INDONESIAN JOURNAL OF LIFE SCIENCES

Volume 5, No. 1, March 2023

REVIEW ARTICLE

Improving the Biodiesel Production in *Saccharomyces cerevisiae:* Metabolic Engineering and Enzyme Engineering

Riahna Kembaren[1]*

[1]Department of Biotechnology, Institut Bio Scientia Internasional Indonesia, Jakarta, Indonesia

**corresponding author: riahna.kembaren@i3l.ac.id*

A B S T R A C T

The yeast *Saccharomyces cerevisiae* can be genetically modified using metabolic engineering techniques to produce biofuels such as biodiesel from renewable carbon sources such as lignocellulose. *S. cerevisiae* metabolize monosaccharide of lignocellulosic hydrolysates to supply fuels/energy globally without competition issue with food supply. *S. cerevisiae* can produce ethanol and free fatty acids (FFAs), two precursors for biodiesel. Expressing the heterologous wax ester synthase or wax ester acyltransferase (WS) in *S. cerevisiae* will establish processes that allow for the production of biodiesel by *S. cerevisiae* from ethanol and fatty acyl-CoA using WS. Here, we review some recent strategies applied to the metabolic and enzyme engineering of *S. cerevisiae* for improving biodiesel yield. The metabolic engineering strategy can be done by directing the carbon flux toward the fatty acid biosynthesis pathway by engineering some genes in the central carbon metabolism and fatty acid synthesis. Combined overexpression genes of Acetyl-CoA synthetase (ACS), Acetyl-CoA carboxylase (ACC1), and Fatty Acid Synthase (FAS) will improve biodiesel synthesis. The biodiesel yield produced by *S. cerevisiae* can also be increased by blocking side reactions. In enzyme engineering, we can remove the allosteric inhibition control of Snf1 to Acetyl-CoA carboxylase (ACC1). This enzyme converts Acetyl Co-A to malonyl Co-A, by mutation of two phosphorylation sites of ACC1 via site-directed mutagenesis, which mutates serine^{1157,659} to alanine^{1157,629}. This results in enhanced carbon flux to fatty acid synthesis and contributes to a higher biodiesel yield.

K E Y W O R D S

Biodiesel; Lignocellulose; Metabolic engineering; Enzyme engineering

H I G H L I G H T S

- There is a global demand for a more environmentally friendly energy source alternative to fossil fuels.
- Biodiesel production from renewable lignocellulose can be used as an energy source.
- *S. cerevisiae* can produce the precursors for biodiesel.
- Metabolic and enzyme engineering of *S. cerevisiae* can improve its biodiesel yield.

INTRODUCTION

The need for energy continues to rise globally. On the other hand, the high consumption of fossil fuels is one of the major causes of environmental pollution and climate change. An alternative solution to this problem is replacing fossil fuels with biofuels such as biodiesel (Mohamad et al., 2017). Biodiesels or

mono-alkyl esters of long-chain fatty acids are obtained from fatty acids (mostly C16-C18) with short-chain alcohols via the transesterification process. Biodiesel has several types, such as fatty acid methyl esters (FAME) (methylic biodiesel), fatty acid ethyl esters (FAEE) (ethylic biodiesel), and fatty acid alkyl esters (FAAE) (alkylic biodiesel). This type of biodiesel depends on the type of alcohol used in transesterification during the forming reaction of biodiesel. However, FAEEs is the most common biodiesel produced in the industry because it uses ethanol which is less toxic and considered more renewable compared to methanol (Issariyakul & Dalai, 2014).

The transesterification process of biodiesel uses vegetable oil, animal fat, or waste cooking oils with ethanol. It is non-toxic and environmentally friendly, but unsuitable for high volume and continuous process production because there is the limitation for oil sources, and for some sources, such as vegetable oil and animal fat, are used as foods (Sundus et al., 2017). Lignocellulose can be used as a renewable resource for the manufacture of biofuels, and polymers substance which can supply energy globally without competition issues with food supply since it is not used for the food product. Lignocellulose can get from the residue of the agriculture sector and pulp mills (Jonsson et al., 2013). Some previous studies have reported using lignocellulose as a substrate for *S. cerevisiae* to produce biodiesel. Before it can be used as a substrate by *S. cerevisiae* to produce biodiesel, this lignocellulose needs to be treated. The pretreatment and hydrolysis process on lignocellulose will produce monosaccharides such as D-glucose, D-galactose, D-mannose, D-rhamnose, D-xylose, and L-arabinose. Monosaccharides from lignocellulosic hydrolysates will be transported into yeast cells using transporters or carriers. The sugar transporter is placed in the cell surface. The native transporter *S. cerevisiae GAL2* is the most efficient for xylose, galactose, ribose, and arabinose (Brink et al., 2021; Young et al., 2011). *HXT*-type transporter will intercede glucose uptake cooperate with glucose receptors such as *SNF3* (high-affinity glucose transporter, sensor of low levels of glucose concentration), and *RGT2* (low-affinity glucose transporter, sensor of high glucose concentrations) (Brink et al., 2021; Young et al., 2011). Glucose transporter in *S. cerevisiae* can transport D-glucose, D-fructose, and mannose (Brink et al., 2021; Young et al., 2011).

The production of fuels or chemicals can be done by using a microbial cell factory in which can meet commercial requirements for yield, productivity, and titer. Biodiesel production using microorganisms is more suitable for high volume and continued process production. The yeast *Saccharomyces cerevisiae (S. cerevisiae*) is the proper microorganism compared to other microorganisms, such as *Escherichia coli* to produce biodiesel because it can produce fatty acids with a chain length of 16-18 carbon (Watsuntorn et al., 2021). *S. cerevisiae* has genetic information and tools to manipulate its metabolic pathways and engineer the enzyme to produce biodiesel in proper amounts (Phukan et al., 2019; Tang et al., 2015). Moreover, *S. cerevisiae* is a good ethanol producer. This ethanol is needed during the esterification of fatty acid during biodiesel biosynthesis. *S. cerevisiae* is able to accumulate ethanol in high concentrations, then producing biodiesel by *S. cerevisiae* will have no limitation by ethanol supply (Lin et al., 2013; Zhou et al., 2014). Moreover, by expressing the heterologous wax ester synthases (WS) in *S. cerevisiae* will establish processes that allow for the production of biodiesel by *S. cerevisiae*.

Metabolic engineering is a purposeful modification of one to several genes to direct the metabolic pathway in a microorganism to achieve the goal that the microorganism can produce a specific product efficiently. Through metabolic engineering, a microorganism also can be engineered to utilize an inexpensive renewable carbon source as a substrate to produce a chemical of interest, even those nonnatives to its metabolism. In biotechnology, metabolic engineering is used to modify the endogenous metabolic network of microorganisms to produce valuable compounds. This method requires overexpression or downregulation of specific enzymes/proteins in a metabolic pathway that can improve the cell to produce desired product (Erb et al., 2017). In enzyme or protein engineering, amino acid sequences are changed by recombinant DNA mutations in order to design proteins or enzymes. By doing this, new metabolites can be produced by modifying the catalytic activity of individual enzymes and also removing the allosteric inhibition control of

specific enzymes (Hong et al., 2018). In this review, we discuss improving the biodiesel production in *S. cerevisiae* by applying various metabolic engineering and enzyme engineering strategies, such as directing carbon flux to fatty acid biosynthetic pathway, expressing the heterologous gene, deleting competing pathways, and modifying or improving enzyme activities by directed evolution.

IMPROVE BIODIESEL SYNTHESIS PATHWAY: METABOLIC ENGINEERING

Directing carbon flux to fatty acid biosynthetic pathway

To produce biodiesel through esterification in *S. cerevisiae, S. cerevisiae* first needs to synthesize ethanol and free fatty acids (FFAs) by itself as the precursors of biodiesel (Figure 1).

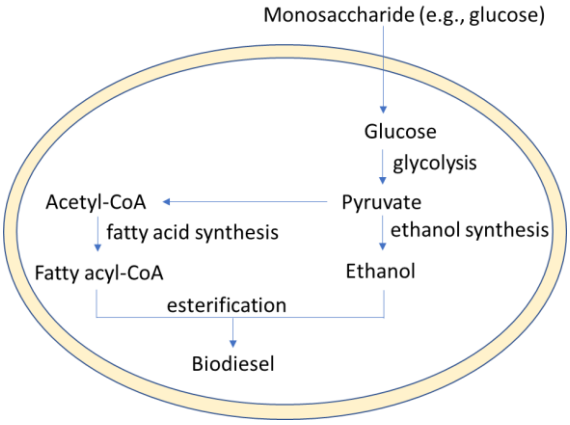

Figure 1. The cell factory produces biodiesel.

After monosaccharides have entered the cell, they will be metabolized by *S. cerevisiae* to produce pyruvate through some sugar catabolic pathway. Glucose will be metabolized directly through the glycolysis pathway. Galactose will be converted to glucose first and then will be metabolized through the glycolysis pathway. Other monosaccharides, such as arabinose, will be converted by *S. cerevisiae* through the pentose phosphate pathway. Some pyruvate will enter mitochondria and be converted to Acetyl-CoA for the tricarboxylic acid cycle (TCA) cycle. In the cytosol, pyruvate will be converted to acetaldehyde catalyzed by pyruvate decarboxylase (PDC), then continue to be converted to acetate catalyzed by aldehyde dehydrogenase (ADH). Acetate will be transformed into Acetyl-CoA by Acetyl-CoA synthetase (ACS) (Li et al., 2014; Tang et al., 2015). The intermediate acetaldehyde also is used in the ethanol production pathway. Non-oleaginous yeast such as *S. cerevisiae* generates Acetyl-CoA in the cytoplasm only from the pyruvate-acetaldehyde-acetate pathway; this limits the level of Acetyl-CoA in the cytoplasm and competes with ethanol production. Whereas to get a high concentration of biodiesel needs a high concentration of Acetyl-CoA in the cytoplasm. Acetyl-CoA in mitochondria cannot be transported to the cytosol directly.

To solve this problem, Yang et al. (2012) subsequently constructed a recombinant *S. cerevisiae*, of which the mitochondrial NAD^+-dependent isocitrate dehydrogenase in *S. cerevisiae* (*ScIDH*) which encodes by *idh1* and *idh2,* was replaced with mitochondrial NAD^+-specific Idh from *Rhodosporidiumtoruloides* (RtIdh) which encodes by RtIDH gene. *Rhodosporidium toruloides* is an oleaginous yeast that can accumulate triacylglycerols as cellular storage lipids up to 70% of the cell mass by assimilating carbohydrates. The original IDH in *S. cerevisiae* (ScIDH) is deleted and RtIDH gene (*idh* gene from *R. toruloides*) is expressed in *S. cerevisiae* (El-rotail et al., 2017; Yang *et al*., 2012). In this recombinant bacterium, RtIDH was expressed under the control of the strong and constitutive TPI promoter. cDNA fragments coding RtIDH will be transcribed to mRNA by RNA polymerase and translated into NAD^+-dependent isocitrate dehydrogenase through the protein synthesis pathway. RtIDH stimulates some further biochemical reactions, which increase the level of

citrate in mitochondria (Figure 2). Knockout of *idh1* and *idh2* genes, which removes the gene from the genome and replaces it with RtIDH gene, will increase ACL (ATP-citrate lyase) and citrate levels in mitochondria. Exceed citrate in mitochondria will strengthen TCA cycle and will be transported to the cytosol *via* the citrate/malate shuttle. ACL then cleaves citrate to oxaloacetate and Acetyl-CoA in the cytoplasm, result in enhancing the supply of the cytosolic Acetyl-CoA (Tang et al., 2015). In the cytosol, Acetyl-CoA will be carboxylated to malonyl-CoA by the acetyl-CoA carboxylase enzyme (ACC1). Acetyl-CoA and malonyl-CoA are then used as precursors for fatty acid synthesis. A sufficient supply of Acetyl-CoA and Malonyl-CoA is needed during fatty acid synthesis (Lin et al., 2013).

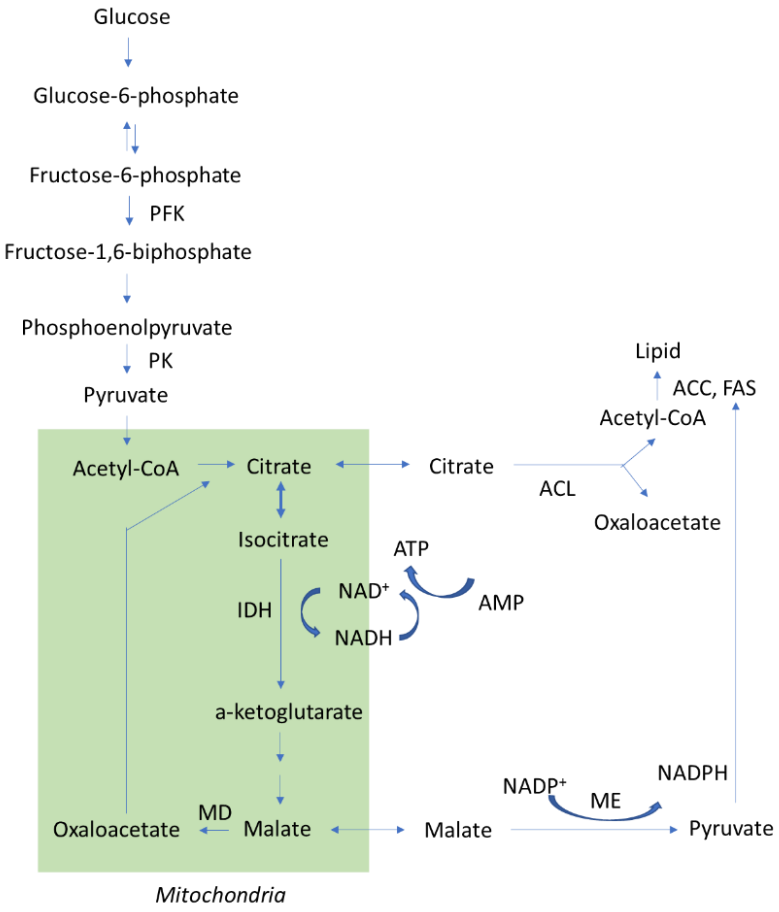

Figure 2. Metabolism influenced by citrate level. PFK = phosphofructokinase, PK = pyruvate kinase, ACL = ATP/ citrate lyase, IDH = NAD^+-dependent isocitrate dehydrogenase, MD = malate dehydrogenase, ME = malic enzyme, ACC = Acetyl-CoA carboxylase, and FAS = fatty acid synthase.

The first step in generating high yields of biodiesel in *S. cerevisiae* is to supply enough fatty acids. In *S. cerevisiae*, the biosynthesis of fatty acids can take place in the cytosol (type I FAS) and the mitochondria (type II FAS). However, type I FAS is responsible for the cytosolic de novo synthesis of fatty acid and biosynthesis of biodiesel. The type I FAS in *S. cerevisiae* comprises two subunits, a-subunit Fas2, and b-subunit Fas1. (Hu et al., 2019; Tang et al. 2015). In cytosol, Acetyl-CoA will be carboxylated to malonyl-CoA by the acetyl-CoA carboxylase enzyme (ACC1). Acetyl-CoA and malonyl-CoA are precursors for fatty acid synthesis. It needs a sufficient supply of Acetyl-CoA and Malonyl-CoA during the fatty acid synthesis process (Lin et al., 2013). Fatty acid synthesis starts when Acetyl-CoA and Malonyl-CoA are charged onto the Fatty Acid Synthase (FAS) complex (Singh et al., 2020). FAS is a dimeric enzyme complex that is responsible for fatty acid synthesis from Malonyl-CoA and Acetyl-CoA in the cytoplasm in *S. cerevisiae*. FAS complex is also responsible for elongation by repeated cycle reaction of reduction-dehydration-reduction. Acyl-ACP thioesterase primarily

establishes the chain lengths of fatty acids. Acyl-ACP thioesterase will control FAS to generate 16- or 18-carbon fatty acids (Figure 3). Acyl-ACP thioesterases can release the fatty acid chain from the FAS (Lin et al., 2013).

Malonyl-CoA is synthesized from Acetyl-CoA by incorporating CO_2, which is catalyzed by Acetyl-CoA carboxylase (ACC1). Following this, Acetyl-CoA is transferred to acyl carrier proteins (ACP) by acyltransferases (AT), and Malonyl-CoA is transferred to acyl carrier proteins (ACP) by malonyl/palmitoyl transferases (MPT). The ketoacyl synthase (KS) condenses them to acetoacetyl-ACP in a malonyl decarboxylation reaction; then, the β-ketoacyl-ACP is reduced by the ketoacyl reductase (KR), followed by a dehydration reaction catalyzed by the dehydratase (DH) and the second reduction reaction catalyzed by the enoyl reductase (ER), results in acyl-ACP. These processes are continued in a repetitive cycle manner using Malonyl-CoA and Acetyl-CoA as building blocks. The chain elongation usually stops at palmitoyl-ACP after seven cycles, which is determined by the ketoacyl synthase. Finally, acyl-ACP and Malonyl-CoA are transformed by malonyl transacylase (MPT) to form fatty acyl-CoA and the activated malonyl-ACP, which is necessary for initiating the next fatty acyl-CoA synthesis. Fatty acyl-CoA then transformed into lipids or FFAs catalyzed by a thioesterase (TE) (Hu et al., 2019) (Figure 4).

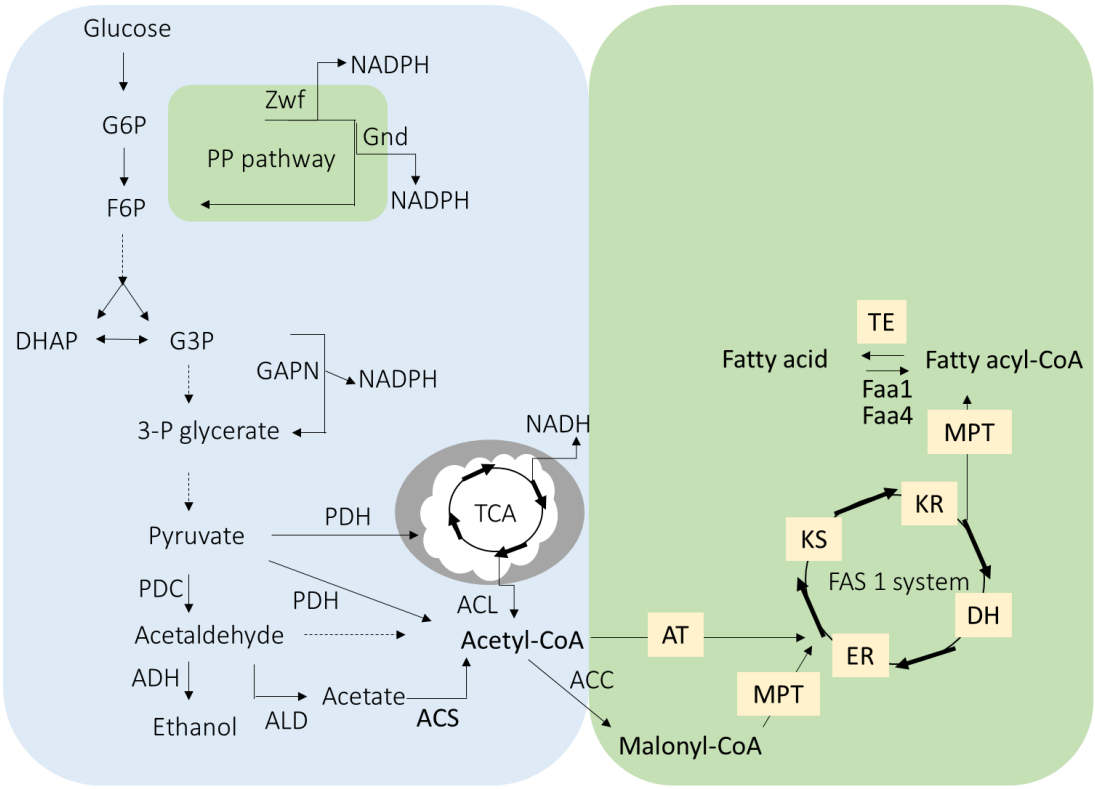

Figure 3. Relation between central carbon metabolism with fatty acid Synthesis. G6P = glucose-6-phosphate; F6P = fructose-6-phosphate; DHAP = dihydroxyacetone phosphate; G3P = glyceraldehyde-3-phosphate; Zwf = D-glucose-6-phosphate dehydrogenase; Gnd = phosphogluconate dehydrogenase; GAPN = glyceraldehyde-3-phosphate dehydrogenase; PDC = pyruvate decarboxylase; PDH = pyruvate dehydrogenase; ADH = alcohol dehydrogenase; ALD = acetaldehyde dehydrogenase; ACS = Acetyl-CoA synthetase; ACL = ATP citratelyase; ACC = acetyl-coenzyme A carboxylase; TE =thioesterase; Faa1/4 = fatty acyl-CoA synthetases; AT = acetyl transferase; MPT = malonyl/palmitoyl transferase; KS = ketoacyl synthase; KR = ketoacyl reductase; CH = dehydratase; and ER = enoyl reductase.

The yield of biodiesel produced by *S. cerevisiae* also can be increased by some metabolic engineering which increases the de novo synthesis of fatty acyl-CoA. The conversion of Acetyl-CoA to Malonyl-CoA by acetyl-coenzyme A carboxylase (ACC1) encoded by the acc1 gene is the rate-limiting step in de novo FAS in

yeast. However, the overexpression of only ACC is not affected fatty acyl-CoA biosynthesis significantly (Lin et al., 2013). The significant improvement is only achieved by combining the overexpression gene of ACS, ACC1, and FAS together. Overexpressing of Acetyl-CoA synthetase (ACSs) which encoded by the *acs1* and *acs2* gene, can increase the concentration of Acetyl Co-A in the cytosol, which then be converted to malonyl CoA by ACC1 (Tang et al., 2015). Overexpression of the FAS complex, which encoded by f*as1* gene also can contribute to a higher yield of biodiesel. The formation of fatty acyl-CoA is determined by acyl-CoA synthetase, which is encoded by the *faa1/4* gene (Figure 3).Overexpression of *faa1/4* gene, which will be translated to acyl-CoA synthetase during the translation protein process will enhance convert of free fatty acids into fatty acyl-CoA (Lin et al., 2013). However, a high concentration of fatty acyl-CoA can give feedback inhibition to the FAS complex. This problem can be solved by overexpression of endogenous acyl-acyl carrier protein (ACP) and overexpression of Acyl-CoA thioesterase (Tang et al., 2015). Runguphan & Keasling (2014) have reported that overexpression of the FAS (by *fas1* and *fas2*), Acetyl-CoA carboxylase (ACC1), and acyl-ACP thioesterase resulted in a higher FFAs production of 400 mg/L in *S. cerevisiae* using glucose as carbon source.

In fatty acid biosynthesis, NADPH is required for reduction in elongation. NADPH can be produced by malic enzyme and the pentose phosphate pathway (Figures 2 and 3). However, overexpressing an NADH-dependent malic enzyme will not be the right way to optimize because the malic enzyme will transform malate from TCA cycle. In general, the malic enzyme may disturb TCA cycle and reduce the ATP production for the cell (Figure 2). In *S. cerevisiae*, NADPH is largely generated from the pentose phosphate pathway (PP). On the PP pathway, ribulose-5- phosphate will be transformed to xylulose-5-phosphate, which is a precursor to the phosphoketolase pathway (PHK). The overexpression gene of phosphoketolase in *S. cerevisiae* will encourage carbon flux to the PHK pathway and increase NADPH concentration in the cytosol. The combination of PP and PHK pathways will increase the NADPH level required to produce fatty acyl-CoA (Tang et al., 2015; Yu et al., 2018). Yu et al. (2018) have reported that *S. cerevisiae* can synthesize 33.4 g/L of FFAs using glucose as a carbon source by increasing the concentration of acetyl CotA and malonyl-CoA and matching the NADPH demand by upregulating the pentose phosphate pathway (PPP) and PHK pathway.

Expressing the heterologous wax ester synthase (WS) in *S. cerevisiae*

Wax ester synthase or also known as wax ester acyltransferases (WS) is an essential enzyme in the last step of biodiesel synthesis. WS catalyzes biodiesel biosynthesis from ethanol and fatty acyl-CoAs (Lin *et al*., 2013). This enzyme is only active in the presence of CoA-activated fatty acids. The expression of heterologous WS in *S. cerevisiae* can successfully enable the strain to produce biodiesel.

A previous study from Shi et al. (2012) showed that heterologous WSs derived from five different microorganisms (*Acinetobacter baylyi* ADP1, *Marinobacter hydrocarbonoclasticus* DSM 8798, *Rhodococcus opacus* PD630, *Mus musculus* C57BL/6 and *Psychrobacter arcticus* 273-4) were successfully expressed in *S. cerevisiae*. They found that all these heterologous WSs can lead to the formation of biodiesel *in S. cerevisiae*. However, the best biodiesel-producing strain was found to be the one expressing WS from *M. hydrocarbonoclasticus* DSM 8798. The *ws2* gene from *M. hydrocarbonoclasticus* DMS 8798 is ligated into vector pSP-GM2 under the control TEF1 promoter. This plasmid was then introduced into *S. cerevisiae*. This introduction gene resulted in a high catalytic rate of WS. It yielded biodiesel with a titer of 6.3 mg/L when this recombinant strain of S. *cerevisiae* was cultured in broth media containing 2% (w/v) glucose. Another study by Shi et al. (2014) also reported that integration of the heterologous wax ester synthase gene (*ws2*) into *S. cerevisiae* chromosomes (integrated into the yeast genome)in multiple copies, established a stable expression system and resulted in an increase in biodiesel production of up to 34 mg/L. Eriksen et al. (2015) have reported that coupled heterologous expression of *ws2* from *M. hydrocarbonoclasticus* with heterologous type I FAS from *Brevibacterium ammoniagenes* yielding biodiesel 6.3-fold higher compared heterologous expression of that ws2 with endogenous FAS of *S. cerevisiae.* A study from de Jong et al. (2014)

reported that coupled heterologous expression of *ws2* from *M. hydrocarbonoclasticus* with overexpression of endogenous genes *ADH2* (encoding alcohol dehydrogenase), *ALD6* (encoding aldehyde dehydrogenase) and *acsSEL641P* from *Salmonella enterica* (encoding acetyl-CoA synthetase containing a point mutation to prevent acetylation) increase biodiesel yield of 2.7-fold.

Deleting competing pathways

The biodiesel yield produced by *S. cerevisiae* can also be increased by blocking side reactions. In *S. cerevisiae*, the production of storage lipids, such as triacylglycerols (TAGs) and sterol esters and the β-oxidation pathway, competes with biodiesel production because they use the same precursors, fatty acyl-CoA. Reducing the activity of diacylglycerol acyltransferase (DGAT) will prevent the direction of fatty acyl-CoA from forming triacylglycerol (TAG). By reducing this enzyme activity, there will be no loss of fatty acyl-CoA converted to side product T AG, and more biodiesel will be produced. DGAT is encoded by the *dga1* gene. By using repressor such as tryptophan in active form, can slow or partially switch off the *dga1* gene. When the repressor binds to the operator, it blocks transcription, result in mRNA and protein encoded by DNA that is not made (Shuler & Kargi, 1992). Knockdown of *the dga1* gene also can be done by the RNA silencing pathway, which uses RNA interface to stop the translation of mRNA gene into protein (Figure 4). Moreover, the knockdown of *are1* and *are2*genes that encode sterol acyltransferases will prevent the direction of fatty acyl-CoA from forming sterol ester (de Jong et al., 2015). A previous study from Valle-Rodriguez et al. (2014) showed that by blocking the conversion of fatty acyl-CoA to triacylglycerol and sterol ester in combination with the expression of *ws2* from *M. hydrocarbonoclasticus* resulted in higher biodiesel production *in S. cerevisiae* with the titer of 17.2 mg/L when its growth in medium contained glucose as carbon source.

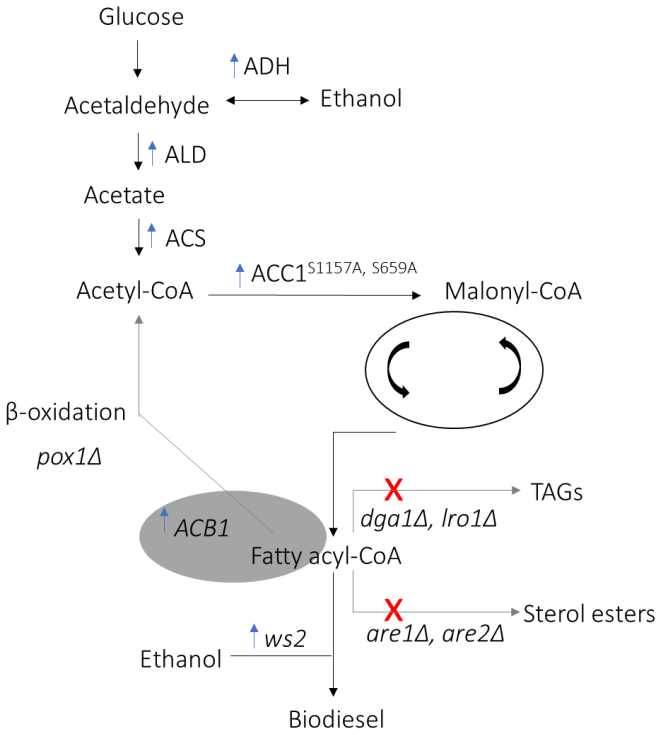

Figure 4. Directing biodiesel biosynthesis by deleting several genes (*dga1, lro1, are1, are2*) that compete on using of fatty acyl-CoA. ADH = alcohol dehydrogenase, ALD = acetaldehyde dehydrogenase, ACS = Acetyl-CoA synthetase, ACC1 $^{S1157A, S659A}$ = mutant version of Acetyl-CoA decarboxylase,*ws2* = heterologous wax ester synthase, and ACB1 = acyl-CoA-binding protein.

Blocking β-oxidation is not the right metabolic engineering target for improving biodiesel synthesis in *S. cerevisiae*. Breakdown of fatty acyl-CoA to Acetyl-CoA catalyzed by β-oxidation enzyme POX1, POX2, POX3 (Runguphan & Keasling, 2014). Deleting *pox1* gene did not increase the production of biodiesel. Breakdown of fatty acids in β-oxidation produces Acetyl-CoA, responsible for the TCA cycle to optimize the central *carbon metabolism.*

IMPROVE BIODIESEL SYNTHESIS PATHWAY: ENZYME ENGINEERING

Enzyme engineering strategy can be used to increase the yield and productivity of biofuels synthesis by increasing the catalytic activity of targeted enzymes and mutating the enzyme's inhibitor or regulator binding sites (Choi et al., 2020). With enzyme engineering also can eliminate feedback inhibition by removing the regulatory domain in the targeted enzyme.

The conversion of Acetyl-CoA to Malonyl-CoA by Acetyl-coenzyme A carboxylase (ACC1) encoded by the *acc1* gene is the rate-limiting step in de novo FAS in yeast. Yeast Snf1 regulates acetyl-CoA carboxylase. From some literature known that the enzyme ACC1 of *S. cerevisiae* is switched off by phosphorylation by Snf1 protein kinase (Figure 5). Regarding the phosphorylation site of ACC1, serine residue ($Ser^{659,1157}$) is identified as the actual target site of Snf1 for phosphorylation. Serine 1157 of ACC1 has a phosphorylation motif (Hyd-X-Arg-XX-**Ser**-XXX-Hyd) which is detected by Snf1 protein kinase. The feedback inhibition by Snf1 protein kinase can be removed, and the activity of Acetyl-Coenzyme A carboxylase in *S. cerevisiae* can be increased by mutation of two phosphorylation sites of ACC1 via site-directed mutagenesis, which mutates $serine^{1157,659}$ to $alanine^{1157,629}$.This modification will remove allosteric control of Snf1 to ACC1 and also increase V_{max} of ACC1 (Shi et al., 2014). This site-directed mutation will change the primary structure of ACC1, but not the folding conformation and active site (tertiary structure) of enzyme. Active-site residues of ACC1 for catalyzingLys-116, Lys-159, His-209, Lys-238, Glu-276, Glu-288, and Asn-290 is not changing then not modify the active site folding, and the function of enzyme (Sloane et al., 2001). Shi et al. (2014) have reported that by engineering acetyl-CoA carboxylase ($ACC1^{S1157A,\ S659A}$) results in partly abolished of posttranslational regulation by phosphorylation of Snf1 in *S. cerevisiae*, resulting in increasing the activity of ACC1 and intracellular malonyl-CoA supply, yield biodiesel of15.8 mg/ L.

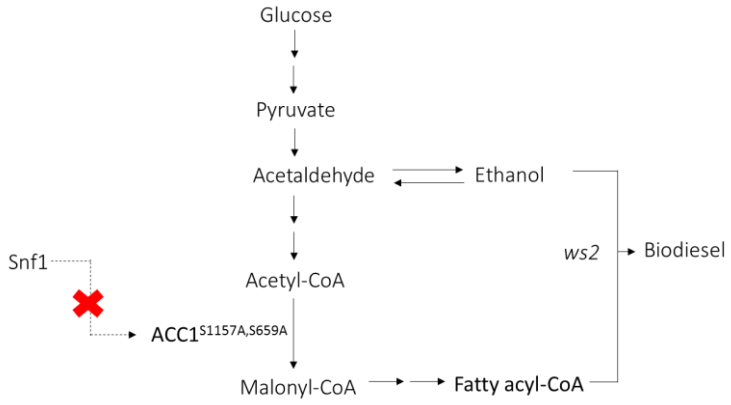

Figure 5. Increasing biodiesel biosynthesis by enzyme engineering

FUTURE CHALLENGES AND RECOMMENDATIONS FOR BIODIESEL PRODUCTION IN *S. cerevisiae*

Biodiesel secretion

Biodiesel secretion becomes a challenge for biodiesel synthesis in *S. cerevisiae*, especially because *S. cerevisiae* is non-oleaginous yeast. Protein Fat1p, which encodes by *fat1* gene, is a plasma membrane-bound

long-chain fatty acid transporter and work together with two other proteins, such as Faa1p and Faa4p, to facilitate the transport of long-chain fatty acid like biodiesel (Lin et al., 2013). It needs further study to investigate the efficient secretion pathway/process for biodiesel in *S. cerevisiae.*

Immobilize cells

Secretion of biodiesel out of the cell will weaken the S-layer and peptidoglycan layers in *S. cerevisiae*. To increase the stability of the outer layer of the cell need to be done by immobilizing the cell (Lin *et al*., 2013). Immobilization is also recyclable the yeast (Mishra et al., 2016). Mishra et al. (2016) reported that immobilized *S. cerevisiae* by entrapment in calcium-alginate could be used up to 40 fermentation batches in a packed bed reactor (PBR). Methods of immobilization are adsorption, covalent attachment, membrane confinement, cross-linking, and entrapment. It needs further study to investigate the immobilization process that can support high cell loadings without disturbing the biochemical activity of yeast.

CONCLUSION

This review has demonstrated the versatility of biodiesel production from lignocellulose using *Saccharomyces cerevisiae.* This product can be increased by applying metabolic engineering and enzyme engineering. The metabolic engineering strategy can be done by directing the carbon flux towards the fatty acids biosynthesis pathway by engineering some genes, expressing the heterologous gene in the central carbon metabolism and biodiesel synthesis, and deleting competing pathways. Expressing the heterologous wax ester synthase or wax ester acyltransferase (WS) in *S. cerevisiae* will establish processes that allow biodiesel production. By combined overexpression genes of Acetyl-CoA synthetase (ACS), Acetyl-CoA carboxylase (ACC1), and Fatty Acid Synthase (FAS) will improve biodiesel synthesis. The yield of biodiesel produced by *S. cerevisiae* also can be increased by blocking side reactions (the production of triacylglycerols and sterol esters). In enzyme engineering, we can remove the allosteric inhibition control of Snf1 to Acetyl-CoA carboxylase ((ACC1), the enzyme that converts Acetyl-CoA to malonyl-CoA) by mutation of two phosphorylation sites of ACC1 via site-directed mutagenesis which mutates serine^{1157,659} to alanine^{1157,629}. This results in enhanced carbon flux to fatty acid synthesis and contribute to a higher biodiesel yield.

REFERENCES

Bailey, J., and Ollis, D. (1987). *Biochemical Engineering Fundamentals*. Second edition. Singapore: McGraw-Hill Book Co.

Brink, D. P., Borgström, C., Persson, V. C., Osiro, K. O., &Gorwa-grauslund, M. F. (2021). D-Xylose Sensing in Saccharomyces cerevisiae : Insights from D-Glucose Signaling and Native D-Xylose Utilizers. *Int. J. Mol. Sci.*, *22*, 12410–12455.

Choi, K. R., Jiao, S., & Lee, S. Y. (2020). Metabolic engineering strategies toward production of biofuels. *Current Opinion in Chemical Biology*, *59*, 1–14. https://doi.org/10.1016/j.cbpa.2020.02.009

de Jong, B.W., Shi, S., Valle-Rodriguez, J.O., Siewers, V., Nielsen, J. (2015). Metabolic Pathway engineering for fatty acid ethyl ester production in Saccharomyces cerevisiae using stable chromosomal integration. Journal of Industrial Microbiology and Biotechnology. 42 (3), pp. 477-486.

Doran, P. M. (1995). Bioprocess Engineering Principle. 2nd edn. London, NW: Academic Press Limited.

El-rotail, A., Shia, G. Y., Rashed, M. M. A., Al-farga, A., Mousa, A., Bakry, A. M., Korma, S. A., Linghuan, Z., &Zhi, G. (2017). Constructing a Library of Stable Mutations for Enhanced Bioethanol Production by Saccharomyces Cerevisiae Using Various Molecular Biology Methods. *International Journal of Research in Agricultural Sciences*, *4*(2), 2348 – 3997.

Erb, T. J., Jones, P. R., & Bar-even, A. (2017). Synthetic metabolism : Metabolic engineering meets enzyme design. *CurrOpin Chem Biol.*, *37*, 56–62. https://doi.org/10.1016/j.cbpa.2016.12.023.Synthetic

Hong, J., Park, S., Kim, S., Kim, S., & Hahn, J. (2018). Efficient production of lycopene in Saccharomyces cerevisiae by enzyme engineering and increasing membrane flexibility and NAPDH production. *Appl Microbiol Biotechnol*, *103*, 211–223.

Hu, Y., Zhu, Z., Nielsen, J., &Siewers, V. (2019). Engineering Saccharomyces cerevisiae cells for production of fatty acid-derived biofuels and chemicals. *Open Biology*, *9*(5), 1–10.

Ibraheem, O., &Ndimba, B. K. (2013). *Molecular Adaptation Mechanisms Employed by Ethanologenic Bacteria in Response to Lignocellulose- derived Inhibitory Compounds*. *9*. https://doi.org/10.7150/ijbs.6091

Issariyakul, T., and Dalai, A.K. (2014). 'Biodiesel from vegetable oils.' Renewable and sustainable energy reviews, 31, pp. 446-471.

Jonsson, L.J., Alriksson, B., and Nilvebrant, N.O. (2013). 'Bioconversion of lignocellulose: inhibitors and detoxification.' Biotechnol Biofuels, 6(1), pp. 16-26.

KEGG. Fatty Acid Metabolisme. http://www.genome.jp/kegg-bin/show_pathway?scale=1.0&query=&map=map01212&scale=0.55&auto_image=&show_description=show&multi_query=&show_module_list=&module_list=map01212_M00082&module_list=map01212_M00083&module_list=map01212_M00085&module_list=map01212_M00415.

Lagunas, R. (1993). 'Sugar transport in Saccharomyces cerevisiae.' FEMS Microbiology, 104(1), pp. 229-242.

Lalonde, S., Boles., E., Hellman, H., Barker., L., Patrick, J.W., Frommer, W.B., and Ward, J.M. (1999). The Dual Function of Sugar Carriers: Transport and Sugar Sensing. The Plant Cell, 11, pp. 707-726.

Li, X., Guo, D., Cheng, Y., Zhu, F., Deng, Z., & Liu, T. (2014). Overproduction of Fatty Acids in Engineered Saccharomyces cerevisiae. *Biotechnology and Bioengineering*, *9999*, 1–12. https://doi.org/10.1002/bit.25239

Lin, H., Wang, Q., Shen, Q., Zhan, J., and Zhao, Y (2013). 'Genetic engineering of microorganisms for biodiesel production.' Bioengineered, 4(5), pp. 292-304.

Liu, X., Xu, W., Mao, L., Zhang, C., Yan, P., Xu, Z. and Zhang, Z.C. (2016). 'Lignocellulosic ethanol production by starch-base industrial yeast under PEG detoxification.' Nature. Scientific report.

Mishra, A., Sharma, A. K., Sharma, S., Bagai, R., Mathur, A. S., Gupta, R. P., & Tuli, D. K. (2016). Lignocellulosic ethanol production employing immobilized Saccharomyces cerevisiae in packed bed reactor. *Renewable Energy*, *98*, 57–63. https://doi.org/10.1016/j.renene.2016.02.010

Phukan, M. M., Bora, P., Gogoi, K., &Konwar, B. K. (2019). Biodiesel from Saccharomyces cerevisiae : fuel property analysis and comparative economics. *SN Applied Sciences*, *1*(2), 1–10. https://doi.org/10.1007/s42452-019-0159-3

Runguphan, W., and Kaesling, J.D. (2014). 'Metabolic engineering of S. cerevisiae for production of fatty acid-derived biofuels and chemicals. Metabolic Engineering, 21(1), pp. 103-113.

Schneiter, R. Hitomi, M., Ivessa, A., Fasch, E.V., Kohlwein, S.D., Tartakoff, A.M. (1996). 'A yeast acetyl Coenzyme A mutant links very-long chain fatty acid synthesis to structure and function of the nuclear membrane-Pore Complex. Molecular and Cellular Biology, 16(12), pp. 7161-7172.

Shi, S., Chen, Y., Siewers, V., and Nielsen, J. (2014). Improving production of malonyl coenzyme A-derived metabolites by abolishing Snf1-dependent regulation of Acc1. MBio. Research Article, 5(3).

Shuler, M.L., and Kargi. (1992). Bioprocess Engineering. Basic Concepts. 1st edn. New Jersey: Prentice-Hall, Inc.

Singh, K., Graf, B., Linden, A., Sautner, V., Urlaub, H., Tittmann, K., Stark, H., & Chari, A. (2020). Discovery of a Regulatory Subunit of the Yeast Fatty Acid Synthase. *Cell*, *180*(6), 1130–1143. https://doi.org/10.1016/j.cell.2020.02.034

Sloane, V., Blanchard, C.Z., Guillot, F., and Waldrop, G.L. (2001). 'Site-directed mutagenesis of ATP binding residues of biotin carboxylase.' Journal of Biology Chemistry, 276(27), pp. 24991-24996.

Sundus, F., Fazal, M.A., and Masjuki, H.H. (2017).'Tribology with biodiesel: A study on enhancing biodiesel stability and its fuel properties.' Renewable and Sustainable Energy Reviews, 70, pp. 399-412.

Tang, X., Lee, J., and Chen, W.N. (2015). 'Engineering the fatty acid metabolic pathway in Saccharomyces cerevisiae for advanced biofuel production.' Metabolic engineering communications, 2(1), pp. 58-66.

Watsuntorn, W., Chuengcharoenphanich, N., Niltaya, P., Butkumchote, C., Theerachat, M., Glinwong, C., Qi, W., Wang, Z., &Chulalaksananukul, W. (2021). A novel oleaginous yeast Saccharomyces cerevisiae CU-TPD4 for lipid and biodiesel production. *Chemosphere*, *280*, 130782–130793. https://doi.org/10.1016/j.chemosphere.2021.130782

Willaert, R., and Baron, G. (1993). 'Growth kinetics of gel-immobilized yeast cells studied by on-line microscopy.' Appl Microbiol Biotechnol, 39(1), pp. 347-352.

Yang, F., Zhang, S., Zhou, Y.J., Zhu, Z., Lin, X., and Zhao, Z.K. (2012). 'Characterization of mitochondrial NAD^+ -dependent isocitrate dehydrogenase of the oleaginous yeast Rhodosporidiumtoruloides.' Applied Microbiology and Biotechnology, 94(4), pp. 1095-1105.

Young, E., Poucher, A., Comer, A., Bailey, A., &Alper, H. (2011). Functional Survey for Heterologous Sugar Transport Proteins , Using Saccharomyces cerevisiae as a Host . *Applied and Environmental Microbiology*, *77*(10), 3311–3319. https://doi.org/10.1128/AEM.02651-10

Yu, T., Zhou, Y. J., Huang, M., Liu, Q., Pereira, R., David, F., Yu, T., Zhou, Y. J., Huang, M., Liu, Q., Pereira, R., David, F., & Nielsen, J. (2018). Reprogramming Yeast Metabolism from Alcoholic Fermentation to Lipogenesis. *Cell*, *174*(6), 1549–1558. https://doi.org/10.1016/j.cell.2018.07.013

Zhou, Y. J., Buijs, N. A., Siewers, V., & Nielsen, J. (2014). Fatty Acid-Derived Biofuels and Chemicals Production in Saccharomyces cerevisiae. *Frontier BIOENGINEERING AND BIOTECHNOLOGY*, *2*(September), 1–6. https://doi.org/10.3389/fbioe.2014.00032