Engineering robust yeast for neutralizing-agent-free production of lactic acid from sugarcane bagasse hydrolysate

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Abstract

From a sustainability standpoint, utilizing lignocellulose-based material for lactic acid production is highly advantageous. By-products generated by pre-treatment of biomass, however, tend to exhibit an inhibitory effect on microbial hosts. Supplementation of neutralizing agents also produces harmful waste for the environment. Here, we report a rapid and non-neutralized fermentation by an original strain, Saccharomyces cerevisiae BTCC3. This robust host was metabolically engineered by the double disruption of PDC1/5 and the introduction of L-LDH. Within only 15 h, 51% of glucose from sugarcane bagasse (SCB) hydrolysate was converted to lactic acid (productivity at 1.69 g·L-1·h-1) without neutralizing agent supplementation at any stage of fermentation. Cultivation using YPD medium under similar conditions achieved productivity of 3.68 g·L-1·h-1 and the strain could still generate lactic acid after several glucose-feeding treatment without neutralization. This study is the first report of lactic acid production from SCB using a genetically modified host in a process that requires neither detoxification nor neutralization. Overall, this method offers an alternative to tolerance engineering that involves extensive genetic manipulations.

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ABSTRACT

From a sustainability standpoint, utilizing lignocellulose-based material for lactic acid production is highly advantageous. By-products generated by pre-treatment of biomass, however, tend to exhibit an inhibitory effect on microbial hosts. Supplementation of neutralizing agents also produces harmful waste for the environment. Here, we report a rapid and non-neutralized fermentation by an original strain,*Saccharomyces cerevisiae* BTCC3. This robust host was metabolically engineered by the double disruption of *PDC1/5* and the introduction of L-*LDH* . Within only 15 h, 51% of glucose from sugarcane bagasse (SCB) hydrolysate was converted to lactic acid (productivity at 1.69 $g{\cdot}L^{-1}{\cdot}h^{-1}$) without neutralizing agent supplementation at any stage of fermentation. Cultivation using YPD medium under similar conditions achieved productivity of 3.68 $g{\cdot}L^{-1}{\cdot}h^{-1}$ and the strain could still generate lactic acid after several glucose-feeding treatment without neutralization. This study is the first report of lactic acid production from SCB using a genetically modified host in a process that requires neither detoxification nor neutralization. Overall, this method offers an alternative to tolerance engineering that involves extensive genetic manipulations.

Keywords:

Lactic acid; lignocellulose; neutralizing agent; acid stress tolerance;

Saccharomyces cerevisiae

INTRODUCTION

Lactic acid is currently one of the essential chemical commodities due to widespread commercial applications in the pharmaceutical, cosmetics, chemical, and food industries (Martinez et al., 2013). With a worldwide production of 400,000 tons per year, lactic acid is considered a top-value platform chemical (Becker et al., 2015; Choi et al., 2015). Lactic acid is the precursor for poly-lactic acid–a popular biodegradable plastic with physicochemical, thermal, and mechanical properties that are competitive with traditional petroleum-based polymers, such as polypropylene (PP) and low-density polypropylene (LDPE) (Ajala et al., 2020; Nduko & Taguchi, 2019). In recent years, about 90% of industrial lactic acid has been manufactured via fermentation rather than chemical synthesis (Dusselier et al., 2013). The former strategy is more environmentally friendly, less energy-intensive, and yields an optically pure product.

Production of lactic acid is even more lucrative when low-cost feedstock is employed in the process. Due to its abundant availability, lignocellulosic biomass has been a prominent material for producing of various bio-based chemicals (Chen et al., 2020; Kim et al., 2019; Tian et al., 2020). Moreover, sugarcane bagasse (SCB) generated by the sugar and alcohol industry is ideal for this objective. Data show that more than 1.8 billion tons of sugarcane were produced worldwide in 2017 (de Matos et al., 2020). About 31.8% composition of sugarcane is bagasse–a residual fraction obtained after the milling process (Cortez et al., 2020). Considering its availability, the utility of SCB has become the subject of numerous studies related to establishing a circular bioeconomy and sustainable industries.

Despite the compelling benefits, the utilization of lignocellulose as a feedstock for bioprocessing has several bottlenecks. The generation of various by-products during the pre-treatment process is one of the challenging issues. These by-products, which include furan derivatives (furfural, 5-HMF, etc.), weak organic acids (formic acid, acetic acid, etc.), and phenolic compounds (vanillin, syringaldehyde, ferulic acid, etc.) (Ling et al., 2014), inhibit microbial metabolism, which renders fermentation and diminishes productivity. Biological, physical, and chemical methods have been explored in a quest to detoxify this process (Palmqvist & Hahn-Hägerdal, 2000). However, these methods necessitate additional equipment, which drives up cost. These processes also decrease the quantity of fermentable sugar (Hahn-Hägerdal et al., 2007). Therefore, employing a stress-

tolerant microorganism in the fermentation step would undoubtedly be more desirable than modifying the operational process.

During lactic acid bioproduction, the product itself may cause additional stress for a microbial host. Many microorganisms, particularly bacteria, suffer growth-rate inhibition under highly acidic conditions. Commonly, neutralizing agent, such as calcium carbonate (Yang et al., 2015), is added to maintain the pH of the medium. In addition to increasing cost, neutralizing agents are often toxic for microorganisms. In addition, calcium salts generally have low solubility in water. Therefore, they can easily mix with biomass and complicate the subsequent downstream process. Calcium salts also react with lactic acid to form calcium lactate. An acidification step using sulfuric acid followed by subsequent purification is needed to obtain the free form of lactic acid. This acidification process generates a harmful by-product known as gypsum, which must be disposed of in landfills (Komesu et al., 2017) and could cause additional environmental issues. Approximately one ton of gypsum is formed per ton of lactic acid production (Dusselier et al., 2013). Hence, from both industrial and environmental perspectives, eliminating the use of neutralizing agents would be advantageous. It can be achieved by employing a robust microbial host during the fermentation process.

Various approaches to obtain a robust microorganism have been proposed. Tolerance engineering by genetic modification is an example of common tools to enhance strain robustness. For instance, the co-expression of both *TAL1* and *ADH1* in *Saccharomyces cerevisiae*enhances ethanol production in a medium containing furfural (Hasunuma et al., 2014). Co-overexpression of both *HAA1* and *PRS3* and disruption of *FPS1* also improve acetic acid tolerance (Cunha et al., 2018; Zhang et al., 2011). Nevertheless, due to the complexity of biomass chemical composition, the successive introduction of a large number of tolerance-related genes could be cumbersome.

On the contrary, rather than performing extensive genetic engineering, the strategy proposed here focuses on increasing the lactic acid production of an originally robust microorganism via a handful of genetic modifications. In the present study, we selected newly isolated yeast, identified as *S. cerevisiae* BTCC3, obtained from the screening of Ascomycota yeasts deposited in Indonesian Culture Collection (InaCC). This strain has the ability to survive at relatively low pH (up to 2.5), as well as the presence of lignocellulose-derived inhibitors such as furfural, formic acid, acetic acid, etc. However, akin to other yeasts, this strain lacks the metabolic pathway for lactic acid production. Therefore, we introduced an exogenous L-*LDH* gene to enable lactic acid fermentation from glucose as an example. This experiment intended to construct a microbial strain with phenotypes suitable for utilizing lignocellulosic biomass as a carbon feedstock, such as SCB. Also, we considered the potential of this recombinant strain to ferment glucose to lactic acid without the use of a neutralizing agent.

MATERIALS AND METHODS

Strains and medium

All strains used in this study are compiled in **Table 1** .*S. cerevisiae* BTCC3 (haploid) was obtained from Indonesian Culture Collection (InaCC, Cibinong Science Center, BRIN, Indonesia). This strain and its derivatives were cultivated using a yeast/peptone/dextrose (YPD) medium (20 g[?]L^{-1} Bacto peptone [BD, San Jose, CA], 10 g[?]L^{-1} Bacto yeast extract [BD], and 50 g[?]L^{-1} glucose [BD]).*Escherichia coli* JM109 (Takara Bio, Shiga, Japan) was used for cloning. Luria-Bertani medium (10 g[?]L^{-1} tryptone [Nacalai, Japan], 5 g[?]L^{-1} yeast extract [BD], and 10 g[?]L^{-1} sodium chloride [Nacalai tesque Inc., Japan]) was used for cultivation.

Plasmid design and construction

All plasmids used in this study are compiled in **Table 1** and illustrated in **Figure 1** , whereas all primers are listed in**Supplementary Table S1** . Codon-optimized L-*LDH* from*Lactobacillus casei* (GenBank accession number MF582630.1),*LcLLDH* , was selected as an *LDH* gene source. DNA fragments were assembled via the NEBuilder HiFi DNA Assembly method (NEB, County Road Ipswich, MA) and transformed into *E. coli* JM109 (*recA1* , *endA1* , *gyrA96* , *thi -1* ,*hsdR17* (r_K^- m_K^+),*e14* $^-$ (*mcrA* $^-$), *supE44* , *relA1* , D(*lac-proAB*

)/F' [*traD36* , *proAB* $^{+}$, *lac* I^{q} , *lacZ* D M15]). Plasmid-harboring transformants were cultivated overnight (37 degC, 190 rpm) using LB medium supplemented with 0.1 g[?]L^{-1} of ampicillin (Sigma-Aldrich, USA) as a selection marker. Plasmids were extracted using a LaboPassTM Plasmid Mini kit (Cosmo Genetech, Seoul, Korea).

Introduction of the *LDH* gene into *S. cerevisiae* genome using a genome-integrated plasmid yields a higher accumulation of lactic acid than using an episomal type plasmid (Ishida et al., 2005). Therefore, the former plasmid type was employed to construct all engineered strains in this experiment. For the simultaneous expression of the *LDH* gene and disruption of *PDC* genes, partial coding sequences of *PDC1* /*PDC5* genes were cloned into plasmids containing an *LDH* expression cassette. The sequence of*LDH* was fused with the *TDH3* promoter and terminator sequences at both its upstream and downstream regions, respectively, to construct constitutively expressed *LDH* systems: pAUR101-TDH3pro-LcLLDH-dPDC1 and pAUR101-TDH3pro-LcLLDH-dPDC5. After the integration of the respective gene-expressing cassettes into the genome, there can be a possibility that both promoters control the expression of the *LDH* gene. The orientation ofP_{TDH3} -*LcLLDH* -$T_{TDH3}$was flipped to avoid that effect, as shown in **Figure 1(B)** . Moreover, pAUR101-BTCC3PDC1-LcLLDH has a sequence of *LDH* gene integrated between the promoter and terminator of *PDC1* gene cloned from the genome, and we intended to utilize this native glucose-dependent promoter. In contrast, pP01-BTCC3PDC5KO contains only the partial coding sequence of *PDC5* and *KanMx* , a marker gene, to distort the expression of the corresponding gene without the introduction of the *LDH* gene.

Breeding yeast

All plasmids were digested by the restriction enzymes (NEB) shown in**Figure 1** to yield the five derived strains mentioned in**Table 1** . Digested plasmids were transformed to a yeast strain by following a LiAc/single-stranded carrier DNA/PEG method (Gietz & Woods, 2002). Each transformant was selected on a YPD agar containing the respective selection marker(s). Sequencing analysis using Applied Biosystems 3500 Genetic Analyzer was performed to confirm that the fragment(s) of interest were successfully inserted.

Fermentation

A yeast strain was initially pre-cultured (30 degC, 150 rpm) using YPD medium until the optical density (OD_{600nm}) reached around 50. Cells were cultivated (30 degC, 90 rpm) in a 100-mL Erlenmeyer flask containing 12 mL of YPD medium supplemented with 50 g[?]L^{-1} sterilized calcium carbonate (Sigma-Aldrich). For fermentation without a neutralizing agent, however, the medium was not supplemented with calcium carbonate.

Furthermore, recombinant yeast was also cultivated using sterile-filtered sugarcane bagasse hydrolysate obtained from a pre-treatment process that mixed liquefied C6 and C5 fractions obtained from sugarcane bagasse following treatment with hot water. This medium (pH 4.5) contained 50 g*L^{-1} glucose and 25 g*L^{-1} xylose with inhibitory chemical compounds (ICC) of furfural (4.5 mM), 5-HMF (3.4 mM), acetic acid (66 mM), formic acid (7.2 mM), and lactic acid (7 mM). After pre-culturing (30 degC, 150 rpm), cells were cultivated (30 degC, 90 rpm) in a 100-mL Erlenmeyer flask containing 12 mL of sterile hydrolysate without the addition of calcium carbonate.

Analysis of fermentation products

The medium broth was periodically sampled. After centrifugation at10,000 rpm for 5 min, the supernatant was filtered through a 0.45-μM syringeless PTFE filter. Concentrations of glucose and other fermentation products were determined via High Performance Liquid Chromatography (HPLC) (LC-20AB, Shimadzu, Japan) using a Coregel-87H3 column (7.8 mm, I.D. x 300 mm Transgenomic) at 80 °C for 40 min using 5 mM of sulfuric acid as eluent (flow rate at 0.6 mL[?]min^{-1}). A refractive index detector was used.

Statistical analysis

The values shown represent the mean of results from three biological replicates + SD (standard deviation). A significant difference was statistically analyzed using a two-tailed Student's t-test at$P < 0.05$.

RESULTS

Effect of pathway manipulation on the metabolism

As previously described, BTCC3 (wild type) strain did not produce lactic acid at a detectable level due to the lack of a metabolic pathway for lactic acid production from sugar. Here, we inserted an exogenous *LDH* gene and disrupted several *PDC* genes. **Figure 2 s** hows how this pathway adjustment affected the product accumulation. As expected, the insertion of LDH genes enhanced the production of lactic acid in all mutant strains. LX1 strain (*PDC1* $^{-}$,*LDH* $^{+}$) generated a 20-fold higher amount of lactic acid compared with the results using LX5 strain (*PDC5* $^{-}$, *LDH* $^{+}$). Our results also revealed that the generation of lactic acid elevated the accumulation of ethanol and glycerol owing to the fact that both mutant strains exhibited higher levels of production compared with the levels from wild-type strains.

Moreover, two *PDC* genes were knocked out to generate LA1 strain (*PDC1* $^{-}$, *PDC5* $^{-}$,*LDH* $^{+}$). This strain exhibited a 1.9-fold increase in lactic acid generation and a 1.8-fold decrease in ethanol accumulation compared with LX1 (*PDC1* $^{-}$,*LDH* $^{+}$) without a noticeable drop-in glucose uptake rate (**Supplementary Figure S1**). Surprisingly, the concentration of lactic acid produced from two copies of the *LDH* gene-harboring strain, namely LA15 (*PDC1* $^{-}$,*LDH* $^{+}$, *PDC5* $^{-}$,*LDH* $^{+}$) and LA1 (*PDC1* $^{-}$,*LDH* $^{+}$, *PDC5* $^{-}$), showed no significant differences ($P < 0.05$).

The effect of promoter strength on the production of lactic acid in this strain was also examined. In addition to the LA1 strain (*PDC1* $^{-}$, *PDC5*$^{-}$,P_{TDH3} -*LDH* $^{+}$) that possesses *LDH* gene integrated to a constitutive promoter, LA2 strain (*PDC1* $^{-}$, *PDC5*$^{-}$,P_{PDC1} -*LDH* $^{+}$) containing a *LDH* gene with glucose-dependent promoter was also constructed. Interestingly, our result revealed that the LA2 strain produced 43.23 g*L^{-1} of lactic acid (approximately two times higher than LA1). This result indicated that the use of a constitutive promoter might not be suitable for our strain, although this type of promoter is commonly used in numerous experiments.

Strain performance without a neutralizing agent

Considering the promising result of the previous experiment, the LA2 strain was selected for further observation. **Figure 3** shows that with and without the addition of calcium carbonate, the LA2 strain produced lactic acid in concentrations of 43.2 and 33.2 g*L^{-1}, respectively. Even though the accumulation of lactic acid declined, the removal of calcium carbonate did not cause a significant decrease in the rate of glucose uptake because the glucose was completely consumed within only 9 h under both conditions. Nonetheless, as shown in **Table 2** , these results were relatively competitive by comparison with those of the microbial hosts described in other studies (Ilmen et al., 2013; Ishida et al., 2005; Mitsui et al., 2020; Park et al., 2018). Despite its lower yield, the LA2 strain maintained the ability to utilize glucose rapidly and exhibited an even more stable level of productivity when the neutralizing agent was removed. With the addition of a neutralizing agent, the productivity was 4.80 g*L^{-1}*h^{-1}. Meanwhile, under a non-neutralized condition, the productivity remained high (3.68 g*L^{-1}*h^{-1}), although the pH of the medium dropped to 3.00 after 9 h of cultivation ([?]pH = + 1.5, compared with a neutralized condition). Other studies included in **Table 2** have exhibited a drop in productivity by as much as 50%, or more, when no neutralizing agent was supplemented into the medium.

Based on the result shown in **Figure 3** , lactic acid generation was terminated because the glucose was completely consumed within 9 h. However, as depicted in **Figure 4** , the LA2 strain could still accumulate lactic acid when glucose was fed to the medium even without supplementation with a neutralizing agent. After being fed with concentrated glucose three times at 6 h, 10 h and 14 h, the productivity of engineered strain stood at 3.45 g*L^{-1}*h^{-1}(C_{LA} = 62.0 g*L^{-1}) and 2.35 g*L^{-1}*h^{-1}(C_{LA} = 42.2 g*L^{-1}) under the neutralized and non-neutralized condition, respectively. Akin to the results from batch cultivation, this strain exhibited high productivity under both conditions, with a drop in value less than 35% when the neutralizing agent was removed from the medium.

Fermentation using bagasse hydrolysate

Previous results show that LA2 exhibited a strong ability to tolerate acidic conditions and produce lactic acid without a neutralizing agent. Subsequently, the robustness of this strain against lignocellulose-derived inhibitors was examined. The hydrolysate obtained from hot water pre-treatment was directly used as a medium without prior treatment of enzymatic hydrolysis, detoxification or neutralization. In addition to glucose, xylose was also present in the hydrolysate. However, our yeast strain possesses no active genes that connect the lactic acid generation and xylose metabolism pathways. Therefore, the concentrations of xylose were not included in any of the calculations.

As can be seen in **Figure 5** , LA2 strain generated lactic acid in a concentration of 25.34 + 3.25 $g*L^{-1}$ at 15 h from an initial glucose concentration of 49.57 + 0.49 $g*L^{-1}$ (yield = 0.51 $g*g^{-1}$ glucose; productivity = 1.69 $g*L^{-1}*h^{-1}$). The productivity of our strain was slightly lower than that of other reports (shown in **Table 3**). In those reports, however, sodium hydroxide had to be added to maintain the pH of the medium during fermentation. Meanwhile, in this experiment, cultivation was performed without the neutralizing treatment at any stage of fermentation. Interestingly, despite a decline in the rate of glucose uptake and productivity, LA2 strain converted about 51% of the available glucose when SCB hydrolysate was used as a medium, whereas cultivation using the YPD medium only converted 33% of the glucose (non-neutralized condition). Also, the ratio of lactic acid to ethanol was remarkably improved (2.67 at 15 h) using SCB hydrolysate compared with cultivation using a YPD medium (0.91 at 9 h). This result indicates that the stress of chemical inhibitors up to particular concentration levels could instead provide a positive impact by shifting the metabolism and accumulating lactic acid in higher concentration.

DISCUSSIONS

Establishing a robust microbial host is crucial for sustainable and greener bio-based chemical production. Rather than employing extensive genetic modification to enhance strain tolerance, we attempted to establish a lactic acid bioproduction process facilitated by a robust microbial platform with high tolerance to acid and numerous lignocellulose-derived inhibitors. For that reason, we used *S. cerevisiae* BTCC3 as a robust yeast platform and enabled its lactic acid production by performing a modest genetic modification. Pathway engineering was conducted to reduce the metabolic flux to ethanol, a major product of fermentation by yeast, by disrupting the pyruvate decarboxylation genes. Triple deletion of *PDC1* , *PDC* 5 and*PDC6* genes inhibited cell growth because these genes are essential for NAD^+ production (van Maris et al., 2004). However, a study has revealed that the *PDC6* gene shows the lowest expression from among the three, and a mere double deletion of*PDC1* and *PDC5* genes would be sufficient to significantly diminish the specific activity of pyruvate decarboxylase (Hohmann, 1991). In addition, another gene candidate for disruption is the*ADH1* gene. However, the deletion of this gene is known to slow the cell growth rate due to the accumulation of toxic acetaldehyde (Tokuhiro et al., 2009). Therefore, only *PDC1* and *PDC5*genes were knocked out in this study.

Although our strain has a high tolerance to various stresses, several negative impacts appeared as the genetic modifications became more extensive. For instance, in all mutants, the accumulations of by-products, such as ethanol and glycerol, were higher than in wild-type strain, which could have been because of the response of the microbial host to cope with acid stress. In fact, the accumulation of ethanol and glycerol induces the generation of NAD^+, which has an essential role in countering the negative impact of various stressors in cells (Kato & Lin, 2014; Massudi et al., 2012). Also, our results revealed that inserting an additional copy of the *LDH* gene into the same locus does not necessarily improve lactic acid production. However, this could be the consequence of employing an identical promoter, i.e. P_{TDH3} , in two different plasmids, pAUR101-TDH3pro-LcLLDH-dPDC1 and pAUR101-TDH3pro-LcLLDH-dPDC5. Promoter rivalry may have resulted in a conflict in the use of transcription factors during the expression of the two *LDH* genes.

Unexpectedly, an engineered strain containing the *LDH* gene under the control of the *TDH3* promoter yielded low lactic acid generation, although this constitutive promoter is commonly utilized in consideration of its high expression (Baek et al., 2016, 2017; Saitoh et al., 2005). Meanwhile, the LA2 strain harboring the *LDH* gene under the control of a glucose-dependent promoter showed the highest lactic acid production. There are several plausible rationalizations for this result. It could simply be because P_{PDC1} is a native

promoter. In addition, although the *TDH3* promoter is categorized as a constitutive promoter, its expression declines in the presence of ethanol (Peng et al., 2015)–one of the major products generated by all strains in this type of experiment. Also, it is worth noting that the employment of constitutive promoters could increase the metabolic load (Balbas & Lorence, 2004), affecting the cell growth rate and metabolic flux of microbial hosts.

Since our strain displayed a high tolerance in acidic condition, we performed the fermentation without adding a neutralizing agent. This strategy is important for the fact that one of the bottlenecks in developing the industrial lactic acid process is the inability to generate a free form of lactic acid that requires no subsequent acidification. In fact, according to the life-cycle assessment and techno-economic analysis of SCB valorization to lactic acid, the removal of neutralizing agents by employing an acid-tolerant host lowers the environmental burden caused by gypsum and reduce total capital investment because the process would no longer require an acidification reactor unit (Daful & Gorgens, 2017). Based on our best knowledge, to date, the utilization of SCB for second-generation lactic acid bioproduction still requisites the neutralizing step, either at the beginning or during the fermentation (Baral et al., 2020; de Oliveira et al., 2019; Unrean, 2018; van der Pol et al., 2016).

Our results show that our engineered *S. cerevisiae* BTCC3 LA2 strain could facilitate a neutralizing-agent-free lactic acid fermentation using the hydrolysate of SCB. This strain could demonstrate a rapid generation of lactic acid at a productivity of 1.69 $g*L^{-1}*h^{-1}$, which is competitive to other studies in which the neutralizing treatment is still included at the beginning or during fermentation. Moreover, non-neutralized fermentation using YPD medium could achieve productivity as high as 3.68 $g*L^{-1}*h^{-1}$. All fermentable glucose in the YPD medium was consumed within only 9 h and higher accumulation of lactic acid could still be accomplished after three times glucose feeding treatment even without any neutralizing agent supplemented.

Based on our best knowledge, no previous reports discuss the metabolic engineering approach to developing a lactic acid bioproduction using SCB as raw material without neutralizing or detoxifying treatment. The genetic modification was performed at a modest level to maintain this strain's robustness, with merely two genes disrupted and one exogenous gene inserted. In contrast, many other studies carried out more complex genetic manipulations in which the number of genes knocked in and out concurrently could reach ten or even more (Baek et al., 2017; Tsuge et al., 2019; Zhong et al., 2019, 2020). Therefore, this strategy is more efficient and potentially generates a more stable host for lactic acid production.

For upcoming experiments, disrupting other gene candidates without severely jeopardizing the rate of cell growth could be accomplished by constructing a switchable metabolic tool (Zhao et al., 2018). Adaptation strategy could also be carried out to attain higher product accumulation when performing fed-batch strategy with more feeding treatments. In addition, the content of xylose in lignocellulosic biomass is the second largest composition after glucose, so the use of xylose as an additional carbon source is essential for efficient production of lactic acid. Therefore, introducing a xylose-assimilating pathway to the microbial host is a good objective for upcoming experiments. In addition, a combination of the utilization of genetically engineered feedstock and the employment of our recombinant strain should lead to the development of a fourth-generation lactic acid.

CONCLUSIONS

Sustainable and greener lactic acid production is possible using lignocellulose biomass as a raw material. In this work, we demonstrated the rapid fermentation of lactic acid from hydrolysate of sugarcane bagasse without the use of a neutralizing agent. This process was catalyzed by an originally robust microorganism engineered through only three metabolic modifications—introduction of exogenous L-*LDH* and double disruptions of *PDC* s—with retaining high tolerance to acid and inhibitor stresses. Compared with other approaches that have attempted to construct a recombinant host with extensive genetic interruptions, this strategy is efficient, more practical and expected to provide a more stable system for industrial lactic acid bioprocess that utilizes lignocellulose biomass as its feedstock.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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AUTHOR CONTRIBUTIONS

Radityo Pangestu: Investigation, Methodology, Data curation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Prihardi Kahar:** Conceptualization, Methodology, Resources, Data curation, Writing – original draft, Writing – review & editing. **Lutfi Nia Kholid & Urip Perwitasari:** Investigation, Data curation. **Ahmad Thontowi,Fahrurrozi & Puspita Lisdiyanti:** Resource.**Yopi:** Resource, Funding acquisition. **Chiaki Ogino:**Funding acquisition, Supervision, Conceptualization, Resources, Project administration, Writing – review & editing. **Bambang Prasetya & Akihiko Kondo:** Funding acquisition.

FIGURE LEGENDS

Figure 1. Maps of the plasmid vectors used for the introduction of *LDH* and disruption of *PDC1/5* . (a) pAUR101-BTCC3PDC1-LcLLDH for disruption of *PDC1* and introduction of *LDH* with expression under the control of an inducible promoter; (b) pAUR101-TDH3pro-LcLLDH-dPDC1 for disruption of *PDC1*and introduction of *LDH* with expression under the control of a constitutive promoter; (c) pPC01-BTCC3PDC5KO for disruption of*PDC5* without introducing *LDH* ; (d) pAUR101-TDH3pro-LcLLDH-dPDC5 for disruption of *PDC5* and introduction of *LDH* with expression under the control of a constitutive promoter. Restriction enzymes indicate the locations for integration to a genome via homologous recombination.

Figure 2. Comparison of major products produced by*Saccharomyces cerevisiae* BTCC3 and all its derived strains. Values represent the average measurement of three biological replicates. Error bars represent the standard deviation of measurements. Symbols (+) and (-) indicate introduction and disruption, respectively.P_{TDH3} and P_{PDC1} represent constitutive and inducible promoters, respectively.

Figure 3. Fermentation profile of *Saccharomyces cerevisiae* BTCC3 LA2 using YPD_{100} medium with and without the addition of neutralizing agent. Concentrations of (a) glucose; (b) lactic acid; (c) ethanol; (d) glycerol; and (e) pH change have been indicated.

Figure 4. Fed-batch fermentation profile of *Saccharomyces cerevisiae* BTCC3 LA2 using YPD_{100} medium with and without the addition of neutralizing agent. Concentrations of (a) glucose; (b) lactic acid; and (c) ethanol have been indicated.

Figure 5. Fermentation profile of *Saccharomyces cerevisiae* BTCC3 LA2 using hydrolysate from the pretreatment of sugarcane bagasse as a medium without the addition of a neutralizing agent. Concentrations of (a) glucose; (b) lactic acid; (c) ethanol; and, (d) pH change have been indicated.

APPENDICES

Supplementary Table S1. List of primers used in this study

Supplementary Figure S1. Comparison of major products produced by *Saccharomyces cerevisiae* BTCC3 and all its derived strains (extended data from Figure 2)

REFERENCES

Ajala, E. O., Olonade, Y. O., Ajala, M. A. & Akinpelu, G. S. (2020). Lactic Acid Production from Lignocellulose - A Review of Major Challenges and Selected Solutions. *ChemBioEng Reviews* , *2* , 1–13. https://doi.org/10.1002/cben.201900018

Baek, S.-H., Kwon, E. Y., Bae, S., Cho, B., Kim, S. & Hahn, J. (2017). Improvement of D-Lactic Acid Production in *Saccharomyces cerevisiae* Under Acidic Conditions by Evolutionary and Rational Metabolic Engineering. *Biotechnology Journal* , *12* (10), 1700015. https://doi.org/10.1002/biot.201700015

Baek, S.-H., Kwon, E. Y., Kim, Y. H. & Hahn, J.-S. (2016). Metabolic engineering and adaptive evolution for efficient production of D-lactic acid in *Saccharomyces cerevisiae* . *Applied Microbiology and Biotechnology* , *100* (6), 2737–2748. https://doi.org/10.1007/s00253-015-7174-0

Balbas, P. & Lorence, A. (2004). *Recombinant Gene Expression*(2nd ed., Vol. 267). Humana Press. https://doi.org/10.1385/1592597742

Baral, P., Pundir, A., Kumar, V., Kurmi, A. K. & Agrawal, D. (2020). Expeditious production of concentrated glucose-rich hydrolysate from sugarcane bagasse and its fermentation to lactic acid with high productivity. *Food and Bioproducts Processing* , *124* , 72–81. https://doi.org/10.1016/j.fbp.2020.08.005

Becker, J., Lange, A., Fabarius, J. & Wittmann, C. (2015). Top value platform chemicals: bio-based production of organic acids. *Current Opinion in Biotechnology* , *36* , 168–175. https://doi.org/10.1016/j.copbio.2015.08.022

Chen, H., Chen, B., Su, Z., Wang, K., Wang, B., Wang, Y., Si, Z., Wu, Y., Cai, D. & Qin, P. (2020). Efficient lactic acid production from cassava bagasse by mixed culture of *Bacillus coagulans* and*Lactobacillus rhamnosus* using stepwise pH controlled simultaneous saccharification and co-fermentation. *Industrial Crops and Products* , *146* (15), 112175. https://doi.org/10.1016/j.indcrop.2020.112175

Choi, S., Song, C. W., Shin, J. H. & Lee, S. Y. (2015). Biorefineries for the production of top building block chemicals and their derivatives. *Metabolic Engineering* , *28* , 223–239. https://doi.org/10.1016/j.ymben.2014.12.007

Cortez, L. A. B., Baldassin, R. & De Almeida, E. (2020). Energy from sugarcane. *Sugarcane Biorefinery, Technology and Perspectives* , 117–139. https://doi.org/10.1016/B978-0-12-814236-3.00007-X

Cunha, J. T., Costa, C. E., Ferraz, L., Romani, A., Johansson, B., Sa-Correia, I. & Domingues, L. (2018). *HAA1* and *PRS3*overexpression boosts yeast tolerance towards acetic acid improving xylose or glucose consumption: unravelling the underlying mechanisms.*Applied Microbiology and Biotechnology* , *102* (10), 4589–4600. https://doi.org/10.1007/s00253-018-8955-z

Daful, A. G. & Gorgens, J. F. (2017). Techno-economic analysis and environmental impact assessment of lignocellulosic lactic acid production. *Chemical Engineering Science* , *162* , 53–65. https://doi.org/10.1016/j.ces.2016.12.054

de Matos, M., Santos, F. & Eichler, P. (2020). Sugarcane world scenario. In *Sugarcane Biorefinery, Technology and Perspectives*(pp. 1–19). Elsevier. https://doi.org/10.1016/B978-0-12-814236-3.00001-9

de Oliveira, R. A., Schneider, R., Vaz Rossell, C. E., Maciel Filho, R. & Venus, J. (2019). Polymer grade L-lactic acid production from sugarcane bagasse hemicellulosic hydrolysate using *Bacillus coagulans* . *Bioresource Technology Reports* , *6* (October 2018), 26–31. https://doi.org/10.1016/j.biteb.2019.02.003

Dusselier, M., Van Wouwe, P., Dewaele, A., Makshina, E. & Sels, B. F. (2013). Lactic acid as a platform chemical in the biobased economy: the role of chemocatalysis. *Energy & Environmental Science* ,*6* (5), 1415. https://doi.org/10.1039/c3ee00069a

Gietz, R. . & Woods, R. A. (2002). Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. In*Methods in Enzymology* (Vol. 350, Issue 2001, pp. 87–96). https://doi.org/10.1016/S0076-6879(02)50957-5

Hahn-Hagerdal, B., Karhumaa, K., Fonseca, C., Spencer-Martins, I. & Gorwa-Grauslund, M. F. (2007). Towards industrial pentose-fermenting yeast strains. *Applied Microbiology and Biotechnology* ,*74* (5), 937–953. https://doi.org/10.1007/s00253-006-0827-2

Hasunuma, T., Ismail, K. S. K., Nambu, Y. & Kondo, A. (2014). Co-expression of *TAL1* and *ADH1* in recombinant xylose-fermenting *Saccharomyces cerevisiae* improves ethanol production from lignocellulosic hydrolysates in the presence of furfural. *Journal of Bioscience and Bioengineering* ,*117* (2), 165–169. https://doi.org/10.1016/j.jbiosc.2013.07.007

Hohmann, S. (1991). *PDC6* , a weakly expressed pyruvate decarboxylase gene from yeast, is activated when fused spontaneously under the control of the *PDC1* promoter. *Current Genetics* ,*20* (5), 373–378. https://doi.org/10.1007/BF00317064

Ilmen, M., Koivuranta, K., Ruohonen, L., Rajgarhia, V., Suominen, P. & Penttila, M. (2013). Production of L-lactic acid by the yeast*Candida sonorensis* expressing heterologous bacterial and fungal lactate dehydrogenases. *Microbial Cell Factories* , *12* (1), 53. https://doi.org/10.1186/1475-2859-12-53

Ishida, N., Saitoh, S., Tokuhiro, K., Nagamori, E., Matsuyama, T., Kitamoto, K. & Takahashi, H. (2005). Efficient production of L-lactic acid by metabolically engineered *Saccharomyces cerevisiae* with a genome-integrated L-lactate dehydrogenase gene. *Applied and Environmental Microbiology* , *71* (4), 1964–1970. https://doi.org/10.1128/AEM.71.4.1964-1970.2005

Kato, M. & Lin, S.-J. (2014). Regulation of NAD^+metabolism, signaling and compartmentalization in the yeast*Saccharomyces cerevisiae* . *DNA Repair* , *23* , 49–58. https://doi.org/10.1016/j.dnarep.2014.07.009

Kim, J., Jang, J. H., Yeo, H. J., Seol, J., Kim, S. R. & Jung, Y. H. (2019). Lactic acid production from a whole slurry of acid-pretreated spent coffee grounds by engineered *Saccharomyces cerevisiae* .*Applied Biochemistry and Biotechnology* , *189* (1), 206–216. https://doi.org/10.1007/s12010-019-03000-6

Komesu, A., Oliveira, J. A. R. de, Martins, L. H. da S., Maciel, M. R. W. & Filho, R. M. (2017). Lactic acid production to purification: A review. *BioResources* , *12* (2), 4364–4383.

Ling, H., Teo, W., Chen, B., Leong, S. S. J. & Chang, M. W. (2014). Microbial tolerance engineering toward biochemical production: from lignocellulose to products. *Current Opinion in Biotechnology* ,*29* (1), 99–106. https://doi.org/10.1016/j.copbio.2014.03.005

Martinez, F. A. C., Balciunas, E. M., Salgado, J. M., Dominguez Gonzalez, J. M., Converti, A. & Oliveira, R. P. de S. (2013). Lactic acid properties, applications and production: A review. *Trends in Food Science & Technology* , *30* (1), 70–83. https://doi.org/10.1016/j.tifs.2012.11.007

Massudi, H., Grant, R., Guillemin, G. J. & Braidy, N. (2012). NAD^+ metabolism and oxidative stress: The golden nucleotide on a crown of thorns. In *Redox Report* . https://doi.org/10.1179/1351000212Y.0000000001

Mitsui, R., Yamada, R., Matsumoto, T., Yoshihara, S., Tokumoto, H. & Ogino, H. (2020). Construction of lactic acid-tolerant*Saccharomyces cerevisiae* by using CRISPR-Cas-mediated genome evolution for efficient D-lactic acid production. *Applied Microbiology and Biotechnology* , *104* (21), 9147–9158. https://doi.org/10.1007/s00253-020-10906-3

Nduko, J. M. & Taguchi, S. (2019). Microbial production and properties of LA-based polymers and oligomers from renewable feedstock. In Z. Fang, R. L. Smith & X.-F. Tian (Eds.), *Production of materials from sustainable biomass resources* (pp. 361–390). Springer Singapore. https://doi.org/10.1007/978-981-13-3768-0_12

Palmqvist, E. & Hahn-Hagerdal, B. (2000). Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxification.*Bioresource Technology* , *74* (1), 17–24. https://doi.org/10.1016/S0960-8524(99)00160-1

Park, H. J., Bae, J. H., Ko, H. J., Lee, S.-H. H. S., Sung, B. H., Han, J. I., Sohn, J. H., Ko, J. B., Ko, H. J., Lee, S.-H. H. S., Sung, B. H., Han, J. I. & Sohn, J. H. (2018). Low-pH production of D-lactic acid using newly isolated acid tolerant yeast *Pichia kudriavzevii* NG7.*Biotechnology and Bioengineering* , *115* (9), 2232–2242. https://doi.org/10.1002/bit.26745

Peng, B., Williams, T. C., Henry, M., Nielsen, L. K. & Vickers, C. E. (2015). Controlling heterologous gene expression in yeast cell factories on different carbon substrates and across the diauxic shift: a comparison of yeast promoter activities. *Microbial Cell Factories* , *14* (1), 91. https://doi.org/10.1186/s12934-015-0278-5

Saitoh, S., Ishida, N., Onishi, T., Tokuhiro, K., Nagamori, E., Kitamoto, K. & Takahashi, H. (2005). Genetically engineered wine yeast produces a high concentration of L-lactic acid of extremely high optical purity. *Applied and Environmental Microbiology* , *71* (5), 2789–2792. https://doi.org/10.1128/AEM.71.5.2789-2792.2005

Tian, X., Hu, W., Chen, J., Zhang, W. & Li, W. (2020). The supplement of vitamin C facilitates L-lactic acid biosynthesis in*Lactobacillus thermophilus* A69 from sweet sorghum juice coupled with soybean hydrolysate as feedstocks. *Industrial Crops and Products* , *146* , 112159. https://doi.org/10.1016/j.indcrop.2020.112159

Tokuhiro, K., Ishida, N., Nagamori, E., Saitoh, S., Onishi, T., Kondo, A. & Takahashi, H. (2009). Double mutation of the *PDC1* and*ADH1* genes improves lactate production in the yeast*Saccharomyces cerevisiae* expressing the bovine lactate dehydrogenase gene. *Applied Microbiology and Biotechnology* ,*82* (5), 883–890. https://doi.org/10.1007/s00253-008-1831-5

Tsuge, Y., Kato, N., Yamamoto, S., Suda, M., Jojima, T. & Inui, M. (2019). Metabolic engineering of *Corynebacterium glutamicum* for hyperproduction of polymer-grade L- and D-lactic acid. *Applied Microbiology and Biotechnology* . https://doi.org/10.1007/s00253-019-09737-8

Unrean, P. (2018). Optimized feeding schemes of simultaneous saccharification and fermentation process for high lactic acid titer from sugarcane bagasse. *Industrial Crops and Products* ,*111* , 660–666. https://doi.org/10.1016/j.indcrop.2017.11.043

van der Pol, E. C., Eggink, G. & Weusthuis, R. A. (2016). Production of L(+)-lactic acid from acid pretreated sugarcane bagasse using*Bacillus coagulans* DSM2314 in a simultaneous saccharification and fermentation strategy. *Biotechnology for Biofuels* , *9* (1), 248. https://doi.org/10.1186/s13068-016-0646-3

van Maris, A. J. A., Geertman, J. A., Vermeulen, A., Groothuizen, M. K., Winkler, A. A., Piper, M. D. W., van Dijken, J. P. & Pronk, J. T. (2004). Directed evolution of pyruvate decarboxylase-negative*Saccharomyces cerevisiae* , yielding a C2-independent, glucose-tolerant, and pyruvate-hyperproducing Yeast. *Applied and Environmental Microbiology* , *70* (1), 159–166. https://doi.org/10.1128/AEM.70.1.159-166.2004

Yang, P.-B., Tian, Y., Wang, Q. & Cong, W. (2015). Effect of different types of calcium carbonate on the lactic acid fermentation performance of *Lactobacillus lactis* . *Biochemical Engineering Journal* ,*98* , 38–46. https://doi.org/10.1016/j.bej.2015.02.023

Zhang, J. G., Liu, X. Y., He, X. P., Guo, X. N., Lu, Y. & Zhang, B. run. (2011). Improvement of acetic acid tolerance and fermentation performance of *Saccharomyces cerevisiae* by disruption of the*FPS1* aquaglyceroporin gene. *Biotechnology Letters* . https://doi.org/10.1007/s10529-010-0433-3

Zhao, E. M., Zhang, Y., Mehl, J., Park, H., Lalwani, M. A., Toettcher, J. E. & Avalos, J. L. (2018). Optogenetic regulation of engineered cellular metabolism for microbial chemical production. *Nature* ,*555* (7698), 683–687. https://doi.org/10.1038/nature26141

Zhong, W., Yang, M., Hao, X., Sharshar, M. M., Wang, Q. & Xing, J. (2020). Improvement of D-lactic acid production at low pH through expressing acid-resistant gene *IoGAS1* in engineered*Saccharomyces cerevisiae* . *Journal of Chemical Technology & Biotechnology* , *August* . https://doi.org/10.1002/jctb.6587

Zhong, W., Yang, M., Mu, T., Wu, F., Hao, X., Chen, R., Sharshara, M. M., Thygesene, A., Wang, Q. & Xing, J. (2019). Systematically redesigning and optimizing the expression of D-lactate dehydrogenase efficiently produces high-optical-purity D-lactic acid in*Saccharomyces cerevisiae* . *Biochemical Engineering Journal* ,*144* , 217–226. https://doi.org/10.1016/j.bej.2018.09.013

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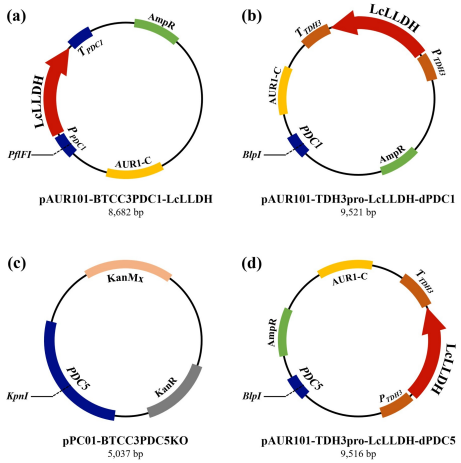
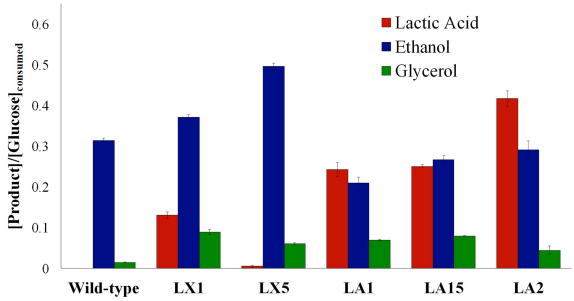


Figure 1



	Wild-type	LX1	LX5	LA1	LA15	LA2
PDC1	+	-	+	-	-	-
PDC5	+	+	-	-	-	-
LDH (# of copy)	-	1	1	1	2	1
Promoter of *LDH*		P_{TDH3}	P_{TDH3}	P_{TDH3}	P_{TDH3}	P_{LDH}

Figure 2

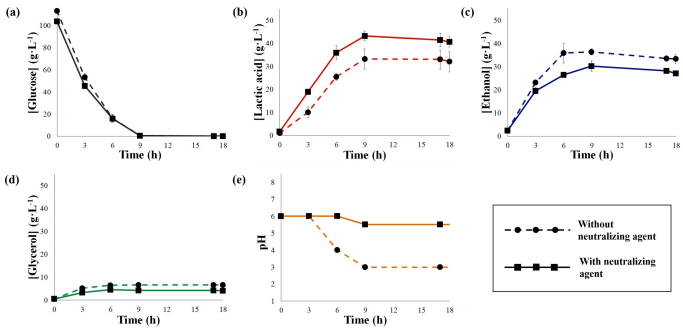


Figure 3

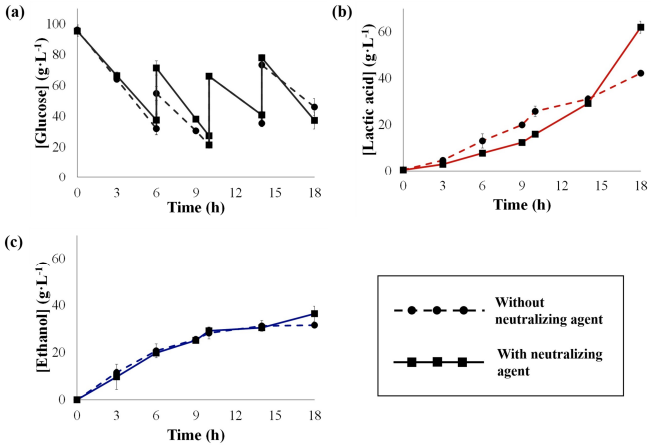


Figure 4

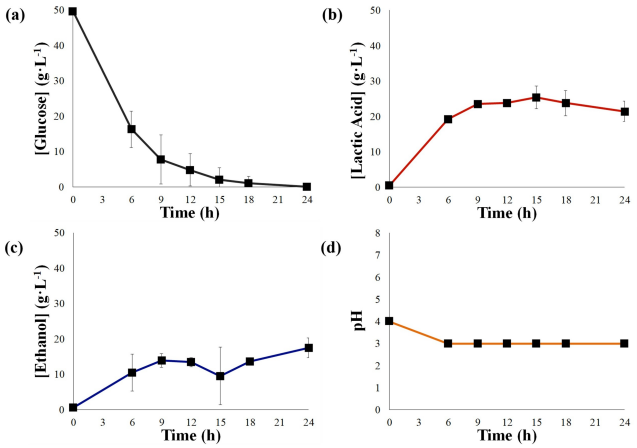
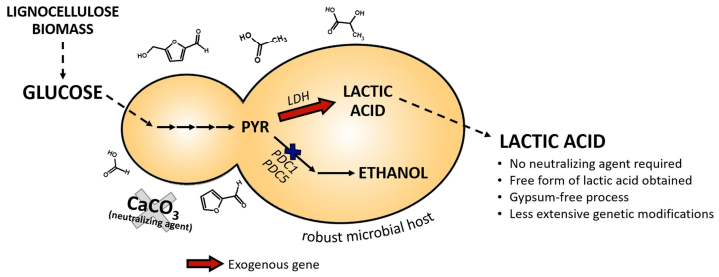


Figure 5



Graphical Abstract