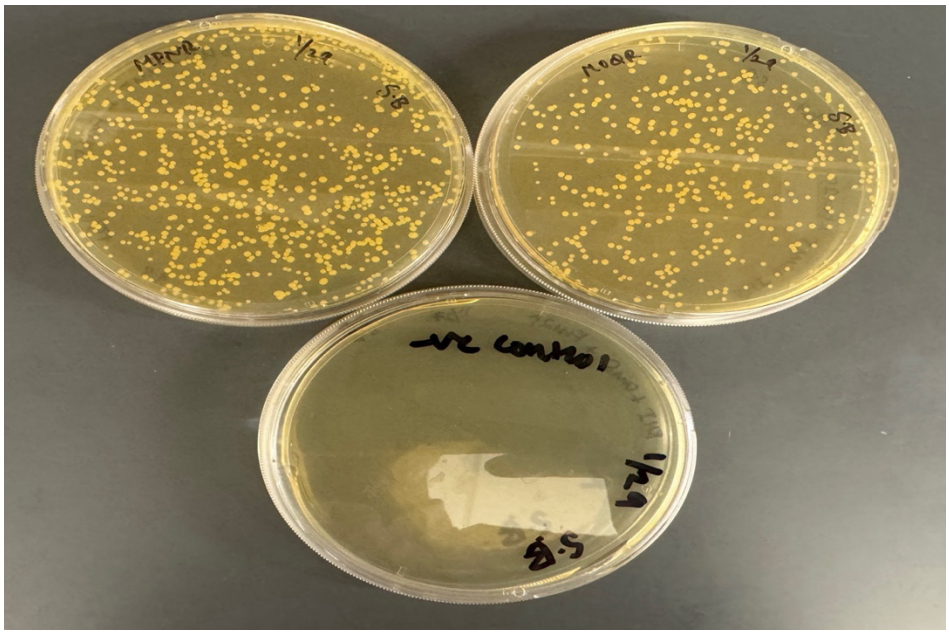

Figure 8: Agar Plate of *C. glutamicum* transformants. Electroporation showed high transformation efficiency, yielding many colonies with positive colony PCR results.

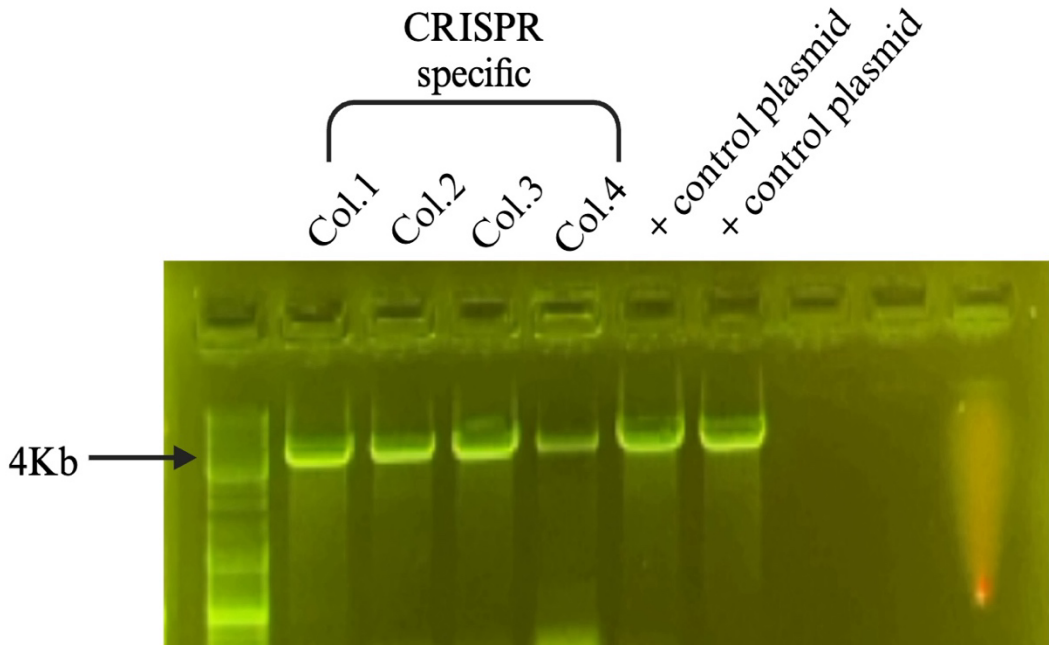

Figure 9: Sample gel electrophoresis from colony PCR confirming plasmids in *C. glutamicum* transformants. Bands obtained from colony PCR show the expected length of DNA.

3.3 Regulating gene expression by induction of IPTG:

After getting the plasmids harboring CRISPR-Cas9 and the homologous repair inside of the cell and confirming it by performing a gel electrophoresis as shown in fig 9 the gene insertion was carried out by inducing Isopropyl β- d-1-thiogalactopyranoside (IPTG). It has been repeatedly observed that Cas9-induced double-strand breakage (DSB) is fatal to bacterial cells because many microorganisms lack the endogenous nonhomologous end joining (NHEJ) mechanism, or the NHEJ is inefficient enough to repair the DSB (39). Due to this CRISPR-Cas9 has been used as a tool to check the lethality of the enzyme on the cells. The Cas9 gene was isolated from *Streptococcus pyogenes* to carry out the gene insertion in this study. Considering the lethality of Cas9 in the cells controlling the expression of the gene is very necessary. The expression of the Cas9 was

under P_{tac} promoter and guideRNA was controlled by P_{trc} promoter. Both guideRNA as well as Cas9 gene expression were repressed by the LacIq to reduce the expression of Cas9 gene to carry out the double stranded break. LacIq is a repressor that triggers the gene expression only when IPTG is induced. The schematic plasmid representation containing the CRISPR-Cas9 and guideRNA and LacIq gene is shown below in the figure (9).

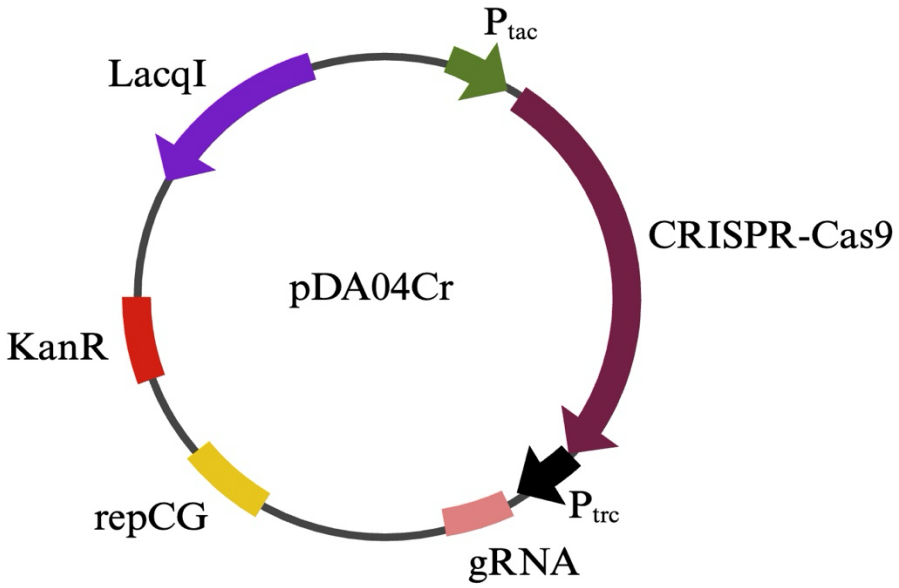

Figure 10: Schematic representation of CRISPR-Cas9 plasmid. The plasmid consists of the Cas9 gene expressed by the Ptac promoter, gRNA is controlled by the Ptrc promoter, KanR is the antibiotic resistance gene, *repCG* is the replication gene of *C.glutamicum,* CRISPR activity is repressed by the *Lacq*I gene.

3.4 CRISPR gene deletion from the chromosome and plasmid-based gene Integration:

As CRISPR-Cas9 acts as a tool to initiate the double-stranded break (DSB) a simultaneous repair is necessary to carry out the insertion of the desired genes into the chromosome of the microbial host. The most preferred way for the insertion of foreign genes into the chromosome is by homologous recombination. The plasmid pDD02 containing the heterologous *ldhA* gene and *acs* gene, as well as 400bp of flanking region upstream and downstream of the native ldhA gene, was first transformed into *C.glutamicum* to create SB01 strain. Later SB01 containing the pDD02 plasmid was made competent to carry out the transformation for pDA04CR1 and pDA04CR2 to get strains SB02 and SB03. The strains were not yet induced with IPTG to improve the transformation efficiency. The resultant strain SB04 after the homologous recombination will carry the *ldhA* and *acs* gene into the chromosome as shown in Figure 11. To check the presence of the pDD02 - pDA04CR1 and pDD02 - pDA04CR2 plasmids in the strain SB02 and SB03 colony PCR was carried out.

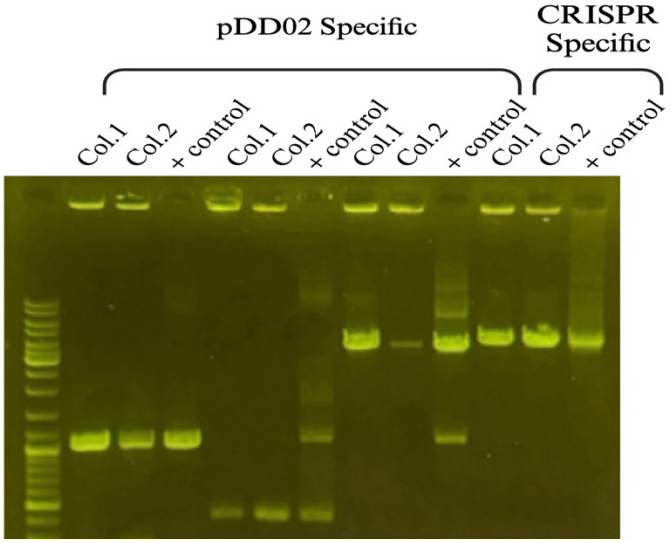

Figure 11: Gel electrophoresis image to confirm both plasmids are inside the cells.

The positive colonies were patched on the plates containing appropriate selection markers and 1mM IPTG. The colonies were screened after incubating for 24-36 h to verify the markerless insertion. No colonies for SB03 strains were obtained. Primer 5'G_FP and 3'G_RP were used to amplify the pre knockout region, primer 5'G_FP and 5'ldhA_RP were used to amplify the region that will get replaced after the gene replacement happens. Schematic representation of the primers and their sets for amplification of the desired region is shown in figure 12. After screening the colonies through gel electrophoresis no positive colonies were obtained.

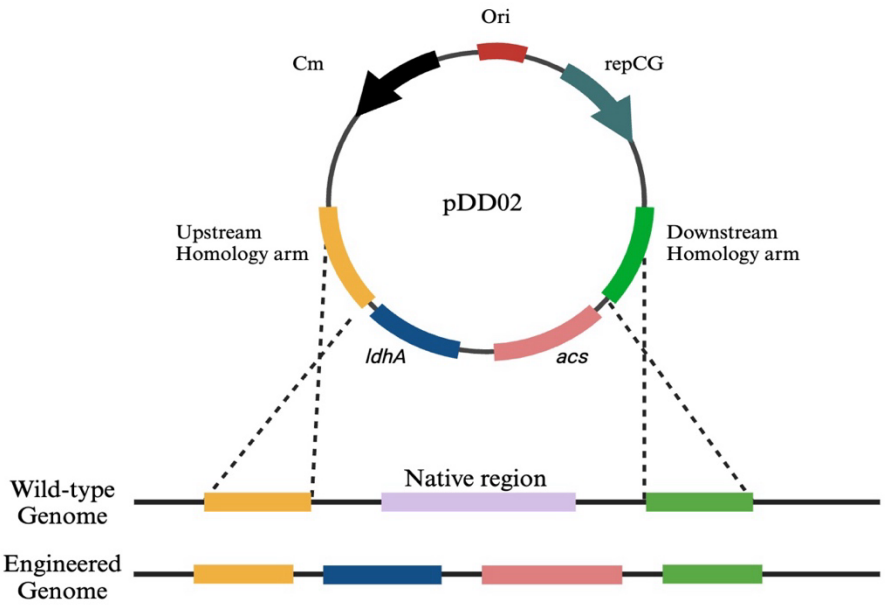

Figure 12: Schematic representation of homologous recombination by homology arms after the DSB provided by CRSIPR-Cas9 enzyme. The native gene is deleted by the DSB and replaced by the new cargo via the homologous recombination method.

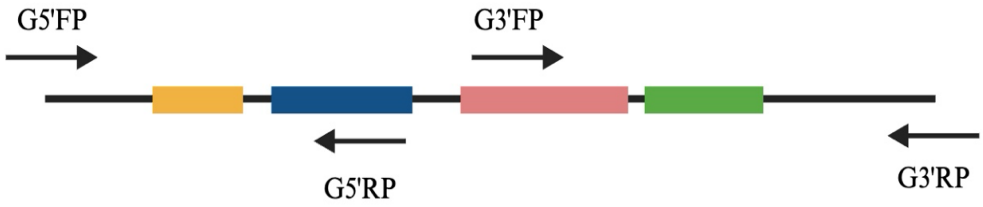

Figure 13: Representation of using different sets of primers to confirm the precise gene integration into the genome of microbial host cells.

3.5 IPTG induction in Liquid cultures:

As gene insertion was not successful through the IPTG induction on the BHI plates, we tried another way of inducing IPTG. Here, picking the colonies which has Cas9, and repair plasmid were grown in a rich media with appropriate selective markers Cm, Km and 1mM IPTG. The cultures were incubated at 30°C at 20 rpm overnight. The cells were grown until the mid-log phase (OD ≈ 0.6) to achieve the cells at a healthy phase. Later the over culture was diluted to obtain single colonies and were patched on the BHI plates with antibiotics and without antibiotics. Single colonies were obtained after 24-36hrs of incubation at 30°C. Single colonies were then screened with colony pcr using the desired primer set to verify the gene insertion into the genome of the microbial host cells. Total 15 colonies were screened for verifying successful insertion. The colony PCR showed that there was no insertion into the chromosome. The study was conducted by disrupting the native *ldhA* gene and *rfp* cassette (781bp) was inserted to check the markerless insertion efficiency. Liu et. al reports that the efficiency in *C.glutamicum* while performing a markerless insertion is very low at 25%.

To increase the possibility of the insertion happening inside the cell's DNA liquid cultures were started overnight with the same conditions before. This time the cells were diluted several folds to obtain single recombinant colonies. The dilution was plated onto the BHI plates containing antibiotic markers and IPTG also dilution was plated onto the BHI plates without antibiotic markers with IPTG. The later was done to select the cells which could possibly have the plasmids and with the IPTG induction insertion could be happening and without antibiotics better chances of the cells with the insertion to grow. Colony PCR was done on the recombinant colonies after 24hrs of incubation at 30°C. Two different primer sets were used to verify the insertion of the foreign genes. 5'G_FP and 5'G_RP was used to amplify from genome and some part of the native gene expected length 1kb, 5'G_FP and 5'ldhA_RP was used to amplify through genome and insert expected length 2kb. Samples fig. 13 and 14 were checked using gel electrophoresis, mixed population of cells were observed with the different sets of the primers.

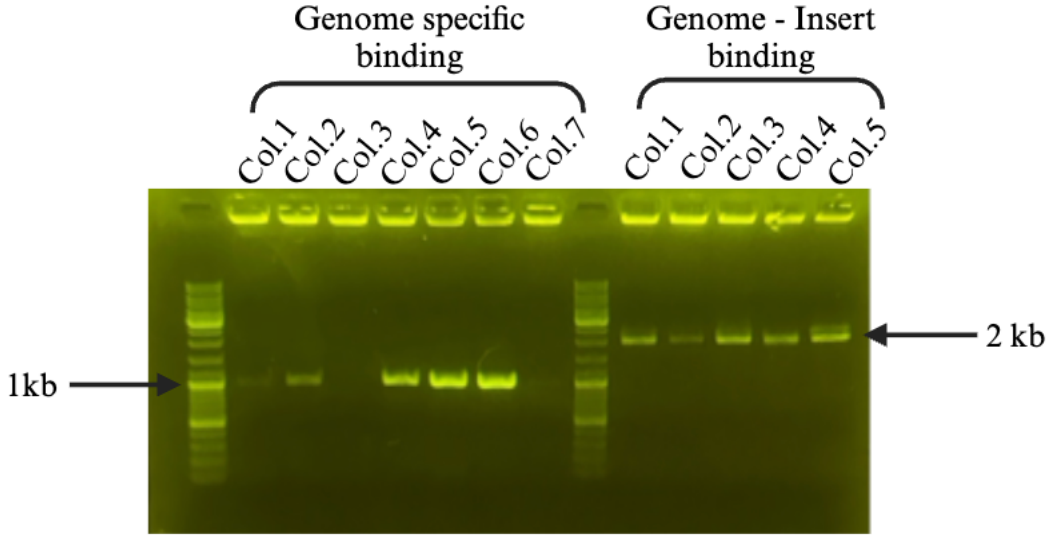

Figure 14: Sample gel electrophoresis from colony PCR confirming gene insertion in *C. glutamicum*. Bands obtained from colony PCR show the expected length of DNA.

To check the possibility of insertion happening downstream of the genome primer specific to downstream region were used to screen the positive colonies further for confirming the complete insertion. Here two different primer sets were used to verify the integration of the genes. 3'G_FP and 3'G_RP primer set was used to amplify the genome region downstream expected band length 1100 bp, primer set 3'ACS_FP and 3'G_RP was used to amplify through genome and insert expected length 2700 bp.

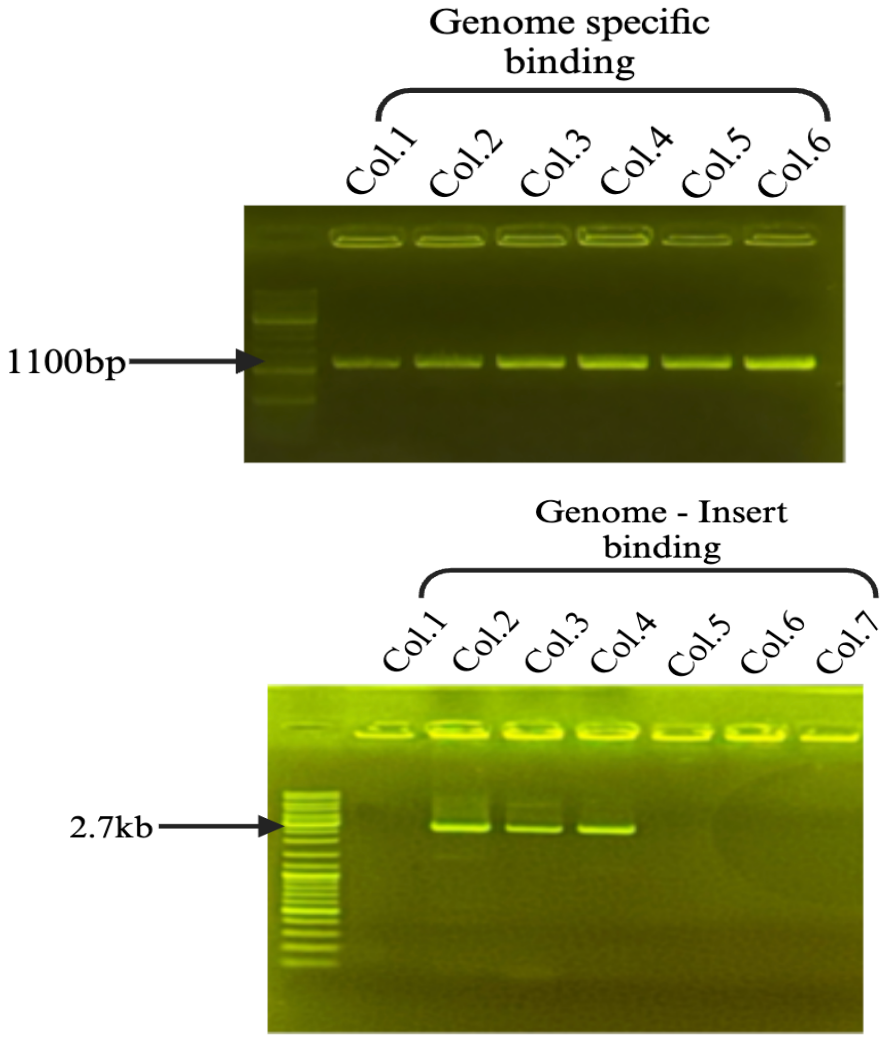

Figure 15: Sample gel electrophoresis from colony PCR on *C. glutamicum*. Bands obtained from colony PCR show the expected length of DNA.

3.6 Different gRNA targets for better insertion:

Different gRNA targets have different efficiency of the gene insertion happening inside the genome of the microbial host. Different gRNA target containing plasmid was used to increase the gene insertion possibilities into the chromosome of the bacteria. Plasmid pDA04Cr1 which contains different gRNA target was used for this experiment. The gRNA target was onto the native *ldh* gene which would disrupt the gene and after the double-stranded break is made with the help of homologous techniques the gene is inserted into the genome.

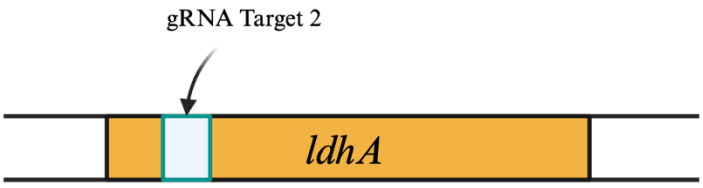

Figure 16: Representation of the gRNA target, targeting the specific region on the native *ldh* gene.

3.7 Acetate as a Carbon source for specific selection:

To reduce the chances of mixed pollution of the cells with and without integration, a single colony with mixed results was picked and cultured overnight in rich media with appropriate antibiotics and 1 mM of IPTG. The overnight culture was then transferred into minimal BTM media with acetate as a carbon source. The overnight grown culture was then inoculated into the minimal BTM media with different concentrations of sodium acetate 5 g/L, 10 g/L and 14g/L. The cultures were started with antibiotics and without antibiotics but with 1mM of IPTG. The hypothesis behind this was that *acs* gene initiates

acetate uptake and converts acetate into acetyl-CoA which helps with better growth of the engineered cells. By feeding acetate, chances of cells with integration of *acs* could be higher, and mixed population possibility could be lowered. The grown cell culture was then serially diluted to achieve single colonies. Single colonies were then screened by colony PCR using a specific primer set. The expected size of the band was 5400 bp, the results show that the DNA within the cells are same as WT which was used as a control. This means that there was no integration happening. The same result was obtained for both gRNA targets.

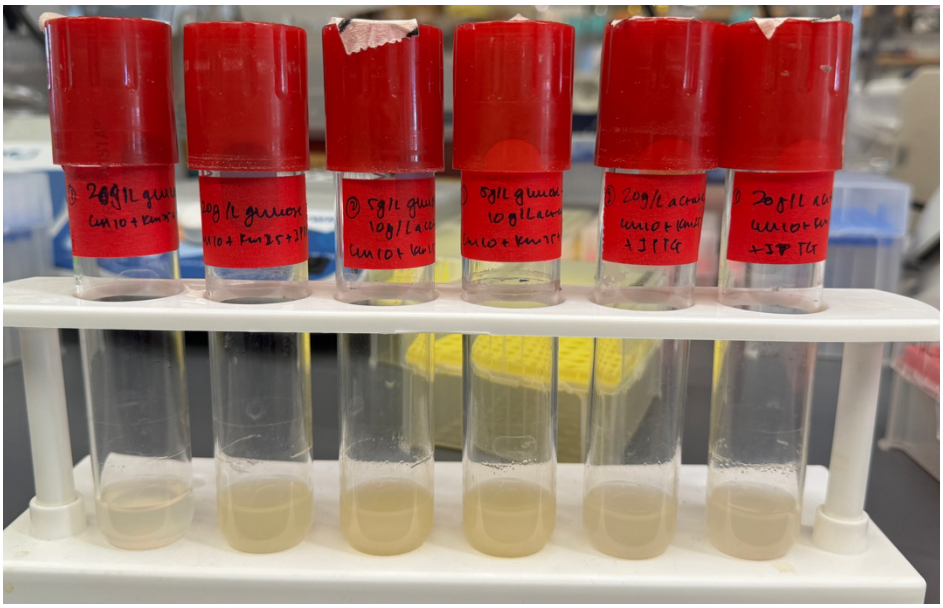

Figure 17: Cell cultures with different acetate concentrations for increasing the chances of insertion of the heterologous genes into the genome of the microbial host.

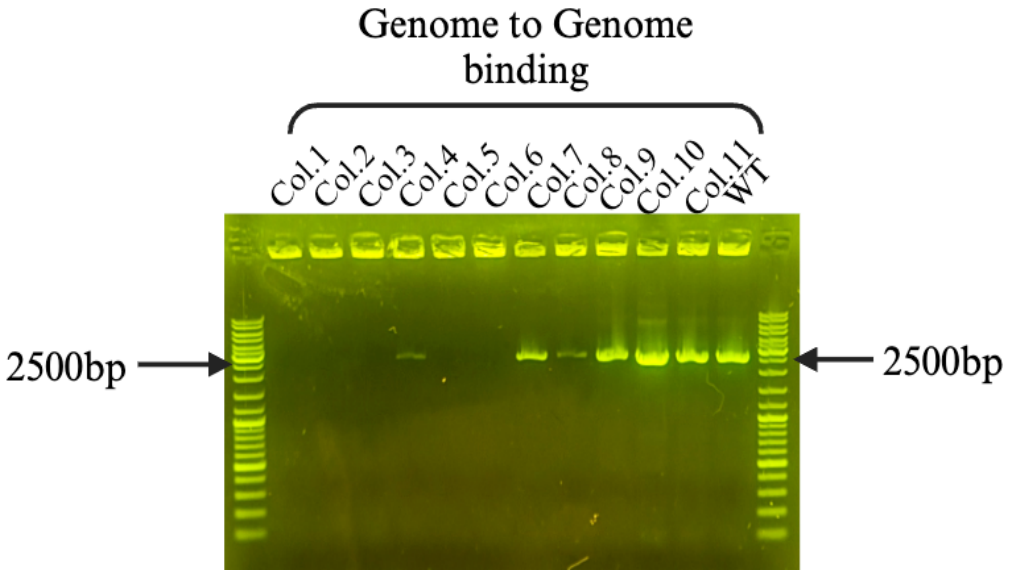

Figure 18: Sample gel electrophoresis from colony PCR with gRNA target 1 to verify the gene insertion within the genome of the *C. glutamicum.*

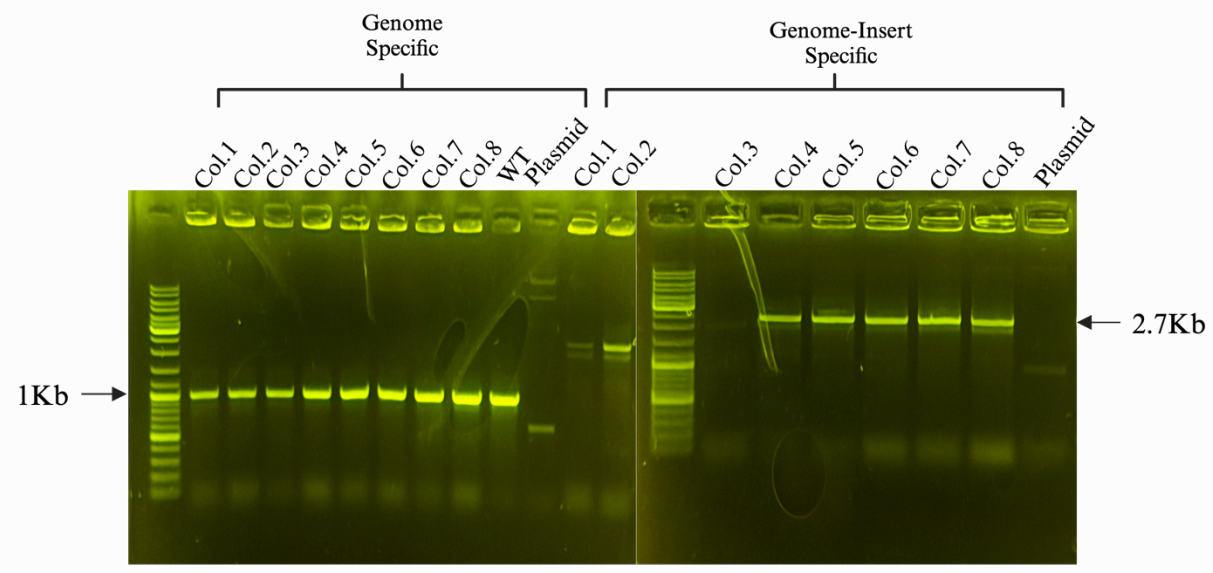

Figure 19: Sample gel electrophoresis from colony PCR with gRNA target 2 to verify the gene insertion within the genome of the *C. glutamicum.*

CHAPTER 4

DISCUSSION

As a gram-positive bacterium with good genomic stability, *C. glutamicum* is more difficult to engineer than genetically tractable hosts such as *E. coli*(39). Gene deletion and insertion through the utilization of plasmid-based editing templates, which are crucial tools for reconstructing and integrating metabolic pathways, continue to be highly sought after(40). *S. pyogenes* Cas9 is considered toxic to *C. glutamicum* and difficult to introduce into *C. glutamicum* (40). CRISPR-Cas9 mediated gene deletion and insertion is more efficient using plasmid-based editing templates. CRISPR/Cas9 system is more suitable for genome-wide scale engineering of *C. glutamicum*. Because Cpf1 utilizes a T-rich PAM and Cas9 utilizes NGG as PAM (40).

Due to the size of the cargo being around 3500 bp, this could be a reason the insertion was not very effective. The larger the cargo size less the chances of the gene insertion happening via homologous recombination. In this study because of the larger gene insertion size, a mixed population of the cells was observed. Another reason the gene insertion was not efficient and yielded lower positive results could be the size of the homology arms. 400 bp of homology arms were used in this study to carry out the homologous recombination after the DSB carried out by the CRISPR-Cas9. More efficient gene insertion could have been possible if homology arms were selected around 1000bp. This would have helped the integration of large cargo inside the chromosome more efficiently.

Future Experiments:

While performing the markerless gene integration, a mixed population of the cells was observed, to check the CRISPR-Cas9 ability to cleave the DNA at a specific location, a lethality experiment will be carried out to check the CRSIPR-Cas9's ability and impact to carry out the double-stranded break. New primers will be designed to mitigate the possibility of non-specific binding of the primers onto the plasmid pDD02. This will help to verify the gene insertion more effectively. To verify the activity of heterologous *ldhA* and *acs* gene real-time quantitative PCR could be performed. To verify any amount of desired enzyme is produced and secreted, protein purification and characterization techniques such as SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) could be used. A Bradford assay could be used to quantify the total amount of protein produced or secreted by the experimental strains as another method to measure and determine whether enzymes are being expressed and secreted.

After the strain is segregated fermentation could be carried out to check the heterologous gene activities. Various fermentation could be carried out with different amounts of glucose as a feed, and it can be compared with the wild-type strain. High-performance liquid chromatography (HPLC) will be carried out on the samples from the fermentation to quantify the amount of lactate and acetyl-CoA inside the cells. This strain could be used to further engineer to produce ethyl lactate by expressing certain genes through the plasmid or incorporating the genes into the genome of the strain.

CHAPTER 5

CONCLUSION

In this study *C. glutamicum* strain was developed to enhance the uptake of acetate and convert it into acetyl-CoA and the native ldh gene found in *C. glutamicum* was replaced with a heterologous ldhA gene to improve the production of lactate from pyruvate. At the current stage of the study, the recombinant strain is still found within the mixed population of the cells without any editing. The segregation of the strain remains the top priority within a few weeks of the experiments. This recombinant strain could be a platform strain for the production of valuable industrial chemicals replacing petroleum-based chemicals. Fermentation using different concentrations of glucose could give a clear insight how the strain is better than the non-engineered strain for the production of acetyl-CoA and lactate. The results from the experiments carried out could considered while integrating a bigger fragment into the chromosome of *C. glutamicum*. These findings are informative for future troubleshooting and for other research to optimize conditions to increase the possibility of insertion of foreign genes. More investigation is needed to verify the efficacy of the CRISPR-Cas9 editing tool for effective gene insertion into *C. glutamicum* platform for high-valued chemical production, but this work creates headway toward the goal of sustainable waste valorization.

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