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Original Research Article

Enhancing 3-hydroxypropionic acid production from recombinant *Saccharomyces cerevisiae* for using rice straw hydrolysate and sugar cane industrial waste as substrate

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ABSTRACT

Rice straw and sugar cane industrial waste are a plentiful source of lignocellulosic biomass with a high polysaccharide content, that is hydrolyzed into sugar for microbial growth and their metabolites. 3-Hydroxypropionic acid (3-HP) is a promising chemical building block that can be produced from renewable resources. The malonyl-CoA pathway is one of the biosynthetic routes for 3-HP production by expressing the malonyl-CoA reductase gene (*mcr*). However, the problem of the activity imbalance between the C and N-terminal causes a low conversion rate of malonyl-CoA to 3-hydroxypropionic acid. This study aimed to balance the bi-functional MCR enzyme by dissecting MCR into two fragments and enhancing the supply of intermediates to increase the production of 3-HP. The recombinant strain harboring the dissected *mcr* gene showed a 21-fold increase in 3-HP titer compared to the strain carrying the full-length *mcr* gene. The addition of cerulenin and acetate to the fermented medium enhanced 3-HP yield by 8 times, in which recombinant yeast produced 3-HP up to 10 g/L (0.201 $g_{product}/g_{substrate}$). The results of using rice straw hydrolysate as a carbon source indicated that *Saccharomyces cerevisiae* S2 produced 3-HP of 4.02 g/L, which was 0.074 $g_{product}/g_{glucose}$ in the diluted hydrolysate. These findings provide an alternative and sustainable strategy for utilizing lignocellulosic biomass for future 3-HP production at an industrial scale.

1. Introduction

Lignocellulosic biomass is an attractive feedstock to produce biofuels and high-value chemicals due to its abundance, inexpensive, and renewable. The primary lignocellulosic elements utilized in biorefineries consist of agricultural wastes such as sugarcane bagasse, wheat straw, maize stalks, pineapple leaves, and woody residue [1,2]. Among various lignocellulosic feedstocks, rice straw is one of the potential renewable energy resources. It contains elevated levels of polysaccharides (70 %), including cellulose (32–47 %) and hemicellulose (19–27 %), which can be converted into sugars for microorganisms via pretreatment and enzyme hydrolysis process [3,4]. The rice straw hydrolysate was widely used as a substrate to produce renewable energy such as ethanol, hydrogen, and organic acid [5,6]. The production process relies on molasses or sugarcane juice, byproducts of sugar manufacturing, as primary raw materials. These feedstocks are characterized by a limited shelf life and susceptibility to degradation, manifesting as color alteration and spoilage, if not promptly processed. Despite these inherent limitations, their utilization presents a compelling opportunity due to the minimal economic value of surplus quantities, thereby substantially reducing production costs and enhancing the commercial competitiveness of the final products. Consequently, these materials demonstrate significant potential for industrial-scale applications. Furthermore, the process embodies a bio-renewable polymer synthesis pathway, aligning with principles of environmental sustainability. This approach contributes to a reduction in pollution stemming from factory wastewater and

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residual molasses, thereby mitigating the environmental impact of sugar processing operations.

3-Hydroxypropionic acid (3-HP) represents a promising bio-platform chemical with potential applications across diverse industries, and its production from renewable agro-industrial wastes offers a sustainable alternative to traditional petrochemical routes [7]. Despite the acknowledged potential of various biomass feedstocks, including agricultural residues like rice straw, biomass hydrolysates, and agro-industrial wastes such as molasses, the existing literature demonstrates a paucity of fundamental data necessary for robust process optimization and scale-up. Consequently, this research endeavors to address this knowledge gap by systematically investigating the fermentative production of 3-HP utilizing rice straw hydrolysates as a primary substrate. Furthermore, the study extends its scope to encompass the evaluation of molasses and sugar factory wastes, thereby providing a comprehensive assessment of readily available and abundant feedstocks for 3-HP biosynthesis through the application of genetically modified yeast strains.

3-Hydroxypropionic acid is a non-chiral three-carbon organic compound with hydroxyl and carboxyl functional groups that can be modified into several high-value substances through chemical reactions. The reduction form of 3-HP gives 1,3-propanediol used in manufacturing cosmetics, foods, and pharmaceuticals. Moreover, the oxidation form of this acid leads to malonic acid that can be used in the flavoring and fragrance industries [8]. 3-HP has various applications in the chemical industry, including surgical biocomposite materials, drug release materials, cross-linking agents for polymer coatings, metal lubricants, and textile antistatic agents [9]. As a result, the US Department of Energy recognized 3-HP as one of the significant building-block compounds that can be generated from renewable resources [10]. The predominant method for producing acrylic acid involves the catalytic oxidation of propene, a petroleum-derived feedstock. In contrast, β-hydroxy and hydracrylic acids can be generated through diverse natural metabolic pathways utilizing glucose or glycerol as substrates by *Klebsiella* spp. However, this biological production route presents significant limitations for industrial scalability. The requirement for substantial quantities of vitamin B12 in the microbial culture medium renders it economically impractical. Furthermore, *Klebsiella* is classified as a Biosafety Level 2 (BSL-2) pathogen, posing additional safety concerns. Consequently, despite reported high yields, the practical implementation of this approach is impeded by substantial challenges related to industrial process design and equipment engineering.

In nature, 3-HP functions as an intermediary in the 3HP-dependent carbon absorption cycle in the metabolism of some thermophilic archaea [11]. This carboxylic acid is generated from malonyl-CoA in two steps by malonyl-CoA reductase (MCR), which catalyzes malonyl-CoA to malonate semialdehyde, and then 3-hydroxypropionate dehydrogenase/3-hydroxyisobutyrate dehydrogenase, which converts malonate semialdehyde to 3-HP. Alternatively, 3-HP can be synthesized using only a bi-functional MCR, which catalyzes malonyl-CoA conversion to 3-HP [12]. In particular, the bi-functional MCR of *Chloroflexus aurantiacus* has two short-chain domains; the N-terminal domain expresses alcohol dehydrogenase and the C-terminal domain expresses aldehyde dehydrogenase, which is commonly used in the construction of the malonyl-CoA pathway for 3-HP production in microorganisms [13]. However, the recombinant strain presented a low 3-HP production yield due to the imbalance in enzyme activity between MCR-N and MCR-C proteins. Lui et al. [14] demonstrated that point mutations (N940V, K1106W, S1114R) and the dissection of MCR into two individual fragments were introduced to improve enzyme activity, which enhanced 3-HP titer 270-fold via the malonyl-CoA pathway in *Escherichia coli*. To increase the 3-HP yield, malonyl-CoA should be sufficient for MCR activity. Hence, many genes are required for overexpression to redirect the metabolic flux including acetyl-CoA synthase (ACS), aldehyde dehydrogenase (ALD), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), pyruvate decarboxylase (PDC), pyruvate dehydrogenase (PDH), and acetyl-CoA carboxylase (ACC) [15]. All these enzymes are necessary to catalyze the conversion of glucose to malonyl-CoA. Moreover, nicotinamide adenine dinucleotide phosphate (NADPH) generation pathways are required for providing reducing power, which is another factor regulating 3-HP synthesis [16,17]. Nonetheless, this approach is challenging to control because the overexpression of a few enzymes may have no effect or a slight impact on the desired product. Furthermore, competing pathways should be knocked out or knocked down, particularly the fatty acid pathway, which is primarily responsible for malonyl-CoA utilization and results in low intracellular malonyl-CoA levels. Chen et al. [18] reported that the downregulation of fatty acid production by deleting the coregulated phospholipid synthesis genes improved the malonyl-CoA-derived products. However, cell growth was significantly decreased in mutant strains, indicating that gene deletion might cause the repression of phospholipid formation and the related pathway, thereby reducing cell growth.

Since fatty acid biosynthesis is important to cell components, development, and energy storage, the enzymes in this pathway cannot be completely abolished. Attenuating the modified pathway leads to a greater malonyl-CoA pool in the cytosol, which promotes 3-HP production. The inducer and inhibitor are an interesting approach to providing intermediate availability and blocking undesirable products. Cerulenin, a powerful fatty acid inhibitor, is widely introduced to decrease unwanted malonyl-CoA consumption. This led to improved malonyl-CoA accumulation in the cytosol and an increase in the synthesis of malonyl-CoA-dependent molecules. For example, introducing cerulenin to *E. coli* and *Corynebacterium glutamicum* improved the formation of polyphenolic polyketides [19,20]. It is reported that the addition of cerulenin could increase malonyl-CoA concentration to 40 % of the total pool [21]. van Summeren-Wesenhagen and Marienhagen [22] demonstrated that the intracellular malonyl-CoA level rose from 2 pmol/mg cell dry weight (CDW) to 105 pmol/mg CDW by adding 200 mM cerulenin, which was more than 50-fold increasing. Cerulenin was also used as a fatty acid suppressor for 3-HP production in recombinant *E. coli* and enhanced 3-HP yield by 12-fold [23]. However, the effect of cerulenin on malonyl-CoA reductase expression is limited. Therefore, the objective of this study is to construct the 3-HP-producing strain by balancing the gene expression between the *mcrN* and *mcrC* genes and to investigate the effect of cerulenin on *mcr* gene expression using real-time PCR. The *mcrC* gene was subjected to site-directed mutagenesis to increase MCR enzyme activity. Acetate was added to the production medium to increase the availability of malonyl-CoA in the cytosol. Cerulenin was used as a biochemical inhibitor for down-regulating fatty acid synthesis. The optimal conditions for 3-HP production from rice straw hydrolysate were evaluated for high-yield titers.

In this research, we are interested in studying the effects of gene mutations in the *mcr* segments truncated into *mcrC* and *mcrN* cloned into *Saccharomyces cerevisiae* and monitoring the effects of 3-HP and fatty acid production as well as growth by using hydrolyzed rice straw as a substrate. The objective is to create the potential of an alternative carbon source that is cheaper than sugar in the market as a source of chemical stability in the industry.

2. Methods and materials

2.1. Strain, plasmid, and medium

S. cerevisiae TISTR5344 was purchased from the Thailand Institute of Scientific and Technological Research (TISTR) and used as a wild-type background strain. *E. coli* DH5α was chosen for all DNA manipulations. The gene encoding malonyl-CoA reductase from *C. aurantiacus* (GenBank: accession no. AY530019.1) was searched in the National Center for Biotechnology Information (NCBI) database. The pCEV-G1-Km vector was a gift from Lars Nielsen and Claudia Vickers (Addgene plasmid # 46,813) [24], which was used as an expression plasmid in yeast. *E. coli* DH5α was cultured in Luria-Bertani broth (LB) at 37 °C and

supplemented with 100 μg/mL of ampicillin for recombinants carrying plasmids. *S. cerevisiae* recombinant strain was grown in Yeast extract–Peptone–Dextrose (YPD) media supplemented with 200 μg/mL of geneticin (G418) at 30 °C.

2.2. DNA manipulation

The *mcr* gene was synthesized with codon optimization and inserted into the pUC19 plasmid (GenScript, USA). The full-length *mcr* gene was digested with *Hind*III and *Bam*HI restriction enzymes before being ligated into an expression plasmid, which was transformed into *S. cerevisiae* TISTR5344 by the standard lithium acetate method [25], resulting in *S. cerevisiae* M1 strain.

Three mutation points at *mcrC* gene (N940V, K1106W, and S1114R) of the MCR enzyme were created by site-directed mutagenesis using a KOD-Plus-Mutagenesis kit (Toyobo, Tokyo, Japan). The mcrSD11-12, mcrSD21-22, and mcrSD31-32 primers for Ser1114, Asn940, and Lys1106 site-directed mutation were listed in Table 1. The PCR amplified fragment was ligated by a reaction with T4 polynucleotide kinase. The ligated PCR products were transformed into *E. coli* DH5α by heat shock. The resultant plasmid carrying *mcrC** gene under the control of the pTEF1 promoter was named pDmcrNC*, which was transformed into *S. cerevisiae* TISTR5344, resulting in the *S. cerevisiae* M2 strain.

The *mrc* gene was separated into two fragments, including *mcrN* (1-549) and *mcrC** (550-1219) by PCR amplification using mcrDC and mcrDN primers. The *mcr*-N fragment was ligated into the pCEV-G1-Km vector under the control of the *pgk*1 promoter, while *mcrC** fragment was ligated into the same plasmid under the control of the *tef*1 promoter. The pMCR-pgkMN-tefMC* was transformed into *S. cerevisiae* TISTR5344, resulting in *S. cerevisiae* S2 strain. The clones with the correct inserts were selected and confirmed by colony PCR and DNA sequencing.

2.3. Effect of cerulenin and acetate on 3-hydroxypropionic acid production

Biosynthesis of 3HP was conducted in minimal medium containing (g/L) 7.5 g $(NH_4)_2SO_4$, 14.5 g KH_2PO_4, 0.5 g $MgSO_4{\cdot}7H_2O$, 2 mL trace elements, 1 mL vitamin solution, and 50 g/L glucose. The trace elements and vitamin solution were prepared by Kildegaard et al. [26]. The glucose, trace elements and vitamin solution were autoclaved separately. The 3HP-producing strain was inoculated in 50 mL of YPD media containing 2 % glucose to reach the late exponential phase. Cells were harvested by centrifugation at 4000 rpm for 5 min at 4 °C and washed by minimal medium. The cell pellet was resuspended in a fresh minimal medium containing 50 g/L glucose. The initial inoculum was determined by measuring the optical density of the culture at 600 nm of 0.5 in 20 mL medium. The various cerulenin levels were added to the media, including 5, 10, 15, and 20 μM. To estimate the effect of acetate on 3HP production, potassium acetate was added to the media at different concentrations, including 10, 20, 30, and 40 mM with optimized cerulenin titers. The cultivations were conducted in a shake flask under aerobic condition at 30 °C and 200 rpm. The fermented broth was taken every 24 h for analyzing growth rate and 3-HP production.

Table 1
Primer list in this study.

Name	Sequence
mcrSD11	GCTAGATTAGCATTGGTTACTCCA
mcrSD12	ACCATCAGACAATGCGATCTTTCT
mcrSD21	AGAGTTGTTTCTGGTGAAACTTTT
mcrSD22	ATCAGCTAAGTAGTAAACTGTTGC
mcrSD31	AGATGGATCGCATTGTCTGATG
mcrSD32	AGCAACTCTGAAATGATGAGTC
mcrDC_F	AAGGAAAAAAGCGGCCGCATGTCTGCAACTACAGGTGC
mcrDC_R	GGACTAGTTTAAACAGTGATTGCTCTACCTC
mcrDN_F	CGCGGATCCATGAGTGGTACAGGTAGATTG
mcrDN_R	CGGGGTACCTTAAATATTAGCTGGAATGTTCAATG
qMN11	GGATGGTTCTGATCCAGTTGC
qMN12	CCTGGACCCAATTCAGCTTC
qMC11	GGTTCTGCAGGTATTGGTGGTC
qMC12	CACAACCTGGAGCAATATGAACTC

2.4. 3-Hydroxypropionic acid production from rice straw hydrolysate

Rice straw (RW) was collected from rice fields in Phichit province, Thailand. The straw was ground and homogenized using a sieve of 2–5 mm. The sieved straw was pretreated with hydrogen peroxide (H_2O_2) to remove lignin and hemicellulose. The pretreatment mixture was performed in a 500 mL bottle containing 15 % w/v sieved rice straw in 7.5 % H_2O_2. The pH was adjusted to 11.0 with sodium hydroxide (NaOH). The bottle of the mixture was incubated at 35 °C with 200 rpm shaking for 24 h. The pretreated RW was rinsed with water and dried at 60 °C. The cellulase enzyme was used for converting lignocellulose to glucose. The enzymatic hydrolysis was conducted using 4 % cellulase (v/v) in 0.1 M potassium acetate (pH 5) and 10 % (dry matter, w/v) pretreated RW was suspended in the solution. To dilute acetate concentration, 50 mM potassium acetate (pH 5) was used in the hydrolysis step. The sample was incubated in a shaker at 200 rpm, 35 °C for 72 h. Rice straw hydrolysate was separated from solid pretreated straw and used as a substrate for 3-HP formation.

To evaluate the 3HP production from rice straw, the 3HP-producing strain was grown in YPD media to provide inoculum. The recombinant cell was inoculated in 20 mL of hydrolysate broth with an initial inoculum density of 2 measured by a spectrophotometer at 600 nm (OD1 correlates to 1.5×10^7 cells/mL). The experiments were divided into three groups including utilizing hydrolysate RW directly, supplementing with minerals, and using RW hydrolysate to generate 3-HP. All experiments were performed in a shake flask incubation at 30 °C and 200 rpm.

2.5. 3-Hydroxypropionic acid production from sugarcane juice

The large particles of suspended materials in sugarcane juice were eliminated by passing several layers of clean cheesecloth. The raw juice was sterilized by autoclave at 110 °C for 20 min. The recombinant strain was inoculated in different percentages of sugarcane juice including 20, 40, 60, 80 % to investigate 3-HP production. The sugarcane juice fermentation was divided into two groups experiment consisting of supplemented with mineral and without mineral addition. All experiment was performed in a shaking incubator at 30 °C under aerobic condition.

2.6. 3-Hydroxypropionic acid production from molasses

To reduce the viscous substance, molasses was diluted to 50 % with distilled water. The diluted molasses was passed through several layers of clean cheesecloth to eliminate the large particles of suspended materials. The sterilized molasses was performed by autoclave at 110 °C for 20 min. This study examined 3-HP production by analyzing the proportion of molasses at 4 levels, including 5, 10, 20, 30 %. The 3-HP synthesis from raw sugar was divided into two groups, consisting of those supplemented with minerals and those without mineral addition. The recombinant *S. cerevisiae* in molasses was carried out in shaking incubator at 30 °C under aerobic condition.

2.7. qPCR analysis

The expression level of *mcrC* and *mcrN* genes were monitored by qPCR. The seed cultures were performed in 250 mL flask with 50 mL of minimal medium for 48 h. The cell pellet was collected by centrifuging at 4000 rpm for 5 min. The total RNA from three recombinant strains was extracted using a NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany) and reverse transcribed into cDNA by ReverTra Ace™ qPCR

RT Master Mix kit (Toyobo, Tokyo, Japan). The qPCR was conducted in triplicate following the Luna Universal qPCR Master Mix Protocol (New England Biolabs, USA). The qMN11-12 and qMC11-12 primers for MCR-N and MCR-C amplification are listed in Table 1. The thermal cycle conditions were started with 1 cycle of initial denaturation at 95 °C for 60 s, followed by 40 cycles of denaturation at 95 °C for 15 s and extension at 60 °C for 30 s, and the melting curve analysis was performed ramping from 65 °C to 95 °C at 0.5 °C/s. The relative expression levels of the target gene were calculated according to the $2^{-\Delta\Delta Cq}$ method [27]. The β-actin gene was used as a reference gene. The student's t-test was used to define gene expression changes that were statistically significant ($p < 0.05$).

2.8. Enzyme activity assay

Cell extracts were prepared using a previously reported fast preparation method for enzyme analysis [28]. Protein concentrations were measured according to the Bradford protein assay and the reaction was monitored at 540 nm. Malonyl-CoA reductase activities of the crude-cell extract were measured using a previously reported spectrophotometric assay [29]. The rate of NADPH oxidation was measured at 365 nm ($\varepsilon_{365nm} = 3.4 \times 10^3\ M^{-1}\ cm^{-1}$) and the oxidation rate of NADPH was used to estimate the enzyme activity.

2.9. Analytical methods

Cell growth was detected by using the microplate reader at the wavelength of 600 nm. The fermented broth was harvested by centrifuging at 10,000 rpm for 10 min. The supernatant was collected for 3-HP, sugar, ethanol, and acetic acid concentration. The 3HP concentration was analyzed by using HPLC (Shimadzu, Japan) with SPD-20 A UV detector at the wavelength of 210 nm. The C18 column was employed for determining 3-HP. The column temperature was at 45 °C, and the mobile phase was 1 mM H_2SO_4 and 8 mM Na_2SO_4 at a flow rate of 0.4 mL/min. The total sugar content in rice straw hydrolysate was analyzed by HPLC using the NH2 column and ELSD detector. The column temperature was 30 °C, and the mobile phase was 75 % acetonitrile and 25 % water at a flow rate of 1.0 mL/min. Ethanol and acetate were analyzed by gas chromatography (GC-2010A Shimadzu, Japan) following Lertsriwong et al. [30]. The 3,5-dinitrosalicylic acid (DNS) method was used to determine the reducing sugar concentration.

2.10. Statistical analysis

The SPSS program (version 28.0) was used to analyze the data. One-way ANOVA was used to evaluate the significance of the mean difference between the experiment groups, with the Tukey post-hoc test accepting significance at the p 0.05 level. Three replicates of data were used to calculate the mean and standard deviation (SD) of the results.

3. Results and discussion

3.1. The expression of mcr gene in the 3-HP-producing strain

The *mcr* gene from *C. aurantiacus* has been reported to be the best candidate for 3-HP synthesis. This protein is a bi-functional form of the MCR protein, which catalyzes the two reaction steps from malonyl-CoA to 3-HP. However, the activity of those proteins was unbalanced, causing a slow conversion rate of malonyl-CoA to 3-HP. To evaluate the gene expression level of bi-functional *mcr*, qPCR was conducted to reveal the relationship between the C and N-terminals in the expression level. Three 3-HP-producing strains were used for this study, including M1 carrying full-length MCR, M2 harboring mutant MCR, and S2 containing dissected MCR. The investigation revealed that the transcription levels of the *mcrC* and *mcrN* genes were imbalanced in the M1 and M2 strains harboring the full-length *mcr* gene. The *mcrN* gene was expressed at a statistically significantly higher level than the *mcr*-C gene, which was 8-fold higher than the *mcrC* gene in the M1 strain, and it was also 6-fold higher than the expression level of the *mcrC* * gene in the M2 strain ($p < 0.05$). This phenomenon was related to the imbalance of the activity of the C and N domains. Due to the lower expression level of the C-terminal, the *mcrC* fragment was separated and independently expressed under the regulation of a strong promoter, which showed a rebalance in transcription levels between the *C and* N-terminal in the S2 strain (Fig. 1). According to comparisons of the transcription levels of the *mcrC* gene in full-length and dissected MCR, the separated *mcrC* gene was expressed at a significantly higher level by 11-fold under the control of the pTEF1 promoter than the *mcrC* gene in the M1 strain ($p < 0.05$). There was no significant change in the expression levels of the *mcrC* gene under the control of the pPGK1 promoter (Fig. 2). Therefore, the pTEF1 promoter was selected for the regulation of *mcrC* expression in this study.

Concerning enzyme activity and 3-HP production (Fig. 3), the M2 strain demonstrated greater enzyme activity and 3-HP yield than the M1 strain harboring the wild-type *mcrC* gene, whereas the M1 strain produced 60 mg/L of 3-HP and the M2 strain generated 330 mg/L of 3-HP titer. It was improved by 5.5 times. It can be assumed that a side-directed

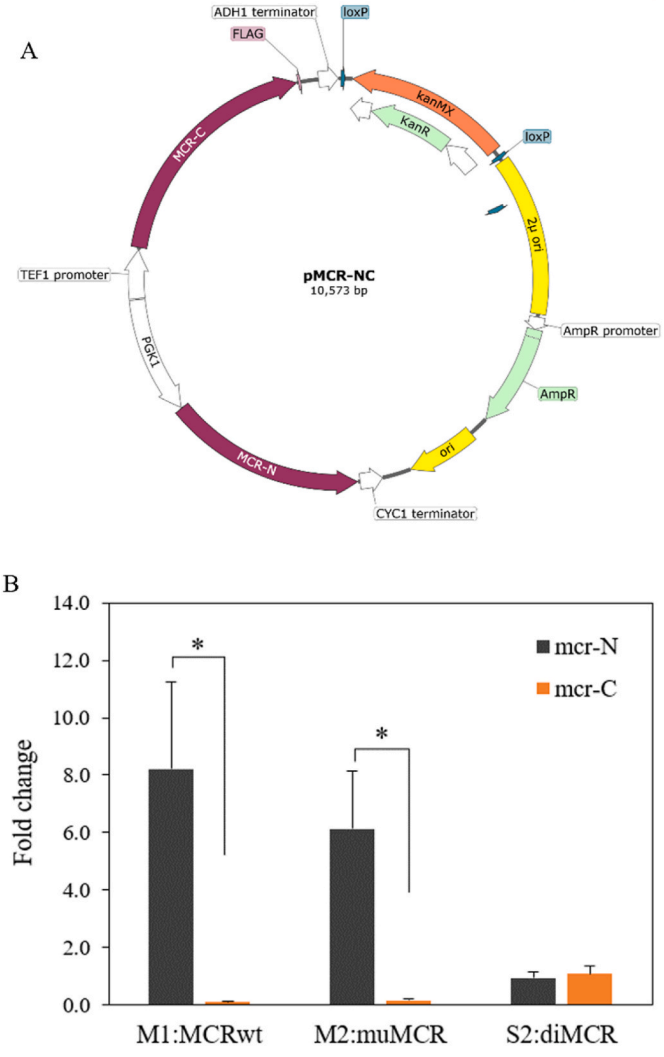

Fig. 1. Schematic representation of the 3-HP plasmid (A) and Comparison of transcription levels of *mcr*-N and *mcr*-C genes in three strains (B), including the M1 strain harboring full-length MCR, the M2 strain harboring mutant MCR, and the S2 strain carrying dissected MCR. The experiments were performed in triplicate, and β-actin was used as a referent gene ($p < 0.05$).

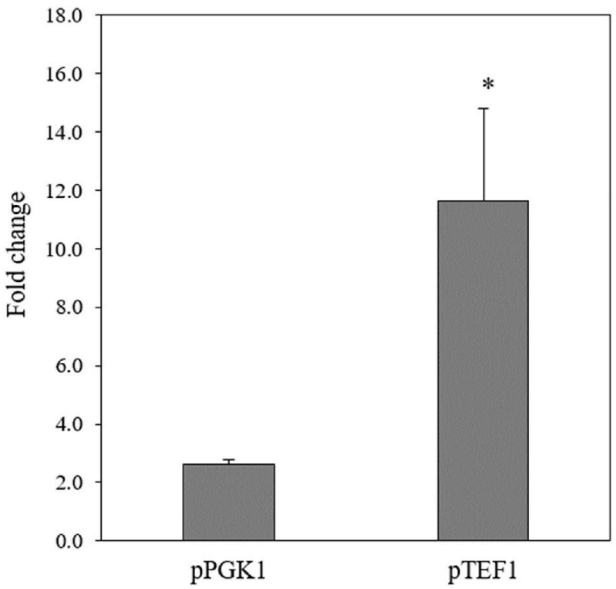

Fig. 2. Relative fold change of the transcription levels of the *mcr*-C gene in the S2 strain carrying dissected MCR under the control of different promoters. The experiments were performed in triplicate, and B-actin was used as a referent gene ($p < 0.05$).

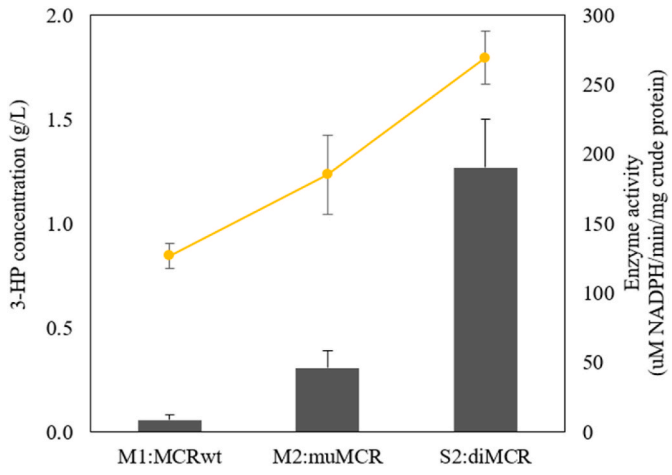

Fig. 3. The oxidation rate of NDNPH and 3-HP yield in different 3-HP-producing strains, including the M1 strain harboring full-length MCR, the M2 strain harboring mutant MCR, and the S2 strain carrying dissected MCR. The line graph represents enzyme activity, and the bar graph represents 3-HP production.

mutation of the *mcrC** gene may have activated enzyme activity and increased production yield but failed to recover the balance of protein expression levels. The balance of enzyme activity of these proteins was not resolved, resulting in a still-lower 3-HP yield. The highest enzyme activity and 3-HP yield were obtained from the S2 strain, in which 3-HP production was achieved at 1.27 g/L. It was enhanced 21-fold compared to the M1 strain carrying the full-length *mcr* gene. These results suggested that the separated expression of the *mcr* gene influenced 3-HP production, and the increment in the *mcrC* gene level caused an increase in carbon flux to 3-HP synthesis. This is consistent with the previous report that *mcrC* is a rate-limiting enzyme that catalyzes the malonyl-CoA to malonate-semialdehyde [31]. The independent expression of *mcrC* and *mcrN*, along with increased *mcrC* expression, was advantageous for this carboxylic acid synthesis [32].

3.2. Effect of cerulenin on 3-hydroxypropionic acid production

The 3-HP production was further improved by adding cerulenin, which inhibited fatty acid synthesis and consequently increased the intracellular level of malonyl-CoA. According to the findings, the engineered S2 strain produced 4.83 and 7.66 g/L of 3-HP by adding 5 and 10 μM cerulenin, respectively (Fig. 4). In comparison to the control without the cerulenin supplement, 3-HP formation was improved by 3.7 and 6 times, respectively. These findings are consistent with a previous report by Lee et al. [23], in which recombinant *E. coli* could achieve a 3-HP titer of 3 g/L. It was higher than the group without cerulenin by 12-fold when 50 μM cerulenin was added to prevent unwanted malonyl-CoA consumption.

However, further increases in cerulenin concentration have negatively impacted cell growth and 3-HP production. As a result, 3-HP yield was slightly decreased when cerulenin was added up to 15 μM concentration compared to 10 μM cerulenin due to a reduced growth rate. When the concentration was raised to 20 μM, cell dry weight decreased by 37 %, accompanied by a moderate reduction in 3-HP synthesis. At 25 μM, the growth rate of the recombinant strain declined by up to 50 %, and 3-HP production was significantly reduced. Although cerulenin effectively enhances 3-HP yield, high concentrations can adversely affect cell viability.

Cerulenin inhibits fatty acid and sterol biosynthesis by binding to the enzyme β-ketoacyl-acyl carrier protein synthases (FabB, FabF, and FabH), which promptly interrupts the proliferation of microbes caused by lipid depletion [33]. Therefore, optimizing cerulenin concentration is critical to balance the benefits of elevated malonyl-CoA with the preservation of cellular viability, enabling efficient production of malonyl-CoA-derived biochemicals.

According to results on the effect of cerulenin on *mcr* gene expression levels, the balance between *mcrC* and *mcrN* gene transcription levels was not disrupted for 48 h, whereas cerulenin suppression affected cell growth during the exponential phase (Fig. 5A). However, the transcription level of the MCR-N fragment was significantly changed in the control group without cerulenin at 96 h, which was 13 times higher in expression than that of the C fragment. The high increase in imbalance expression may be related to a low rate of 3-HP production in cultivation without cerulenin. Surprisingly, there was a moderate change in the expression levels of the *mcrC* and *mcrN* genes in the cerulenin supplement group at 96 h (Fig. 5B), resulting in higher 3-HP production in the cerulenin supplemented medium. The outcome implied that cerulenin did not have much effect on the *mcr* expression level but had an influence on the quantity of malonyl-CoA that was provided as a substrate for the formation of 3-HP. Davis et al. [34] reported that cerulenin supplementation enhanced malonyl-CoA levels by up to 10 % in strains with normal ACC levels without harming ACC function.

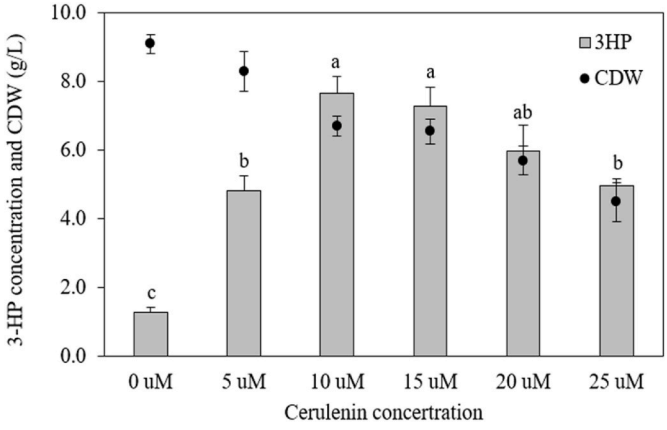

Fig. 4. 3HP-production of recombinant *S. cerevisiae* S2 strain in mineral medium with different concentrations of cerulenin ($p < 0.05$).

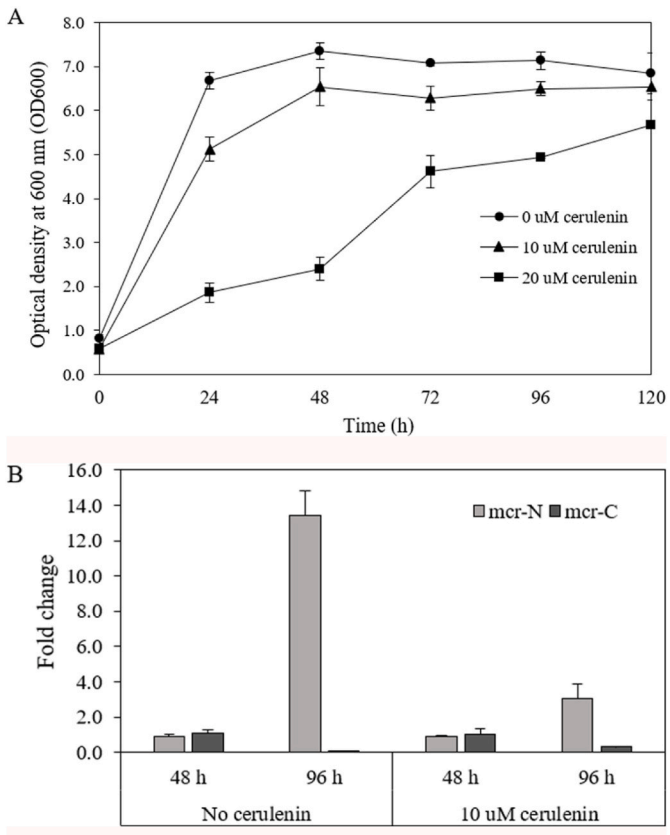

Fig. 5. Growth rate of *S. cerevisiae* S2 strain in mineral medium with 10 μM and without cerulenin (A). RT-qPCR analysis of the fold change in transcription levels (B). The expression levels of *mcr*-N expression levels on the *mcr*-C gene were compared between the control without cerulenin and 10 μM cerulenin supplemented. The experiments were performed in triplicate, and B-actin was used as a referent gene ($p < 0.05$).

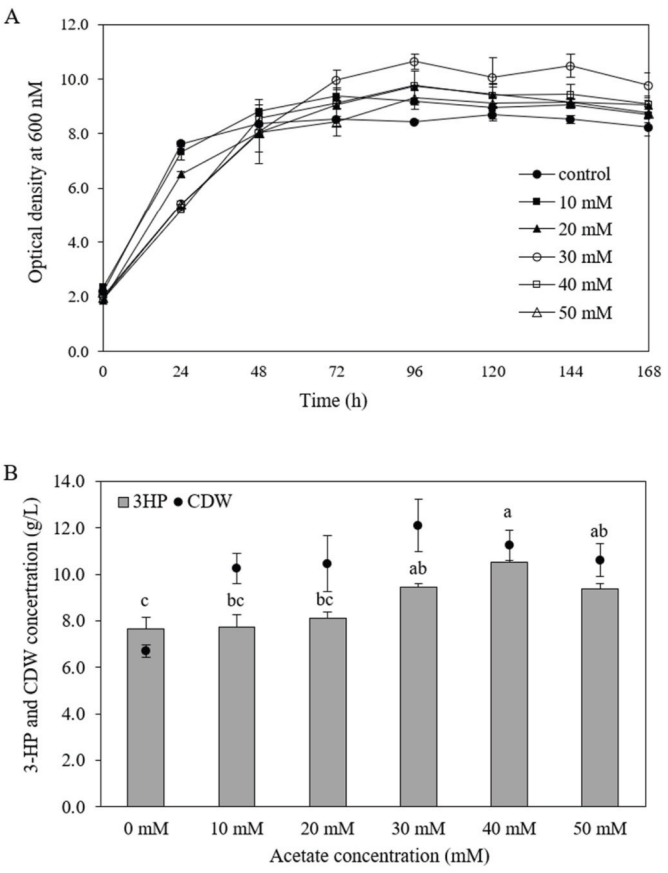

Fig. 6. Cell growth (A) and 3HP-production (B) of recombinant *S. cerevisiae* S2 strain in mineral medium with 10 μM cerulenin and different concentrations of acetate ($p < 0.05$).

3.3. Effect of acetate on 3-hydroxypropionic acid production

The effect of acetate on cell growth is shown in Fig. 6A. The growth of the recombinant was affected by adding acetate during the exponential phase (24-48 h), resulting in a lower cell density than the control without acetate. In a culture containing acetate at 20, 30, 40, and 50 mM, the yeast began to grow fast after 48 h and achieved a maximum growth at 96 h. The cell dry weight of those in a medium containing acetate was higher than the control without acetate in cultivation. The acetate addition at 30 mM presented the highest CDW at 12.1 g/L, while the maximum 3-HP titer was obtained from 40 mM acetate in cultivation (Fig. 6B). The 3-HP yield was improved by 37 % by presenting 40 mM acetate, resulting in a yield of 0.201 $g_{product}/g_{substrate}$. The results demonstrated that adding acetate to cultivation could enhance cell dry weight and 3-HP production in this strain.

Under aerobic conditions, *S. cerevisiae* can consume acetate as an alternative carbon source by converting acetate into acetyl-CoA via acetyl-CoA synthetase or transforming it into intermediates in the glyoxylate pathway [35]. However, the high concentration of weak acids, such as acetate and 3-HP, is commonly toxic to cell development and restrains the generation of acids in fermented broth [36]. Therefore, using acetate as a sole carbon source for 3-HP synthesis becomes challenging due to inefficient cell growth on the acetate, indicating low product. According to Lama et al. [37] finding, the use of glucose and acetate, in which glucose was consumed for cell division and acetate was used to generate 3-HP, was a more effective approach.

3.4. The production of 3-hydroxypropionic acid from rice straw hydrolysate, cane juice, and molasses

After the pretreatment and enzyme hydrolysis processes, the sugar content in rice straw hydrolysate was 60–70 g/L, consisting of 50–55 g/L glucose and 5-10 g/L of other sugars. The acetate was detected at 7.67–6.53 g/L in the RW hydrolysate and 3.11–2.74 g/L in the diluted-acetate hydrolysate (Table 2). The cell growth was low for 72 h and then slowly increased after 72 h (Fig. 7A). Nevertheless, no 3-HP was detected in the induction of hydrolysate directly as a substrate for 3-HP synthesis. To improve 3-HP yield, cerulenin and minerals were supplemented to RW hydrolysate cultivation. The recombinant strain generated 0.48 g/L by supplementing cerulenin and 0.83 g/L by adding cerulenin and minerals. The 3-HP production in dilute acetate hydrolysate was 2.24 g/L with cerulenin addition, which was improved by 4.6 times compared to non-dilute RW hydrolysate. The highest 3-HP titer was obtained from the dilute acetate hydrolysate supplemented with cerulenin and minerals, which produced 4.08 g/L of 3-HP, increasing by 2-fold and 5-fold compared to without mineral addition and non-dilute RW hydrolysate, respectively (Fig. 7B). The results indicated that rice straw hydrolysate could be a viable alternative renewable carbon source for producing 3-HP. The better condition for 3-HP synthesis was the utilization of hydrolysate with a low concentration of acetate. When the buffer for enzyme hydrolysis was diluted, the final titer of acetate in the hydrolysate was decreased to 50-55 mM. This finding was consistent with the above results, whereas 40 mM acetate was beneficial for the three-carbon acid formation.

The application of lignocellulosic hydrolysate as feedstock for microorganism fermentation should be carefully monitored for the concentration of acetate. It was produced during the hydrolysis of

Table 2
Sugar, acetate content, and 3-HP yield from using rice straw hydrolysate (RW) as carbon source.

Parameter	Mineral medium	RW (high-acetate)		RW (low-acetate)	
	cerulenin/acetate	cerulenin	cerulenin/mineral	cerulenin	cerulenin/mineral
Reducing sugar content in medium (g/L)	50	50	55	52	55
Acetate content in medium (g/L)	2.8	7.67	6.53	2.74	3.11
Sugar consumption (g/L)	50	45	49	52	55
CDW (g/L)	11.25	3.9	4.1	4.9	4.8
3-HP (g/L)	10.54	0.48	0.83	2.24	4.08
Yield ($g_{product}/g_{glucose}$)	0.201	0.011	0.017	0.043	0.074

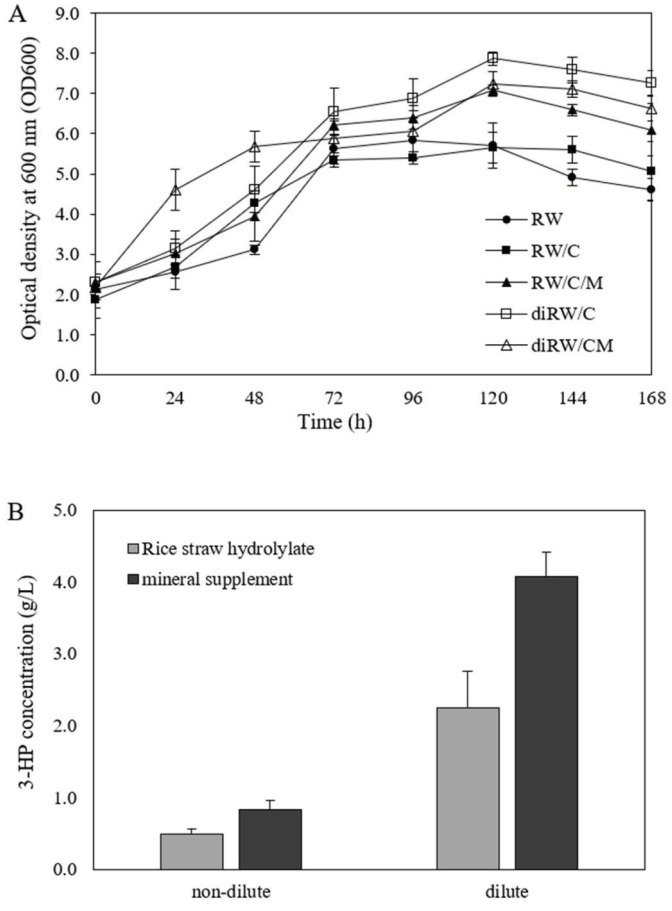

Fig. 7. Growth rate (A) and 3HP-production (B) of recombinant *S. cerevisiae* S2 strain in rice straw hydrolysate and diluted rice straw hydrolysate with 10 μM cerulenin, whereas RW: rice straw hydrolysate; RW/C: rice straw hydrolysate supplemented with cerulenin; RW/C/M: rice straw hydrolysate supplemented with cerulenin and mineral; diRW/C: diluted rice straw hydrolysate supplemented with cerulenin; diRW/C/M: diluted rice straw hydrolysate supplemented with cerulenin and mineral.

lignocellulose, which was contained in cellulosic hydrolysates over 10 g/L [38]. According to previous reports, the addition of 20–80 mM acetic acid can cause *S. cerevisiae* to develop programmed cell death [39]. The cell growth was significantly inhibited, and increased levels of ROS were observed in yeast cells, resulting in the metabolic function and strength of the cell membranes being lost in the yeast cells [40]. Additionally, minerals and vitamins were considered for lignocellulosic hydrolysate usage because rice straw is deficient in appropriate amino acids, vitamins, and minerals for optimal microbial development and enzyme activity. Utilizing rice straw without adding the other necessary nutrition sources will result in the microbe performing poorly.

This study investigated the potential of sugarcane juice as a feedstock for 3-hydroxypropionic acid (3-HP) production using *S. cerevisiae* S2 under optimized conditions. The sugarcane juice used contained a total sugar concentration of 112 g/L, which was diluted to achieve an optimal concentration for 3-HP production. Fig. 8 illustrates the cell growth and 3-HP production profiles, demonstrating the strain's robust growth and significant 3-HP accumulation in sugarcane juice. Notably, a maximum 3-HP titer of 8.98 g/L was achieved with 40 % sugarcane juice supplemented with minerals. In contrast, 60 % sugarcane juice without mineral supplementation yielded 8.23 g/L, indicating a marginal reduction in 3-HP production. However, a substantial decrease in 3-HP production to 5.16 g/L was observed with 60 % sugarcane juice supplemented with minerals, representing a 1.7-fold reduction compared to the 40 % juice condition. Furthermore, the study revealed significant ethanol production, with yields ranging from 10 to 17 g/L, observed in 60 % sugarcane juice with mineral supplementation and 80 % sugarcane juice cultivation, respectively. These results highlight the complex interplay between sugarcane juice concentration, mineral supplementation, and the partitioning of carbon flux between 3-HP and ethanol production. The bioconversion of molasses to 3-hydroxypropionic acid (3-HP) exhibited a diminished titer, ranging from 0.93 to 3.45 g/L (Fig. 9), in the absence of mineral supplementation. This observation suggests a positive influence of mineral components on 3-HP production utilizing molasses as a

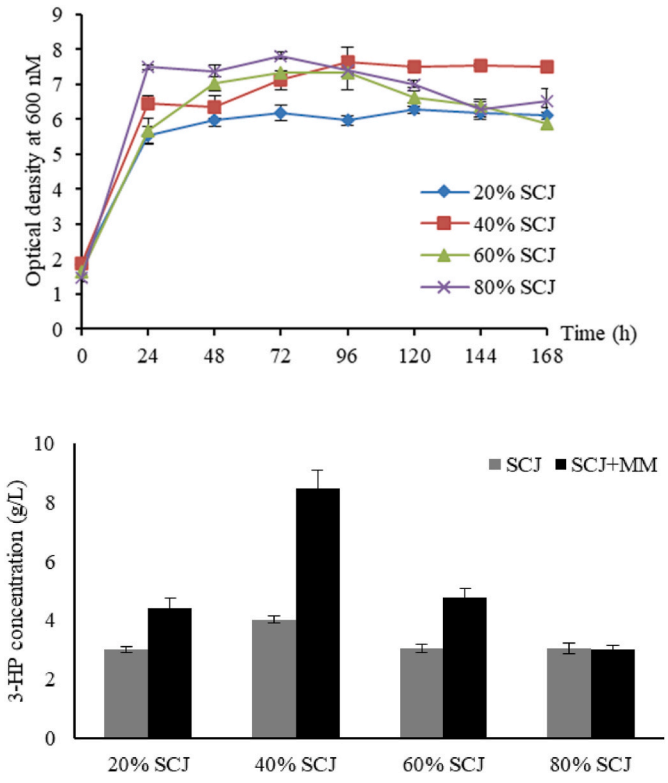

Fig. 8. Growth rate (A) and 3HP-production (B) of recombinant *S. cerevisiae* S2 strain in diluted sugar cane juice supplemented with minimum mineral and 10 μM cerulenin, whereas SCJ: sugar cane juice; SCJ + MM: diluted sugar cane juice supplemented with minimum mineral and cerulenin.

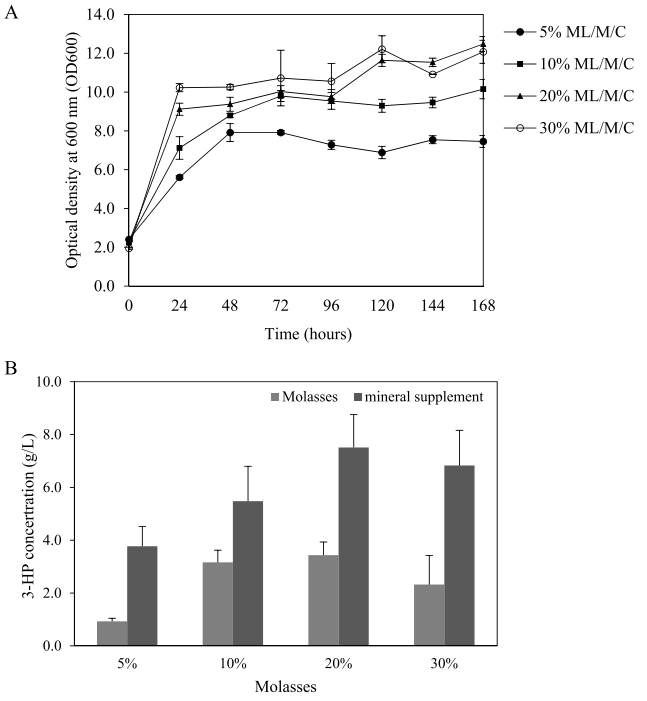

Fig. 9. Growth rate (A) and 3HP-production (B) of recombinant *S. cerevisiae* S2 strain in diluted molasses supplemented with minimum mineral and 10 μM cerulenin, whereas ML/M/C: diluted molasses supplemented with cerulenin and minimum mineral.

substrate. While molasses provides a rich source of organic nutrients, including proteins and vitamins, and inherent mineral constituents, the presence of inhibitory compounds such as copper, potassium, and calcium ions, as well as hydroxymethylfurfural (HMF), appears to compromise *S. cerevisiae* metabolic activity. Consequently, the reduced 3-HP titer without mineral supplementation may be attributed to both the absence of essential cofactors and the exacerbation of inhibitory effects by the contaminants (see Table 3).

The production process utilizes raw materials, specifically molasses from sugar factories or sugarcane juice, which exhibit a restricted shelf life and are susceptible to discoloration and spoilage if unprocessed. These materials present a compelling opportunity for utilization, as surplus quantities possess minimal intrinsic value, thereby significantly mitigating production costs and enhancing the commercial viability of the resultant products. Consequently, these materials demonstrate substantial potential for industrial and commercial applications. Moreover, the process constitutes a bio renewable polymer production method, aligning with environmentally sustainable practices. This approach further contributes to the reduction of pollution generated by factory wastewater and molasses byproducts.

Table 3
Sugar content and chemical properties of molasses.

Test Item	Method	Result	Unit
BOD 5 Days	APHA: 4500-O©5210 B	7600	mg/L
COD	APHA: 5220C	12,144	mg/L
Total Sugars	In-house method SU-056-TM based on AOAC (2016) 977.20	27.28	%
Total nitrogen (Kjeldahl methods)	APHA: 4500-Norg (B)	23	mg/L
Manganese	APHA: 3030 E, 3111B	6.3	mg/L
Copper	APHA: 3030 E, 3111B	0.12	mg/L
sulfide	APHA: 4500-S(F)	1.3	mg/L

Currently, the main approach to producing 3-HP is chemical synthesis using fossil feedstocks, but chemical manufacturing is expensive and toxic to the environment [41]. In response to the growing increase in demand for renewable energy, sustainable development in the chemical industry needs to shift to renewable resources as raw materials [42]. This study explored the production of 3-HP from *S. cerevisiae* using rice straw as feedstock. Rice straw is abandoned on the rice paddy field after the harvest season. Cheewaphongphan et al. [43] reported that about 26 million metric tons of rice straw were generated after the harvesting of rice grain in Thailand, and 685.24 million tons of rice straw were produced worldwide [44]. The straw is usually burned to prepare fields for new plantings. However, the burning of leftover straw releases greenhouse gases into the atmosphere, contributing to air pollution and global warming. The burning of rice straw is reported to emit 1.2–2.2 g of CH_4 and 0.03–0.07 kg of N_2O per kilogram of rice straw, as well as generate other pollutants such as CO_2, SO_2, and particulate matter [45,46]. Based on this experiment, 15 g of dry rice straw could generate 4.08 g/L of 3-HP. Therefore, using 1 kg of rice straw ought to provide approximately 272 g/L of 3-HP. The generation of this outstanding carboxylic acid production from rice straw, which is abundant, renewable, and inexpensive, is a promising alternative for improving long-term prospects and reducing negative environmental effects.

In relation to these findings, Table 4 compares the 3-HP production performance of the engineered *S. cerevisiae* S2 strain from this study with the previous research. It is observed that the 3-HP titter achieved from the alternative carbon sources, particularly rice straw hydrolysate, is quite competitive, particularly, considering the sustainability and low cost of feedstock. This implies that further study in metabolic engineering, indeed, has effectively enhanced the strain's efficiency.

Table 4
Comparison of 3-HP-producing strains.

Microorganism	Substrate	Fermentation mode	3-HP (g/L)	3-HP yield (g/g)	Ref.
SC (*mcr, gapn, acs, adh2, ald6, acc1, Δmls1*)	Glucose	Batch	0.46	0.023	Chen et al., 2014 [13]
SC (*mcr, acc1*)	Glucose	Batch	0.28	0.014	Shi et al., 2014 [28]
SC (*mcr, pmp1, tpi1*)	Glucose	Batch	1.00	0.050	Li et al., 2015 [47]
SC (*mcr, fas1, fapR*)	Glucose	Batch	0.80	0.054	David et al., 2016 [48]
SC (*mcr, acc1*, pdc1, ald6, acs**)	Glucose	Batch Fed-batch	1.80 9.80	0.081 0.070	Kildegaard et al., 2016 [26]
SC (*kdcA, mdlC, mmsB, cdc28, gcr2, glc7, ptc5, ptc7, smk1*)	Glucose	Batch	18.10	0.125	Tong et al., 2021 [49]
SP (*mcr, acc1*)	Glucose	Batch	7.60	0.038	Suyama et al., 2017 [50]
SP (*acc*, mcr-C, mcr-N, acs, atd1, ptk1, bgl, Δadh*)	Glucose Glucose	Batch Fed-batch	3.50 11.20	0.063 0.120	Takayama et al., 2018 [51]
	Glucose*	**Batch**	**10.54**	**0.201**	
SC (*mcr-C*, mcr-N*)	**Cane juice**	**Batch**	**8.23**	**0.11**	**This study**
* supplemented minerals	**Molasses***	**Batch**	**7.51**	**-**	
	Rice straw*	**Batch**	**4.08**	**0.074**	

Furthermore, these results indicate a promising direction for future study aimed at optimizing production processes for bio-based chemicals.

In terms of scalability and industrial feasibility, the use of rice straw is well-aligned with global initiatives promoting the circular economy and carbon footprint reduction. It not only reduces raw material costs but also addresses the environmental issues associated with agricultural waste disposal by reducing open-field burning and associated emissions. Converting this low-value agricultural byproduct into high-value chemicals presents clear economic and ecological advantages.

One challenge to consider is the nutritional profile of rice straw hydrolysate, which may require supplementation to promote microbial growth and metabolic activity. While this could increase production costs, strategic optimization, such as co-fermentation with nutrient-rich byproducts, could mitigate these expenses.

4. Conclusions

This study demonstrated the 3-HP production from *S. cerevisiae* S2 through the malonyl-CoA pathway, whereas the only *mcr* gene was overexpressed. The results indicated that the balancing between C and N domain expression is an important feature for 3-hydroxypropionic acid synthesis of the malonyl-CoA route. Cerulenin significantly improved 3-HP biosynthesis through fatty acid inhibition increasing the malonyl-CoA availability. The maximum 3-HP yield of 10.54 g/L (0.201 $g_{product}/g_{substrate}$) was achieved from the addition of cerulenin and acetate to cultivation via the expression of dissected *mcr* gene, which represented a large amount of 3-HP synthesis obtained in *S. cerevisiae* by expressing only a single *mcr* gene. Moreover, this study revealed the application of low-cost raw materials and easy-to-control processes to produce 3-HP by using rice straw biomass as a feedstock. The product yield was 0.074 $g_{product}/g_{glucose}$. These findings suggested that rice straw hydrolysate, which is a renewable feedstock, has the potential to produce 3-hydroxypropionic acid.

CRediT authorship contribution statement

Supattra Lertsriwong: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Phatcharaphong Khaosaart:** Investigation, Data curation. **Nassapat Boonvitthya:** Supervision, Investigation, Funding acquisition. **Warawut Chulalaksananukul:** Supervision, Resources, Investigation. **Chompunuch Glinwong:** Writing – review & editing, Supervision, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Nassapat Boonvitthya is currently employed by PTT Public Co. Ltd. Innovation Institute, PTT Public Company Limited provided financial support for this research.

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