Production of a Biochemical from Food Waste Through Integration of Anaerobic Digestion and Fermentation Processes

Xiaochao Xiong, Shulin Chen

Department of Biological Systems Engineering, Washington State University,

Pullman, WA 99164-6120, USA

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List of Abbreviations

2-BES	2-bromoethanesulfonate
ACS2	acetyl-CoA synthase
AD	anaerobic digestion
Btu	British thermal unit
CH_4	methane
CO_2	carbon dioxide
DMSO	dimethyl sulfoxide
eGFP	enhanced Green Fluorescent Protein
EPA	eicosapentaenoic acid
GC	gas chromatography
GC-MS	gas chromatography mass spectrometry
GHG	greenhouse gas
GR	glyoxylate reductase
GRAS	Generally Recognized As Safe
H_2	hydrogen gas
HCN	hydrogen cyanide
HPLC	high-performance liquid chromatography
MCA	monochloroacetic acid
MS	malate synthase
MTS	mitochondrial targeting sequence
NaOH	sodium hydroxide
PLGA	poly(lactic-co-glycolic acid)
rpm	rotations per minute
TCA	tricarboxylic acid
VFA	volatile fatty acid
YNB	yeast nitrogen base

Abstract

Recovering and reusing organic wastes as a feedstock for anaerobic digestion (AD) can conserve resources, reduce greenhouse gas emissions, diminish odors, and stabilize waste. Currently, AD represents a well-developed technology that is viable for commercial-scale waste utilization, but economics can be challenging. While AD alone can produce methane, a renewable energy source, using the digester as a platform to create higher value products, such as renewable biochemicals, can make the economics for more favorable. Likewise, the low or even negative cost of organic waste streams could overcome one of the primary hurdles to the production of bio-based products: high production costs. To this end, an innovative technical route was developed to make glycolic acid from organic wastes by integrating AD and aerobic cultivation. This value-added chemical has broad industrial applications and already has a sizeable market. The AD process was adjusted to funnel the waste into simple carbon molecules called volatile fatty acids (VFAs). The VFAs generated from AD were then used to efficiently biosynthesize glycolic acid using a genetically engineered non-conventional yeast, *Yarrowia lipolytica*. This innovative biorefinery benefits both from the capacity of the anaerobic microbial consortia to handle complex waste, and from ability of the engineered cell factory to biosynthesize the target molecule. This technical pathway has potential to be further leveraged to generate other biochemicals with high yield and at low cost from organic wastes.

1. Introduction

1.1 Significance

This project aims to demonstrate the production of bio-based glycolic acid, an important α-hydroxy acid with annual market size of over $280 million, from organic wastes, including food waste (Research and Markets, 2018). Glycolic acid has important industrial applications for making cleaning agents, polymers, and personal care products. Currently, glycolic acid has been mainly produced by chemical processes that have disadvantages, namely, use of toxic materials, generation of undesirable by-products, and operation under harsh conditions (Salusjärvi et al., 2019). This technology offers a more sustainable and potentially economical technical route to produce glycolic acid.

The re-use of materials currently considered wastes is a critical strategy for sustainable growth in a resource-constrained world. The US has the potential to use 77 million dry tons of wet waste per year, which could contribute about 1.079 quadrillion British thermal units (Btu) of energy (USDOE, 2017). These waste streams include biosolids, animal manure, food waste, and fats, oils, and greases. Utilizing these resources will contribute to sustainability by supplying energy, fostering economic growth, reducing greenhouse gas emissions, and contributing to food waste diversion goals. While anaerobic digestion (AD) is well developed practical technology for wet waste utilization at the commercial level, the economics of AD have proven challenging because of the low price for biogas. Integration of processes to produce a high value biochemical with AD would improve the economics of AD and provide a sustainable route for biochemical production.

One promising approach that has been identified for production of high-value biofuels and bioproducts from wet waste streams involves arresting methanogenesis. In arrested methanogenesis, the microbial processes that normally occur during AD are “arrested” after volatile fatty acids (VFAs) are produced from complex organic wastes. The VFAs can then be converted into the desired product (USDOE, 2017). With the funding received through the Waste to Fuels Technology partnership during the 2015-17 biennium, the team made substantial progress to initiating a VFA platform for waste bioconversion. During the current biennium, the team further refined the key process components, including VFA production, yeast cell factory engineering, and production glycolic acid, a high-value bioproduct. Glycolic acid (hydroxyacetic acid) has important industrial applications for making cleaning agents, polymers, and personal care products. The global glycolic acid market is projected to grow from $288.9 million in 2017 to $406.4 million in 2023, with a compound annual growth rate of 6.83% (Research and Markets, 2018). The current chemical processes through which glycolic acid is produced have disadvantages, namely, use of toxic materials, generation of undesirable by-products, and operation under harsh conditions (Salusjärvi et al., 2019).

As shown in Figure 1, this technology is developed by integrating two processes: (1) converting complex waste materials into a group of simple molecules, VFAs mainly consisting of acetate, through acidogenesis in AD, and (2) transforming the resultant VFAs to the target products in a separate bioreactor by a metabolically engineered yeast strain. This novel waste to biochemical

technology benefits both from the anaerobic microbial consortia's capacity to handle complex waste and from the engineered cell factory for the biosynthesis of the target molecule.

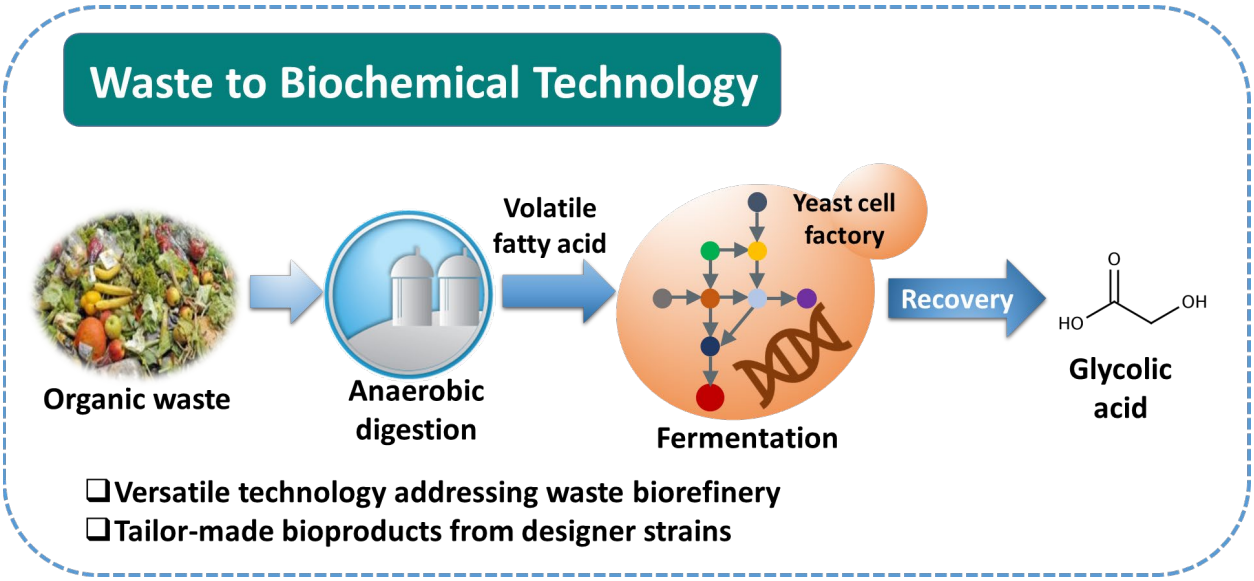

Figure 1: Production of bio-based glycolic acid from waste by integration of acidogenesis in anaerobic digestion and fermentation processes.

Our project addresses the opportunity for waste valorization by providing a cost-effective route to convert negative or low-value waste to bio-based products with high value. Although this project focuses on the production of glycolic acid, the technical platform can be tailored to deliver a variety of commodity chemicals by constructing different metabolic pathways in the microbial host (Figure 1). Therefore, developing and demonstrating this technology can potentially have broad impacts for the biofuel and bioproduct industries.

1.2 Approach and State-of-the-art Technology

Anaerobic digestion is a well-established waste management and treatment process for converting organic wastes to bioenergy (Zamri et al., 2021). The AD process involves the degradation of organic matter to methane (CH_4), carbon dioxide (CO_2), and mineralized nutrients mediated with the mixed culture of symbiotic bacteria (Rasapoor et al., 2020). Typically, AD processing of solid wastes includes multiple steps: (1) *Disintegration* of the waste biomass by physicochemical processes to break down the initial solid biomass into separate components; (2) *Hydrolysis*: the breakdown of relatively large organic compounds, lipids, carbohydrates, and proteins to long-chain fatty acids, monosaccharides, and amino acids, respectively; (3) *Acidogenesis*: a syntrophic process for converting VFAs other than acetate, such as propionate and butyrate to acetate and producing hydrogen gas (H_2) simultaneously; and (4) *Methanogenesis*: the last step of the anaerobic conversion of organic wastes to CH_4. Formic acid, acetic acid, methanol, and H_2 can all be used as energy sources by the various methanogens. In such a multi-step complex process, the kinetics of the slowest step will drive the overall kinetics. Hydrolysis is often the rate-limiting step if the substrate is complex organic solids, while in the

digestion of soluble organic matter, the rate-limiting step has been identified as methanogenesis (Di Capua et al., 2020).

Alternatively, the production of VFAs has been extensively explored either as end products or as precursors for production of biofuels or industrial chemicals (Lu et al., 2020). VFA production can be improved by enhancing the hydrolysis of waste through physical or chemical pretreatments and supplement of enzymes, and by enhancing acidogenesis rates through pH control, temperature control, redox potential, and inoculum optimization. In addition, chemicals such as 2-bromoethanesulfonate (2-BES) can be added to inhibit methanogenesis (Lukitawesa et al., 2020). Various organic wastes, including wheat straw (Awasthi et al., 2018), corn stover, fruit and vegetable waste, food waste (Jones et al., 2021), and manure, have been used to produce VFAs with different concentrations. These studies show that VFAs can be efficiently produced from organic wastes by arresting methanogenesis in AD.

Although VFAs themselves are useful chemicals, separating these chemicals from the bulk liquid presents a significant cost barrier (Shi et al., 2018). To bypass this barrier, another logical approach is the utilization of the VFA rather than optimizing the purification of a single VFA chemical, such as acetate, from the AD effluent. After separation or concentration, VFA as an intermediate can be upgraded into fuels and chemicals by chemical processing (Strazzera et al., 2018). However, this approach still requires costly liquid extraction of products, energy-intensive thermochemical conversion, and supplementation with exogenous H_2 (Huq et al., 2021).

Microorganisms can use VFAs to synthesize more complex molecules through aerobic cultivation. For example, microbial lipids are precursors for producing biofuels with high energy density (e.g., biodiesel), and can be generated from VFAs, including acetic acid. Microbial lipids biosynthesized from acetic acid by a recombinant yeast reached a titer (concentration) of over 100 g/L (Xu et al., 2017). Although microbial lipid production achieved a high titer, the overall yield was only 0.16 g lipid/g acetic acid (Xu et al., 2017). Long-chain hydrocarbon molecules, including lipids, are reduced substances with much lower oxygen content than the substrate, acetic acid. Consequently, both theoretical and empirical lipid yield from acetate is low (around 0.25 g/g) mainly due to loss of CO_2 for generation of reducing and energy equivalents for carbon chain elongation (Hu et al., 2016).

Glycolic acid (hydroxyacetic acid) is one of the smallest organic molecules with both acid and alcohol functionality (Figure 1). Its unique set of properties make it ideal for a broad range of applications. As a biodegradable, non-toxic, non-volatile, and phosphate-free chemical, glycolic acid repents a good environmental choice. Glycolic acid can be used as an efficient cleaning agent with many added benefits such as negligible odor, high solubility in water, and ease of rinsing. Glycolic acid has been used as a cleaner in dairy processing plants for many years because most equipment can be cleaned in place with glycolic acid formulations. Glycolic acid can be used as a building block to produce many other chemicals, such as biopolymer poly(lactic-co-glycolic acid) (PLGA) either by chemical synthesis or biosynthesis (Choi et al., 2016). The global glycolic acid market is projected to grow from $288.9 million in 2017 to $406.4 million in 2023, with a compound annual growth rate of 6.83% (Research and Markets, 2018).

Despite its vital role in commercialization and wide applications, glycolic acid occurs naturally only as a trace component in some plants such as sugarcane, beets, grapes, and fruits. Different methods have been explored for chemical synthesis of glycolic acid, including carbonylation of

formaldehyde with synthesis gas, hydrogenation of oxalic acid, and hydrolysis of the cyanohydrin derived from formaldehyde. These methods involve using toxic materials such as formaldehyde and hydrogen cyanide (HCN) to prepare cyanohydrin, operation under harsh conditions such as hydrogenation, and formation of undesirable by-products. Recently, monochloroacetic acid (MCA) reaction with sodium hydroxide (NaOH) followed by re-acidification has been employed to produce glycolic acid. However, the primary market for MCA is agricultural chemicals, making the supply chain very volatile. MCA itself is a hazardous alkylating agent, and MCA-produced glycolic acid, especially technical grade product, has elevated levels of chlorides, including dichloroacetic acid, and has proven to be very corrosive. Therefore, development of a new, reliable, scalable, and safe pipeline for the production of glycolic acid is a great opportunity.

Biosynthesis of glycolic acid can potentially overcome the limitations and disadvantages of the chemical process. Metabolic engineering of the model organisms *Escherichia coli* and *Saccharomyces cerevisiae* has been explored for the biosynthesis of glycolic acid from glucose and xylose (Cabulong et al., 2018; Salusjärvi et al., 2017). Other organisms, including *Kluyveromyces lactis* and *Corynebacterium glutamicum*, were genetically engineered to produce glycolic acid from sugars (Koivistoinen et al., 2013). Although recombinant *E. coli* was further employed to produce glycolic acid from acetic acid, it could grow in acetic acid with a content lower than 5 g/L (Li et al., 2019). A recombinant *E. coli* developed by metabolic engineering of both tricarboxylic acid (TCA) cycle and glyoxylate shunt can produce 65.5 g/L glycolic acid from glucose via fed-batch fermentation (Deng et al., 2018). Using renewable feedstock such as cellulosic sugars to make bio-based products is a clear advancement over petroleum-based chemicals. However, a low-cost and readily supply of cellulosic sugar is still a primary challenge as demonstrated by the lack of progress in the cellulosic ethanol industry.

1.3 Innovations in the Waste to Biochemical Approach

Our technology integrates two novel processes, including (1) thermophilic (70 ºC) acidogenic digestion of waste materials for VFA production, and (2) bioconversion of resultant VFA into bioproduct by engineering of new pathways in a non-conventional yeast *Yarrowia lipolytica* with advanced metabolic engineering strategies. Our innovations and technical approach advance the current knowledge and state-of-the-art technology detailed below.

(1) Reprogramming the AD process for the production of VFAs

We developed a novel thermophilic AD operating at 50-70 ºC to produce VFAs from waste streams. Aside from the generally accepted advantages of AD processes (no sterile conditions or expensive enzymes required, mixed microbial communities that can handle complex and variable organic waste streams), thermophilic AD adds unique benefits for producing VFAs (Saber et al., 2021). At these temperatures, methane production ceases as methanogens are not thermo-tolerant. Higher temperatures allow more complete digestion of the feedstock, higher VFA yields, and decreased solid retention times (Qiao et al., 2013). AD operated at higher temperatures is also more capable of pathogen removal and destruction of antibiotics, generating high-quality residues as biofertilizers.

(2) *Y. lipolytica* as a platform organism for bioproduct production

Y. lipolytica is uniquely advantageous for transforming VFAs into high-value products with the advancement in synthetic biology. As a Generally Recognized As Safe (GRAS) organism, *Y. lipolytica* has been widely used for industrial production of a suite of chemicals and has been metabolically engineered for producing lipid mainly consisting of triacylglycerol (TAG) and other lipid-derived molecules such as eicosapentaenoic acid (EPA) (Xue et al., 2013). Non-lipid compounds such as lycopene can also be produced by metabolic engineering of *Y. lipolytica* (Matthäus et al., 2014). *Y. lipolytica* can accumulate citrate and isocitrate (Yuzbasheva et al., 2019), the precursor for the biosynthesis of glycolic acid. *Y. lipolytica* is tolerant to high concentrations of VFAs (100 g/L of propionate) and has been cultured with both synthetic and food waste-derived VFAs for lipid production (Park & Nicaud, 2020). Use of acetate for lipid production in *Y. lipolytica* was studied using metabolic flux analysis (MFA) by Prof. Gregory Stephanopoulos's group from Massachusetts Institute of Technology (MIT) (Liu et al., 2016). This study provided a deep insight into the metabolism of *Y. lipolytica* cultured on acetate. Our previous research developed a comprehensive set of molecular biology tools for genetic manipulation of *Y. lipolytica* (Xiong & Chen, 2020). We have successfully genetically engineered *Y. lipolytica* to produce lipid, free fatty acid (Ghogare et al., 2020), long-chain dicarboxylic acid, fatty alcohol (Wang et al., 2016), and wax ester. *Y. lipolytica* is a desirable biological platform for biochemical production from waste materials.

(3) Pathway design for producing bio-based glycolic acid

The problem of low yield represents a significant barrier in biofuel and biochemical production from VFAs. For example, both theoretical and empirical lipid yields from acetate are low, mainly due to loss of CO_2 through reduction reactions (Xu et al., 2017). Selecting glycolic acid as the target product overcomes the low yield barrier in biofuel and biochemical production because of the similar carbon and oxygen contents between the product (glycolic acid) and substrate (acetic acid).

As shown in Figure 2, only one heterologous gene encoding glyoxylate reductase (GR) (purple line) needs to be introduced into *Y. lipolytica* to produce glycolic acid from acetate through the glyoxylate shunt in this designed pathway (Koivistoinen et al., 2013). Acetate can be converted to acetyl-CoA through the native acetyl-CoA synthase (ACS2) in *Y. lipolytica* at a loss of two moles of ATP equivalents, as ATP is transformed into AMP. Citrate is formed by the combination of acetyl-CoA and oxaloacetate and is then converted to isocitrate. Isocitrate is cleaved by isocitrate lyase to generate glyoxylate and succinate, and the former is accumulated when malate synthase (MS) is disrupted. Oxalate can be combined with acetyl-CoA to start the next run of biosynthesis of glyoxylate.

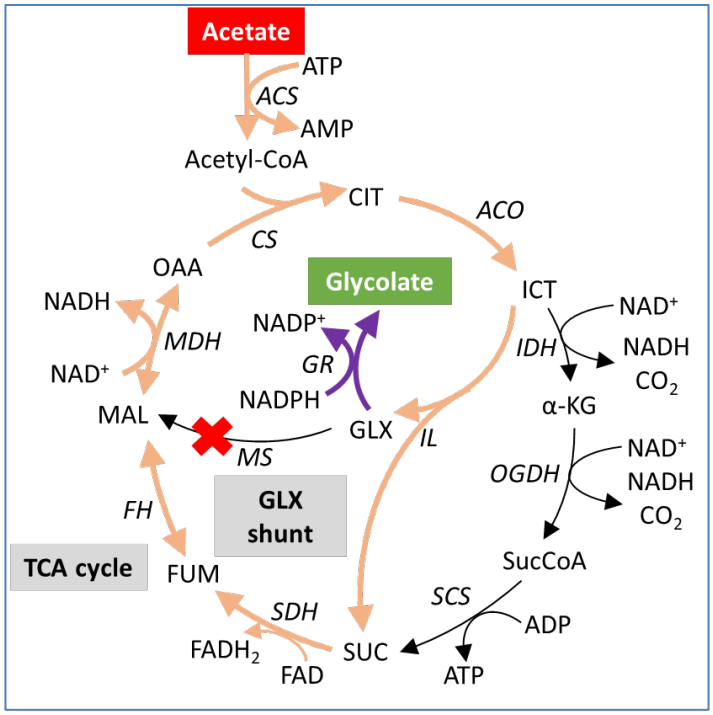

Figure 2: Pathway design for the biosynthesis of glycolic acid (glycolate) from acetic acid (acetate) in yeast cells.

(4) Compartmentalizing metabolic pathways for biosynthesis of glycolate

We employed the strategy of pathway compartmentalization in biological design to isolate and concentrate the substrates, regulate pathway fluxes and eventually generate a high yield of the targeted product. In yeast, the glyoxylate shunt and TCA cycle reactions are highly connected, involving different cellular compartments, including cytosol, peroxisomes, and the mitochondria (Figure 2). Pathway compartmentalization as a novel metabolic engineering strategy allows for enrichment of the precursors and avoidance of metabolic crosstalk (Avalos et al., 2013). Furthermore, it is possible to re-wire the transport of metabolites across the different organelles by manipulating the genes encoding the corresponding transporters such as mitochondrial citrate carrier in *Y. lipolytica* to modulate the metabolic flux (Yuzbasheva et al., 2021). While traditional pathway engineering uses cytoplasm, both mitochondria and peroxisomes can also be engineered as the production units in yeast cells.

In summary, we devised a unique technical route with multiple innovations, including arresting methanogenesis in the AD process for the production of VFA at a high rate, engineering *Y. lipolytica* as a desirable host that is tolerant to high concentrations of VFAs and capable of producing organic acids, and compartmentalizing metabolic pathways in the yeast cell factories. In addition, our approach builds upon sound rationale because (1) the AD process is very effective in converting various organic wastes to VFA, and (2) VFA is particularly suitable for the production of glycolate because VFA is particularly suitable for the production of glycolate because expression of the enzymes involved in the glyoxylate shunt, which plays a role in the metabolism of two-carbon substrates (e.g., acetate) and the replenishment of tricarboxylic acid (TCA) cycle intermediates essential for the production of biomolecules. The glyoxylate shunt,

the biochemical pathway for generating the precursor, is known to be up-regulated (expression is increased) in yeast when the strains are grown on a VFA, such as acetic acid (Walsh & Koshland Jr, 1984). Our technology not only uses waste as a feedstock, but also has a great potential to achieve high concentration, productivity, and yield. This integrated biorefinery makes it possible to deliver triple bottom lines associated with waste valorization: producing a renewable chemical, reducing energy consumption by replacing the petroleum feedstock, and generating environmental benefits.

To achieve the project goal and incorporate these innovations, we designed two specific technical tasks for this project.

Task #1: Metabolic engineering of Y. lipolytica for biosynthesis of glycolic acid from VFA

We aimed to engineer the yeast cell factory for producing glycolic acid with high value. We designed the pathways to synthesize this biochemical from acetic acid (Figure 2). We identified and expressed the genes for producing glycolic acid.

Task #2: Development of a bioprocess for producing biochemical from waste streams

We produced VFAs from organic wastes, including food waste and dairy manure. The resultant VFAs were used as substrates for glycolic acid production using the genetically engineered yeast strains.

2. Methods and Materials

2.1 Strains, Culture Conditions, and DNA Techniques

In this project, *Y. lipolytica* PO4f developed by our lab from the strain *Y. lipolytica* PO1f (ATCC MYA-2613) was used as a microbial host for metabolic engineering to produce glycolic acid (Wang et al., 2016). Compared with *Y. lipolytica* PO1f, the gene encoding protein Ku70 in *Y. lipolytica* PO4f was disrupted to increase homologous recombination efficiency, facilitating further genetic manipulation, specifically deletion of a targeted gene. *Y. lipolytica* PO4f was cultured in shake flasks using a rich medium, YPD, consisting of 10 g/L of yeast extract (Difco), 20 g/L of peptone (Difco), and 20 g/L of glucose at 28 °C with a rotation speed of 200 rotations per minute (rpm). Agar plates of YPD were made by supplement of 15 g/L agar (Difco). *Y. lipolytica* PO4f was an auxotrophic strain. It could not grow on the synthetic media without uracil or leucine unless transformed to prototrophy with a functional marker gene such as *ura3* or *leu2*. Synthetic media for yeast growth was composed of 20 g/L glucose, 6.7 g/L yeast nitrogen base (YNB) without amino acid and with ammonium sulfate (US Biologicals), supplemented with 2.0 g/L of the complete supplement of amino acids lacking either leucine or uracil (US Biologicals). Agar plates of selective media were prepared by adding 20 g/L agar to the broth. *E. coli* TOP10 (Invitrogen) was used to construct and propagate recombinant plasmids. *E. coli* strains bearing plasmids were cultured at 37 °C on LB medium added with 100 μg/ml ampicillin.

The general procedures for conducting molecular biology experiments, such as PCR and gene cloning, are described in some of our previous publications (Wang et al., 2016; Xiong & Chen, 2020).

2.2 Strains Engineered for Glycolic Acid Production

(1) Deletion of two genes encoding MS in *Y. lipolytica*

As shown in Figure 2, to block the consumption of glyoxylate, which is the precursor for the biosynthesis of our target product glycolic acid, the genes encoding MS need to be deleted. Two genes *MS1* (YALI0D19140g) and *MS2* (YALI0E15708g) encoding MS were found in the genome of *Y. lipolytica* PO1f. We have developed a procedure for generating a gene knockout of *Y. lipolytica*. As shown in Figure. 3, the process includes: constructing a plasmid containing the upstream and downstream homologous arms in place of the desired knockout gene and a selection marker *uar3*, transforming *Y. lipolytica* PO4f with the linearized plasmid, verifying a targeted gene deletion, and removing and recycling marker gene *ura3* for further engineering. Through this iterative gene integration and marker curation process, we can generate combinational gene knockout of *Y. lipolytica*.

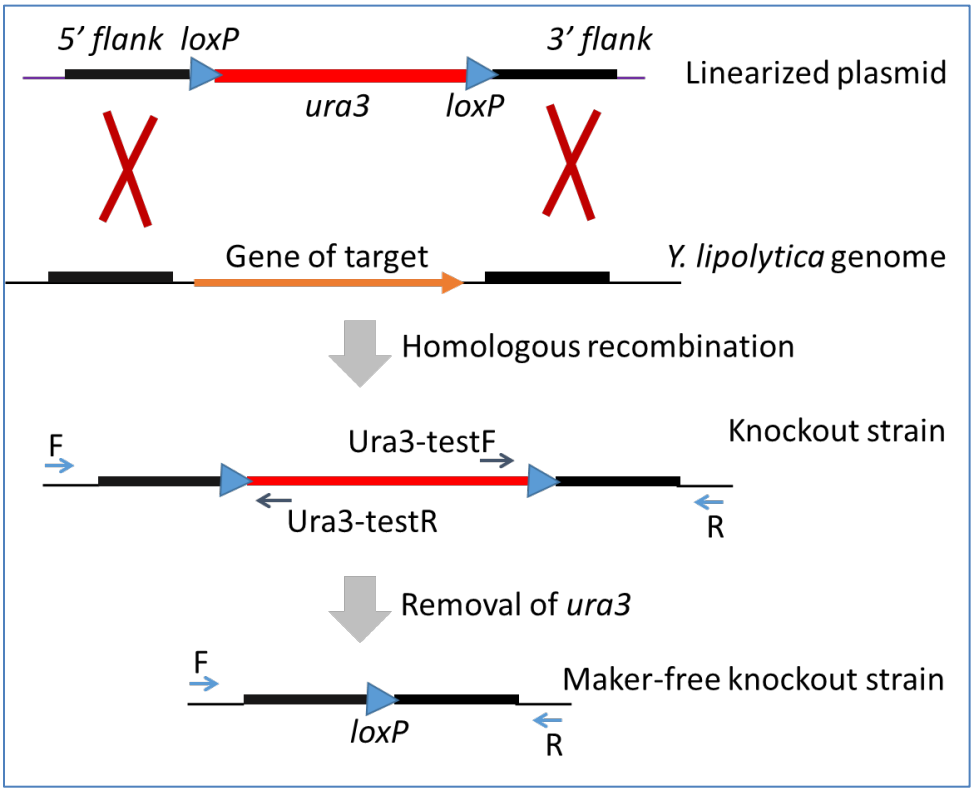

Figure 3: Workflow for deletion of a targeted gene in Y. lipolytica.

As shown in Figure 3, to delete gene *MS1*, we first need to construct the plasmid containing both 5' and 3' flanks of the targeted gene. The DNA sequences of the primers used to amplify 5' and 3' flanks of *MS1* gene by PCR were listed in Table 1. These two DNA fragments were cloned into plasmid pUra3loxp containing a selection marker *uar3* flanked with 34-bp *loxP* sites, and the resultant plasmid was pMS1UpDo. As shown in Figure 3, the linearized plasmid DNA was used to transform *Y. lipolytica* PO4f. The yeast transformants were grown on agar plates of

synthetic media without uracil. After growth for two days, the single colonies on the agar plates were picked and further cultured in YPD broth at 28-30 °C. At the same time, the colonies were replicated on YPD agar plates. After cultivating for two days, one ml of yeast culture was used to extract genomic DNA. The diagnosis PCR was carried out to verify the gene deletion (Figure 3), and the knockout strain *Y. lipolytica* ΔMS1 was obtained. After transforming with the plasmid expressing *Cre* recombinase, the marker *ura3* in *Y. lipolytica* ΔMS1 was removed (Wang et al., 2016). The strain without *ura3* could be used for further engineering (Figure 3).

Similarly, the plasmid pMS2UpDo was constructed to knock out *MS2* in *Y. lipolytica*. After transforming marker-free *Y. lipolytica* ΔMS1 with linearized pMS2UpDo, the double knockout strain *Y. lipolytica* ΔMS1ΔMS2 was developed. The resultant strain *Y. lipolytica* ΔMS1ΔMS2 bearing disrupted *MS1* and *MS2* genes could be further engineered to produce glycolic acid.

(2) Expressing GR in *Y. lipolytica* ΔMS1ΔMS2 for producing glycolic acid

As shown in Figure 2, to produce the target product, we need to introduce a foreign gene encoding GR into *Y. lipolytica* ΔMS1ΔMS2 for converting glyoxylate into glycolate. The 0.94-kb gene *ycdW* encoding GR from *E. coli* K12 was amplified by using PCR with the primers YcdWF1 (5'-CGGCGGCTGCAGATGGATATCATCTTTTATC-3', PstI) and YcdWR1 (5'-TATATATTCCCGGGTTAGTAGCCGCGTGCGC-3', SmaI) (Koivistoinen et al., 2013). The *ycdW* gene was digested with PstI and SmaI and inserted into an expression vector pJN34 developed by our lab. The plasmid for expression of *ycdW* was designated as pYCDW.

Another gene encoding glyoxylate reductase GLYR1 (At3g25530.1) from the plant *Arabidopsis thaliana* was characterized (Koivistoinen et al., 2013). To express *GLYR1* in *Y. lipolytica* for producing glycolic acid, we had the gene *GlyR1* from *A. thaliana* synthesized by Integrated DNA Technologies based on the codon usage preference of *Y. lipolytica*. In this synthesized *GLYR1* gene, the C-terminal tripeptide SRE was removed (Ching et al., 2012), and 34 amino acids derived from the C-terminus of *Y. lipolytica* isocitrate lyase (ICL1, YALI0C16885g) was added. The DNA sequences of codon-optimized *GLYR1*were shown in Table 2.

Table 1: Primers used to generate knockouts of MS1, MS2, and ACS2.

Primers	Seqeunce (5' to 3')
MS1-up1	AGGGCGAATT GGGCCCGACG TCAGCACGTT CGATCTAGCA
MS1-up2	CCATGCTTAG TTACAATGCT TAGCCGATCT AAAAGTGGAG
MS1-Do1	TAAGCATTGT AACTAAGCAT GGTAGGTGGG ATGACGAAGA
MS1-Do2	GGAGCTCTCC CATATGGTCG ACTCCATGTC ACAGTTTCGC
MS1-testF	CAAGGGCATC AAACTAGCTG
MS1-testR	GTTTAACACA GCCAGATGGG
MS2-up1	AGGGCGAATT GGGCCCGACG TCCTATTGTT CGATTCGGCG
MS2-up2	CCATGCTTAG TTACAATGCT TATGTGCAGG TACAACGGAA
MS2-Do1	TAAGCATTGT AACTAAGCAT GGAAGCTCTA AGCGCGATGT
MS2-Do2	GGAGCTCTCC CATATGGTCG ACTGATTCTG TCGCCCAACT
MS2-testF	CCATATGATT CTGTGCCTGC
MS2-testR	CGAGGAGTAT CCTTCCACCA
ACS2-up1	GGCGAATTGG GCCCGACGTC ATTATCGCGT GCTGACCAAC
ACS2-up2	ATGCTTAGTT ACAATGCTTA GTGAGTGCTG GAAAATGTGC
ACS2-Do1	ATACAATGGT AAGCAATCGC TGTCGATCTG GGGTAAAACG
ACS2-Do2	AGCTCTCCCA TATGGTCGAC TGCCACCCAT CTCACTGTTG
ACS2-testF	CAACTTGTCA GCTTTTCGCC
ACS2-testR	ACGAGAAGAC GAGGAGACGA
uar3-testF	TCCTGGAGGC AGAAGAACTT
uar3-testR	AGCCCTTCTG ACTCACGTAT

Table 2: DNA sequences of synthesized GLYR1 from *A. thaliana*. The gene was synthesized based on the codon usage of *Y. lipolytica*. Restriction sites were underlined. The start codon ATG was shown in a frame, and stop codon TAA was depicted with red color. The 34 amino acids from the C-terminus of ILC1 were shown with character shading.

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1       AAGCTTATGG AGGTCGGCTT CCTCGGACTG GGTATTATGG GCAAGGCTAT
51      GTCGATGAAC CTGCTCAAGA ACGGCTTTAA GGTCACTGTG TGGAACCGAA
101     CCCTGTCTAA GTGTGACGAG CTGGTGGAGC ACGGCGCTTC TGTCTGTGAG
151     TCCCCCGCCG AGGTCATCAA GAAGTGCAAG TACACCATTG CCATGCTGTC
201     TGACCCCTGT GCCGCTCTGT CCGTGGTCTT CGACAAGGGC GGAGTGCTGG
251     AGCAGATCTG CGAGGGCAAG GGATACATTG ACATGTCTAC CGTCGACGCT
301     GAGACTTCTC TGAAGATCAA CGAGGCCATT ACCGGAAAGG GTGGCCGATT
351     CGTGGAGGGT CCCGTCTCTG GATCCAAGAA GCCCGCCGAG GACGGTCAGC
401     TGATCATTCT GGCCGCTGGA GACAAGGCTC TGTTCGAGGA GTCTATCCCC
451     GCCTTCGACG TGCTGGGAAA GCGATCCTTC TACCTGGGTC AAGTGGGTAA
501     CGGCGCCAAG ATGAAGCTGA TCGTCAACAT GATTATGGGA TCTATGATGA
551     ACGCTTTCTC CGAGGGTCTG GTCCTGGCCG ACAAGTCTGG CCTGTCTTCC
601     GACACCCTGC TGGACATTCT GGACCTGGGT GCTATGACCA ACCCCATGTT
651     CAAGGGAAAG GGTCCCTCCA TGACCAAGTC TTCCTACCCT CCCGCCTTCC
701     CCCTGAAGCA CCAGCAGAAG GACATGCGAC TGGCCCTGGC TCTGGGCGAC
751     GAGAACGCTG TGTCCATGCC TGTGGCCGCT GCCGCTAACG AGGCCTTCAA
801     GAAGGCTCGA TCTCTGGGCC TGGGCGACCT GGACTTCTCC GCCGTGATCG
851     AGGCTGTCAA GTTCGCCGAG TACATCGACG GAATTCTGCG AATGGTCACC
901     GGAGGTATTA CTTCTACCGC CGCTATGGGT GCTGGTGTGA CCGAGGATCA
951     GTTCAAGTCT AAGCTGTAAC CCGGG
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The GlyR1 enzyme could be expressed in the different yeast organelles for producing glycolic acid. The episome plasmid pJN34 bearing a constitutive promoter P_{TEF} was used to express the gene *GLYR1*(Ghogare et al., 2020). After digestion of *GLYR1*consisting of 34 amino acids from the C-terminus of *ICL1* with HindIII and SmaI (Table 2), the gene fragment was cloned into pJN34 to develop the vector pSynGLYR1. By transforming *Y. lipolytica* ΔMS1ΔMS2 with pSynGLYR1, the expressed enzyme GlyR1 was in yeast peroxisome because the added C-terminus amino acids from ICL1 had a signal peptide for peroxisomal localization (Barth & Scheuber, 1993). The vector pGLYR1 was constructed to express the gene *GLYR1*without C-terminus amino acids from ICL1, and the gene product was in yeast cytosol without any specific signal peptides.

To enable the expressed enzyme to target yeast mitochondria, we need to identify and characterize a mitochondrial targeting sequence in *Y. lipolytica*. We tested the leading peptide from 2-oxoglutarate dehydrogenase E1 component (OGDC1, YALI0E33517g) from *Y. lipolytica* as Mitochondrial Targeting Sequence (MTS). OGDC1 is a mitochondrial enzyme in yeast. In this study, 36 N-terminus amino acids of OGDC1 have been predicted as a cleavage peptide by an online tool, TargetP-2.0. We investigated the function of mitochondrial targeting by visualizing the cellular localization of enhanced Green Fluorescent Protein (eGFP) fused with the selected leading peptide of OGDC1. After verifying the translocation of eGFP to the yeast mitochondria, the new expression vector pMT-GLYR1 containing MTS was constructed to express *GlyR1*. The enzyme GLYR1 was present in mitochondria in yeast transformants carrying the plasmid pMT-GLYR1.

(3) Investigation of the role of *acs2* gene in the utilization of acetic acid

As shown in Figure 2, to utilize acetic acid, the major component of VFAs, the first step is activating acetate into acetyl-CoA by ACS with consuming ATP in the cells. One gene (*ACS2*, YALI0F05962g) encoding acetyl-CoA synthetase has been found in *Y. lipolytica*, while *S. cerevisiae* has two genes *ACS1* and *ACS2*. To investigate the role of *ACS2* in the utilization of acetic acid by *Y. lipolytica*, we deleted this gene by following the procedure shown in Figure 3. The primers used to generate the single knockout strain *Y. lipolytica* ΔACS2 were listed in Table 1. The expression vector pJN34-ACS2 was developed by cloning the native gene *ACS2* into the plasmid pJN34. *Y. lipolytica* ΔACS2 was transformed with pJN34-ACS2, and the transformant was characterized for the growth on acetate as a sole carbon source.

2.3 Producing VFAs from Organic Waste

Two types of organic wastes, food waste and dairy manure, were tested for the production of VFAs in this study. We collected the anaerobic sludge inoculum from a primary sedimentation tank at the wastewater treatment plant in Pullman, Washington. The sludge was transferred into sterile bottles purged with nitrogen gas to ensure anaerobic conditions and then stored at 37 ºC for one week to minimize the degradation of organic compounds in the sludge. Food waste was obtained from a student cafeteria at Washington State University (WSU) in Pullman, Washington. The food waste consisted of rice, noodles, meat, and all kinds of vegetables and fruits. The dairy manure was collected from Knott Dairy Center at WSU. The sludge inoculum and waste materials were characterized in terms of total solid (TS) and volatile solid (VS) contents.

Methanogenesis has to be blocked for the production of VFAs rather than biogas from organic wastes during the AD process. VFA production process was conducted using a 7.5-liter fermenter (NBS Bioflo-110) with a 5-liter working volume. The mixed liquor was adjusted to contain 15% total solids of 2,500 g of waste materials (e.g., food waste) and 2,500 g of anaerobic sludge. The mixture was purged with nitrogen for 20 minutes and capped tightly with butyl rubber to maintain anaerobic conditions. The AD process was carried at a controlled temperature, agitation speed at 300 rpm, pH at 7.0, and without aeration. The temperature used for the production of VFAs was 70 ºC. Samples were collected from the bioreactor each day and used to determine the content and composition of VFAs produced.

2.4 Analytical Procedures

We analyzed the structure of the product using gas chromatography-mass spectrometry (GC-MS) and quantified the content of glycolic acid using high-performance liquid chromatography (HPLC). To confirm the glycolic acid produced by the engineered yeast strains, the supernatant was dried and used for methanolysis with methanol at 95 ºC for eight hours. After extracting with hexane, the organic phase was used for analysis with GC-MS 7890A (Agilent Technologies, Inc. Santa Clara, CA) equipped with 5975C mass selective detector. The content of glycolic acid was qualified by using HPLC. The cell cultures with a volume of 1.5 ml were harvested by centrifuging at 13,000 rpm for 10 minutes. After filtration, the supernatant was collected for the determination of the glycolic acid secreted by the engineered strains. Glycolic

acid was analyzed by using a Pro-Star 230 HPLC system equipped with an Aminex HPX-87H ion exclusion column equipped with an ultraviolet detector. The column was maintained at 65 ºC. The mobile phase was 0.005 M sulfuric acid with a flow rate of 0.6 ml/min. A standard curve of the area versus the concentration of glycolic acid (Sigma) was also generated using the same HPLC system.

We have investigated the subcellular localization of protein expressed in yeast cells using staining. Staining of cells was performed with MitoTracker Red (Invitrogen) to track the cellular localization of eGFP expressed in *Y. lipolytica* (Bakin & Jung, 2004). Cells grown for two days were harvested, washed twice, and suspended with sterile 50 mM Tris-HCl, pH 7.5. The cells in Tris-HCl buffer were mixed with MitoTracker Red with a stock concentration of 1 mg/ml dissolved in dimethyl sulfoxide (DMSO), and the final concentration of MitoTracker Red for staining was 1 μg/ml. After incubating the cells with MitoTracker Red for 10 minutes at room temperature, the samples were washed and then observed using a Leica TCS SP8 confocal microscope located at the Franceschi Microscopy and Imaging Center, WSU. The fluorescence of MitoTracker Red was excited at a wavelength of 543 nm and detected in the range of 550-650 nm. GFP was excited at 488 nm, and fluorescence emission ranging 500-535 nm was detected. Finally, we merged the fluorescence images of MitoTracker Red and GFP to verify their co-localization.

VFAs produced from organic wastes were quantified using Agilent 7890A Gas Chromatograph (Agilent Technologies, Inc. Santa Clara, CA) equipped with a flame ionization detector and Famewax column (30 m × 320 μm × 0.25 μm) (Restek Corporation). The procedure was modified from a previously developed protocol (Federation, 2005). One ml of supernatant was collected from the bioreactors for waste digestion and then diluted with water. The solution of 85% phosphoric acid was used to adjust the pH of the liquid to around 2.0. After centrifuging and filtering with a 0.45 μm filter, the supernatant was used for GC analysis. VFAs produced from the organic waste were quantified using the standard curves of individual organic acid compounds with different contents (Sigma).

The cell growth was quantified by measuring absorbance at 600 nm (OD_{600}) of the culture using a Shimadzu UV-visible spectrophotometer (UV-2550) and dry cell weight. For dry cell weight measurement, 5 ml of cell culture was collected and centrifuged at 13,000 rpm for 10 minutes. The pellets were washed twice with 5 ml of distilled water and dried at 104 ºC, until a consistent weight was obtained (approximately 24 hours). Weight of dried cell biomass was recorded.

3. Results and Discussion

3.1 Metabolic Engineering of Yeast Strains

We aimed to engineer the yeast cell factory of a non-conventional yeast *Y. lipolytica* to convert VFAs into glycolic acid as the targeted biochemical in this project. *Y. lipolytica* has several advantages, including a high VFAs tolerance and the capability to utilize VFAs, as a microbial host for metabolic engineering in this study. *Y. lipolytica* was cultured and genetically engineered to biosynthesize various organic acids, including α-ketoglutaric acid, citric acid,

succinic acid, and itaconic acid. The production of glycolic acid from acetate, especially VFAs generated from organic wastes, has not been fully explored.

As shown in Figure 2, we have designed a pathway for the production of glycolic acid from acetic acid. In this designed pathway, only one heterologous encoding GR needs to be introduced into *Y. lipolytica* for producing glycolate from glyoxylate, which can be biosynthesized from acetate through the glyoxylate cycle (Li et al., 2019). However, glyoxylate can be consumed by the native enzyme MS in *Y. lipolytica*. Single knockouts *Y. lipolytica* ΔMS1 and ΔMS2 were developed to block cellular consumption of glyoxylate, the precursor for the biosynthesis of glycolate. Double knockout *Y. lipolytica* ΔMS1ΔMS1 was further generated. Deleting the genes encoding MS will prevent the cell from consuming glyoxylate and allow the organism to funnel all glyoxylate towards glycolate synthesis. The double knockout strain ΔMS1ΔMS2 did not show observable defects of growth on acetate as a sole carbon source. Thus, it represents a successful first step towards the engineering of strains for the production of glycolic acid.

In the strains engineered for the biosynthesis of glycolic acid, one of the commonly used and readily available genes was *ycdW* from *E. coli* (Deng et al., 2018). However, after we cloned and expressed *ycdW* from *E. coli* in the double knockout strain *Y. lipolytica* ΔMS1ΔMS1, the recombinant could not produce glycolic acid. This result was consistent with Koivistoinen et al. (2013), in which the yeast *S. cerevisiae* bearing *ycdW* from *E. coli* could not produce glycolic acid either.

We looked into an alternative gene encoding GR to produce glycolic acid. We investigated the expression of codon-optimized GLYR1 from *A. thaliana* for glycolic acid production in the mutant *Y. lipolytica* ΔMS1ΔMS1 (Koivistoinen et al., 2013). Glycolic acid was detected in the supernatant of the strain bearing *GLYR1* by HPLC, and the methanolysis product, methyl glycolate, was further confirmed by GC-MS analysis. The resulting mass spectrum is shown in Figure 4. Because each component of the mass spectra is unique, the fragments are used to determine the mass weight and structure of a compound in a conclusive and an accurate way. Expression of GLYR1 could be optimized to improve glycolic acid production by *Y. lipolytica*.

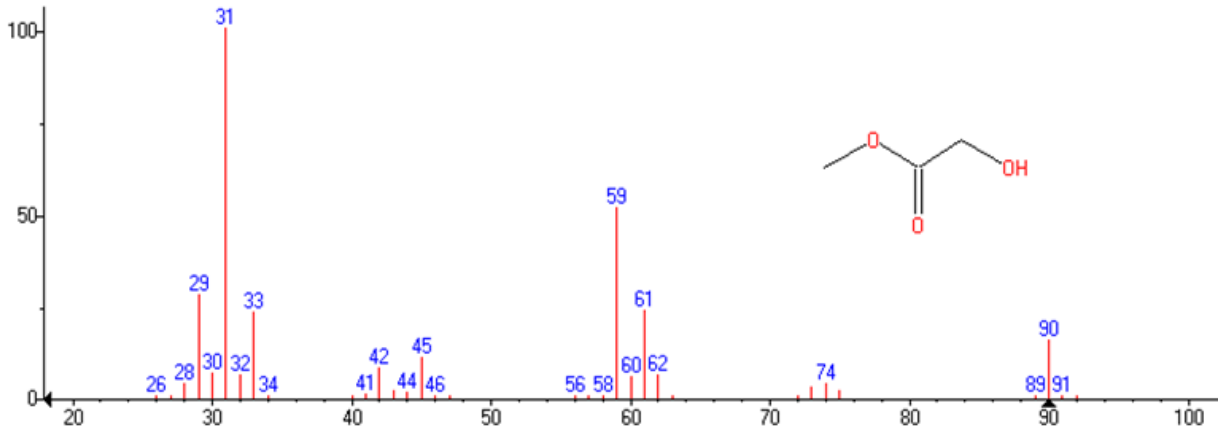

Figure 4: Analysis of product produced by the strain expressing GLYR1 using GC-MS. This mass spectrum has been used to verify the presence of glycolic acid by comparison of the chemical structure from a standard library.

As a eukaryotic cell, yeast has different cellular compartments, including cytosol (the water-based solution inside cells, in which the organelles, proteins, and other cell structures float), peroxisome, and mitochondria (two important organelles). The previous studies indicated that the target product, glycolic acid, could be produced from glyoxylate, which was the primary metabolite produced in the glyoxylate cycle. The reactions for the glyoxylate cycle mainly occur in yeast peroxisome. In contrast, the steps of TCA cycle for the generation of isocitrate, the substrate for the formation of glyoxylate, localize in the mitochondria (Figure 2). Therefore, it is necessary to take the pathway compartmentalization into account for the engineering of a yeast cell factory for the biosynthesis of glycolic acid.

To target an enzyme in yeast mitochondria, the expression gene of interest needs to be fused with a functional MTS. We evaluated the leading peptide from a mitochondrial enzyme, OGDC1 in *Y. lipolytica* as MTS, by investigating eGFP localization within the cells. As shown in Figure 5, mitochondrial localization of GFP fusion protein was confirmed by merging red and green fluorescence images. The results indicated that eGFP could be targeted to *Y. lipolytica* mitochondria using 36 amino acids in N-terminus from OGDC1.

The synthetic biology tools for genetic manipulation of this important microbial host, *Y. lipolytica* were expanded to enable pathway compartmentalization in the yeast organelles by testing the different signal peptides, including MTS (Figure 5). For the expression of enzymes in the peroxisome, we used 34-amino acid in C-terminus from a native peroxisomal enzyme, ICL1 (Barth & Scheuber, 1993). We then targeted the enzymes for producing glycolic acid in the peroxisome and mitochondria within the double knockout strain, *Y. lipolytica* ΔMS1ΔMS2. These tools developed can be used to express genes in this study and further expand the molecular toolbox for synthetic biology of *Y. lipolytica*.

We found that the production of the enzyme GYLR1 in different organelles, including cytosol, peroxisome, and mitochondria, resulted in the production of glycolic acid at the different titers (Figure 6A). Using 30 g/L of acetate, one of the major components in VFAs, as the sole carbon

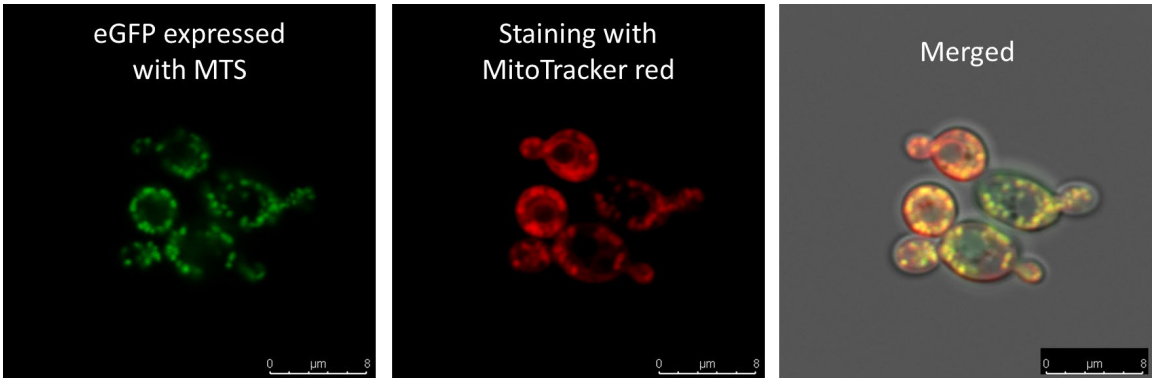

Figure 5: Subcellular localization of GFP in Y. lipolytica mitochondria with leading peptide from OGDC1 observed under a fluorescence microscope. The fluorescence between GFP and MitoTracker Red, which specifically stained the yeast mitochondria, was merged to verify the mitochondrial localization of the GFP.

source, the strain *Y. lipolytica* ΔMS1ΔMS2 expressing GYLR1 in peroxisome produced the highest titer, 3.4 g/L of glycolic acid after cultivation for 96 hours at 28 ºC in a shaking flask.

Under the same culture conditions, the stain bearing GYLR1 in mitochondria produced 2.5 g/L glycolic acid, whereas expression of GYLR1 in cytosol only led to the production of 1.8 g/L glycolic acid (Figure 6A). The strains secreted the glycolic acid to the supernatant of culture media. During cultivation, the pH in the culture media increased from 6.6 to 9.2, mainly due to the utilization of acetate. We further investigated the cell growth of *Y. lipolytica* ΔMS1ΔMS2 expressing GYLR1 in peroxisome on 30 g/L of acetate, and there was no noticeable difference after genetic engineering compared with the parent strain (Figure 6B). This result highlights that pathway compartmentalization in different cellular compartments can be used as an essential strategy for designing and engineering a yeast cell factory to produce glycolic acid. Furthermore, we can improve glycolic acid production from acetate by both pathway engineering and optimizing fermentation by controlling pH and other factors.

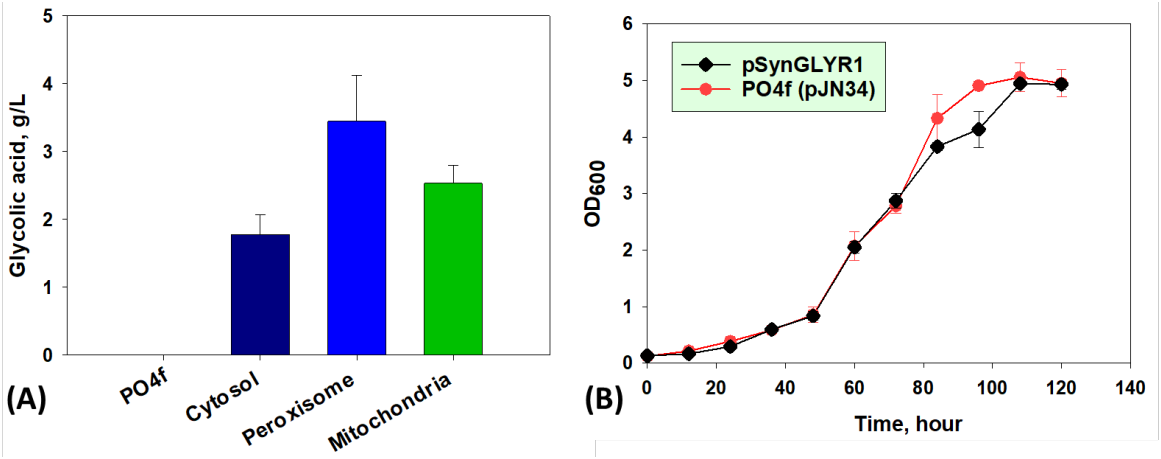

Figure 6: (A) Glycolic acid production from 30 g/L of acetate by Y. lipolytica ΔMS1ΔMS2 expressing GYLR1 in different subcellular organelles, including cytosol, peroxisome, and mitochondria, and (B) Cell growth of Y. lipolytica ΔMS1ΔMS2 bearing plasmid GYLR1 and Po4f bearing plasmid pJN34 on 30 g/L of acetate.

Y. lipolytica can utilize acetate as a sole carbon source for its growth (Figure 6), and we can further engineer the strain to improve the capability of acetate utilization. To understand the role of *acs2* of *Y. lipolytica* in acetate utilization, we first deleted this gene, and then overexpressed *acs2* in this knockout strain *Y. lipolytica* ΔACS2. As shown in Figure 7A, plasmid pJN34-ACS2 was developed to express *acs2* under the control of promoter P_{TEF}. After deletion of *acs2* in *Y. lipolytica*, the mutant *Y. lipolytica* ΔACS2 exhibited growth deficiency on acetate (Figure 7B). OD_{600} of *Y. lipolytica* ΔACS2 bearing pJN34 was around half of the value of the control strain, Po4f. Transforming *Y. lipolytica* ΔACS2 with pJN34-ACS2 restored the growth on acetate. The result indicated that *acs2* of *Y. lipolytica* played an essential role in acetate utilization. The single knockout *Y. lipolytica* ΔACS2 still could use acetate as a sole carbon for growth. This suggested that there might be other enzymes, such as short-chain fatty acyl-CoA synthetase, responsible for converting acetate into acetyl-CoA. We can overexpress *acs2* to improve acetate and other VFA compounds utilization by *Y. lipolytica*.

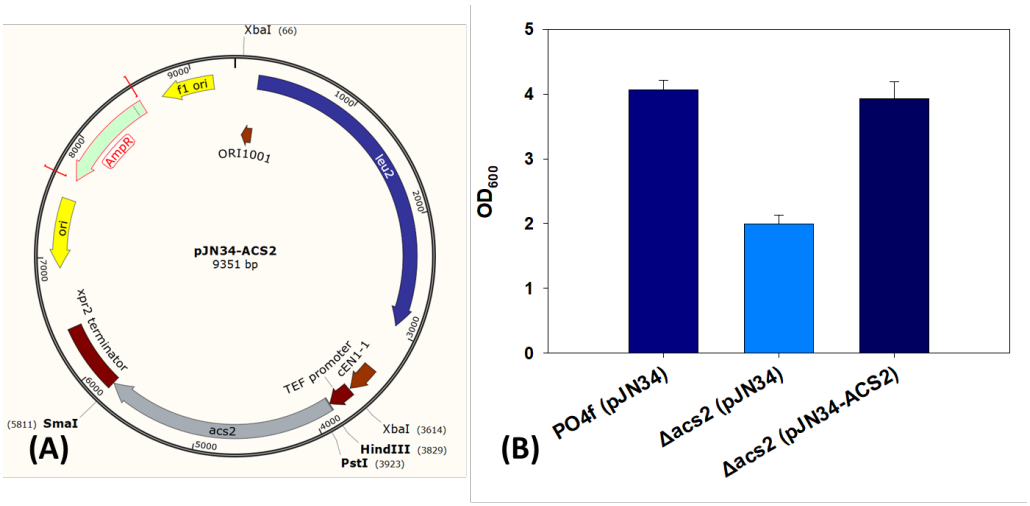

Figure 7: (A) The schematic map of expression vector pJN34-ACS2, and (B) Cell growth of Y. lipolytica ΔACS2 bearing plasmid pJN34 or plasmid pJN34-ACS2 on 20 g/L of acetate for four days. The parent strain bearing pJN34 grown under the same conditions was used as a control.

3.2 Production of VFAs from Organic Wastes

Two types of organic waste, food waste and dairy manure, were used as feedstock to produce VFAs in this project. Because the total solid (TS) and volatile solid (VS) contents of waste materials affected the efficiency of AD, we measured these two parameters for food waste and inoculum sludge (Table 3).

Table 3: Total solid (TS) and volatile solid (VS) contents and pH of inoculum sludge and food waste.

Parameter	Inoculum sludge	Food waste
TS (%)	1.58 ± 0.2	27.42 ± 0.3
VS (%)	1.12 ± 0.1	26.19 ± 0.3
VS /TS (%)	70.9	95.5
pH	7.4	-

We developed a novel AD process operated at high temperature (70 °C) for efficient production of VFAs from waste streams by arresting methanogenesis and accelerating acidogenesis. By controlling pH at 7.0, VFAs mainly consisting of acetate were efficiently produced from food waste in an anaerobic digester. As shown in Figure 8A, more than 50 g/L VFAs were produced from food waste by this novel AD process within five days. Food waste was considered as an easily digested substrate for AD. We also evaluated VFA production from dairy manure. As shown in Figure 8A, the highest acetate titer reached 27.0 g/L after five days and then decreased when using dairy manure as feedstock. We also investigated the composition of VFAs produced from dairy manure. As shown in Figure 8B, acetate was the dominant component of VFAs, although we detected both propionate and butyrate. This result demonstrated that VFAs could be efficiently produced from wastes by pH and temperature control without expensive chemicals such as 2-BES to inhibit methanogenesis. VFA production can be further improved by enhancing the hydrolysis and acidogenesis rates through physical or chemical pretreatments, the addition of enzymes, pH control, temperature control, redox potential, and inocula optimization.

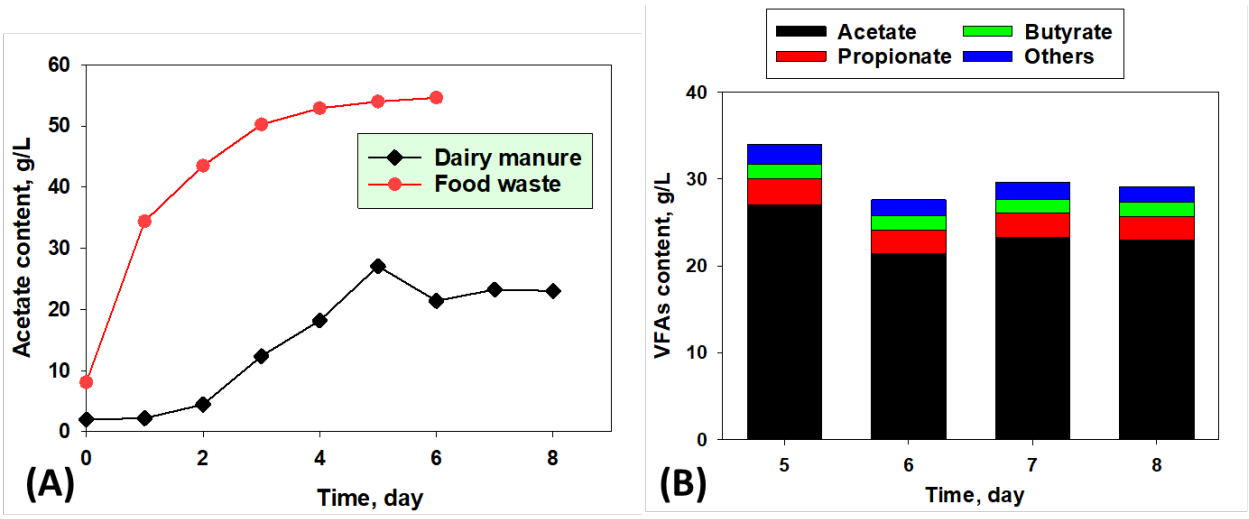

Figure 8: (A) Acetate production from AD of food waste and dairy manure, and (B) the composition of VFAs produced from AD of dairy manure for 5-8 days.

Although we have successfully engineered the strains for producing a targeted chemical from acetate (Figure 6A), the production of glycolic acid from VFAs generated from waste materials has not yet been demonstrated. Because the use of food waste as feedstock led to higher titers of VFAs than dairy manure (Figure 8A), we used VFAs generated from food waste to culture the engineered strain expressing peroxisomal GYLR1to produce glycolic acid. The liquid effluent was separated from the product of food waste digestion. The effluent enriched with VFA was used to culture the glycolic acid-producing strain. As shown in Figure 9, the strain produced more than 3.6 g/L glycolic acid in a shaking flask for 120 hours. During cultivation, pH increased from 7.0 to 9.5. This hybrid process succeeded in production of glycolic acid from organic waste by incorporating the AD process with yeast cultivation.

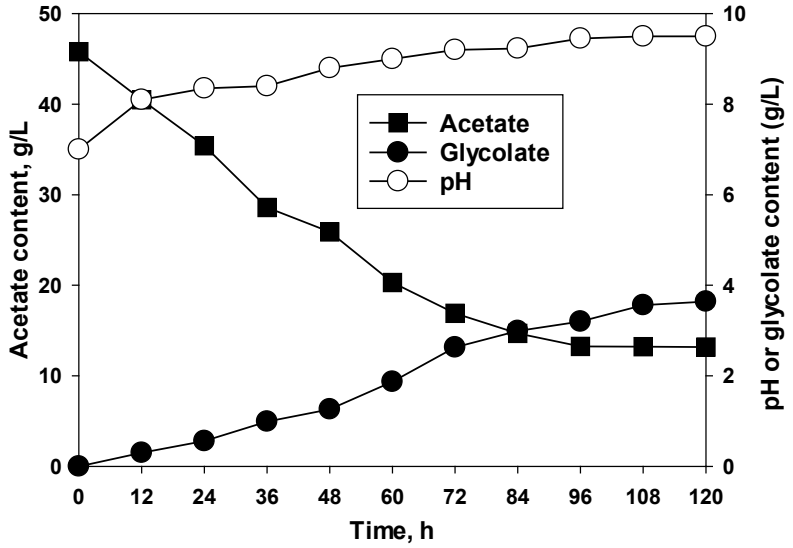

Figure 9: Production of glycolic acid from VFAs generated from food waste by the genetically engineered yeast strain.

4. Conclusions & Future Direction

Using organic wastes as feedstock offers a promising option because of the benefits in environmental protection and resource conservation. It also provides the opportunity for cost-effective production for production of biofuels and renewable bio-chemicals. In this project, we demonstrated the feasibility of the production of the biochemical glycolic acid, a biochemical with broad industrial applications, from organic waste using an innovative technology route. The AD process was operated at a high temperature to produce VFAs from organic wastes. The titer of acetate, the primary component in VFAs, reached 50 g/L by using food waste as a feedstock. We successfully engineered a non-conventional yeast, *Y. lipolytica,* as a cell factory to biosynthesize glycolic acid from acetate as a sole carbon source. *Y. lipolytica* is an ideal microbial host for biochemical production from VFAs. Potential exists for other biofuels and

bioproducts to be made using the design and engineering of this versatile cell factory with other genetic modifications. Success in this project has the potential to advance enabling technologies, accelerate the development of bio-based industry, and maximizing biochemical production from organic waste streams.

Future efforts will focus on optimizing the developed processes and incorporating them into a functional biorefinery to achieve the technical and economic performance targets. To advance the bio-economy for converting waste streams into bio-based products, engineering the microorganisms to reach high TRY performance (Titer, production Rate, and Yield), remains a top priority. We will employ the strategies of overexpressing genes for enhancing precursors supply ("push") and product formation ("pull") to develop a productive strain for generating glycolic acid.

After further improvement of performance of the strains developed in this project for glycolic acid production, their potential for use in a bioreactor setting will be enhanced by further work in four areas:

1. Further optimization of the thermophilic AD process for generation of VFAs;
2. Developing an optimal fermentation process, including a feeding strategy;
3. Optimization of product separation and purification; and
4. Development of a techno-economic analysis.

Although this project focuses on the production of glycolic acid, the technical platform can be tailored to deliver a variety of commodity chemicals by constructing different metabolic pathways in the microbial host. Therefore, developing and demonstrating this technology has the potential for broad impacts for the biofuel and bioproduct industries.

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