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Research on metabolic regulation and optimal design of novel biofuels

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Abstract Biofuels, as a renewable energy source, are gradually replacing traditional fossil fuels. However, the current yield and conversion efficiency of biofuels still need to be improved. Through metabolic regulation and optimal design, microbial cell factories can be modified to enhance the expression of key enzymes, optimize the supply of precursors, and improve the fermentation conditions, thus significantly increasing the production efficiency of biofuels. In this study, we explored an effective strategy to enhance biofuel production through metabolic regulation and optimal design. Saccharomyces cerevisiae (S. cerevisiae) was used as the host cell, and the biofuel production engineering strain was constructed by LiAc transformation method, and systematic research was carried out to optimize the expression of key enzymes of the synthetic pathway, the supply of precursor substances and the fermentation conditions. The study showed that the best growth state of Saccharomyces cerevisiae was achieved at a glucose concentration of 30 g/L, with an OD600 of up to 2.897, a biofuel yield of 2366.52 mg/L, and a lipid content of more than 55%. The precursor addition experiment showed that the addition of phosphoenolpyruvic acid (PEP) increased the biofuel yield to 202.545 mg/L, which was about 3.3-fold higher compared with that of the no precursor addition group. The results of simultaneous saccharification co-fermentation (SSCF) showed that the recombinant strain optimized for metabolic regulation had a high biofuel yield of 86.91% when using rice straw as feedstock, which was 18.07% higher than that of the original strain. The experimental results confirmed that metabolic regulation and optimal design are effective ways to improve biofuel yield, which provides a scientific basis for the development of an efficient biofuel production process.

Index Terms Saccharomyces cerevisiae, Metabolic regulation, Biofuel, Precursors, Simultaneous saccharification co-fermentation, Fermentation optimization

I. Introduction

In recent years, the global energy crisis has intensified. The World Energy Outlook 2022 report released by the International Energy Agency begins with the statement that “this is the first truly global energy crisis, and the breadth and complexity of its impact is unprecedented” [1]. The report clearly points out that the global demand for fossil fuels such as oil and coal is increasing due to the shortage of natural gas supply, and the global crude oil market price continues to remain high, and the future energy supply will continue to be tight [2]-[4]. However, as non-renewable resources, fossil fuels are not only characterized by unsustainability, but also by the greenhouse effect and global climate change caused by their combustion emission of large quantities of carbon-containing gases, which will lead to great changes in the earth's ecological environment, and the future of the global agriculture, economy, and political stability will be affected [5]-[8]. Therefore, expanding the capacity of renewable energy, broadening its industrialized application routes, and increasing the proportion of renewable energy application in various industries can effectively alleviate the problems of energy shortage and environmental pollution [9]. Among them, biofuels are attracting global attention as a sustainable energy source to replace traditional fossil fuels [10].

The first generation of biofuels, which are mainly produced from food crops such as corn, sugarcane, and soybeans, are converted into ethanol and biodiesel through a fermentation or refining process [11]. The production process of this generation of biofuels is simple, but its sustainability has been questioned due to competition with food supplies for land and resources [12]. In response to this challenge, the second generation of biofuels was developed, which mainly utilizes non-food plants or waste biomass, such as straw, bark and waste plants [13], [14]. The use of these feedstocks reduces the impact on food supply and biodiversity and improves the environmental friendliness of biofuels [15]. The third-generation biofuels, which follow closely behind, use specially cultivated algae as feedstock, and the high lipid content of algae makes them ideal for biodiesel production. More importantly, algae can be grown on non-arable land, further alleviating the need for agricultural land [16]-[18].

At the forefront is the fourth generation of novel biofuels, which combines gene editing and nanotechnology [19]. This generation of new biofuels not only extracts energy from biomass, but also effectively captures and stores atmospheric carbon dioxide to combat climate change [20]-[23]. With the evolution from the first to the fourth generation, new biofuels have made significant progress in reducing dependence on food crops, lowering the impact on ecosystems, and increasing energy efficiency [24]. This evolution reflects technological advances that have driven the shift in biofuels from the first generation, which relied on food crops, to advanced forms that utilize non-edible biomass, algae, and even gene editing and nanotechnology, which not only demonstrates technological innovations, but also reflects the growing emphasis on environmental sustainability and energy security [25].

The excessive dependence of human beings on fossil energy has led to serious energy shortage and environmental pollution problems, and the search for renewable and clean energy has become an important direction of contemporary scientific research. Biofuels are considered as one of the most promising options to replace fossil fuels due to their renewable and low-pollution characteristics. The production of biofuels by microbial fermentation has become an important area of biofuel research due to its advantages of abundant raw materials, simple process and environmental friendliness. Currently, microbial production of biofuels still faces challenges such as low yield and high cost, which limits its large-scale commercialization. Metabolic regulation and optimal design of microbial cell factories are key strategies to increase biofuel production. Modification of microbial metabolic networks through genetic engineering can enhance target product synthesis pathways and reduce by-product generation, thereby increasing biofuel yield and conversion efficiency. Saccharomyces cerevisiae, as an ideal host cell with clear genetic background, complete metabolic network, and easy operation, is an important platform for biofuel production research. The metabolic pathway of biofuels involves the synergistic action of multiple enzymes, and the biofuel yield can be significantly improved by optimizing the expression level of key enzymes, balancing the supply of precursor substances and optimizing the fermentation conditions. In this study, we will systematically explore the effective strategies to improve biofuel yield by optimizing the expression of key enzymes of the synthetic pathway, optimizing the supply of precursors and optimizing the fermentation conditions. Firstly, the engineering strains were constructed by molecular biology; then, the effects of different glucose concentrations on the growth and biofuel production of Saccharomyces cerevisiae were investigated; then, the effects of precursor additions on the accumulation of biofuels were examined; finally, the biofuel production performance of the original strain and the recombinant strain during the simultaneous saccharification co-fermentation was compared, which provided a scientific basis for the development of an efficient biofuel production process.

II. Novel biofuel metabolism regulation methods

II. A.Strain culture methods

LB medium: peptone 10g/L, NaCl 10g/L, yeast 5g/L. Dissolve with 800mL of deionized water, then make up to 1000mL, dispense 25mL in 250mL conical flasks, and then autoclave at 121ºC for 30mins. 2% agar powder was added to the solid medium. All antibiotics were added to the medium at a final concentration of 50 μg/mL and as required.LB medium was used for activation, fermentation and preservation of *E. coli*.

YPD medium: tryptone 20 g/L, glucose 210 g/L, yeast 10 g/L. Dissolve with 800 mL of deionized water, then make a volume of 1000 mL, dispense 25 mL into 250 mL conical flasks, and then autoclave at 121ºC for 30 min. 2% agar powder was added to the solid culture medium. Preservation.

(1) Escherichia coli (*E. coli*)

a) Solid culture: The preserved *E. coli* glycerol bacteria at -80℃ were left to melt completely at room temperature, and in the aseptic operating table, a small amount of bacterial liquid was dipped into a sterile inoculation ring and streaked on the LB solid culture plate, which was placed in a constant-temperature incubator and cultivated for 12h at 37℃.

b) Liquid culture: in the aseptic operating table, pick the single colony of *E. coli* on the LB solid plate or aspirate the *E. coli* bacterial liquid preserved in the glycerol tube, inoculate it into the LB liquid medium, the inoculum amount is 3%, put it into a constant temperature shaking incubator at 37℃, and cultivate it for 12h under the condition of 280rpm.

(2) Saccharomyces cerevisiae (*S. cerevisiae*)

a) Solid culture: After the preserved -80℃ S. cerevisiae bacterial liquid was completely melted at room temperature, in the aseptic operating table, use the aseptic inoculation ring to dip a small amount of bacterial liquid to delineate on the solid culture plate of YPD, put it in the constant temperature incubator, and cultivate it for 24h at 30℃.

b) Liquid culture: In the aseptic operation table, pick the single colony of *S. cerevisiae* on the solid plate of YPD or aspirate the bacterial liquid of *S. cerevisiae* preserved in the glycerol tube, inoculate it into the liquid medium of

YPD according to the inoculum amount of 3%, place it into the constant temperature shaking incubator at 30℃, and cultivate it for 24h under the condition of 280rpm.

II. B.Shake flask biphasic fermentation culture of recombinant bacteria

(1) Activation of primary seed liquid: single colony of *S. cerevisiae* was picked with a sterile inoculation loop in a shaker tube containing 5mL YPD medium, and incubated for 24h at 30℃, 280rpm on a shaker to obtain primary seed liquid.

(2) Secondary seed liquid culture: primary seeds were inoculated with 5% ($v \cdot v^{-1}$) of inoculum into a shaker containing 50mL of YPD liquid medium at 30℃, 280rpm, shaking the bed for 24h, and cultivated until the early logarithmic growth to obtain the secondary seed liquid.

(3) Fermentation and inoculation: According to the inoculation ratio of 10% ($v \cdot v^{-1}$), the secondary seed liquid cultured to the early logarithmic growth was inserted into the fermentation medium, and 10% ($v \cdot v^{-1}$) concentration of extractant was added to the medium for biphasic fermentation, and cultured in a shaker at 30℃, 280rpm for 5-7d.

II. C.Experimental methods in molecular biology

(1) PCR reaction conditions and reaction system: colony PCR was performed in accordance with the instruction manual of 2×Taq PCR mix DNA polymerase from Novozymes, and the setting of PCR reaction steps and reaction system was performed in accordance with the instruction manual of Primer star Max high fidelity DNA polymerase from Takara, and the reaction conditions of Taq enzyme/Max enzyme were as follows: pre-denaturation 95℃ for 3min, denaturation 95℃ for 10s, annealing 55℃ for 15s, and extension 72℃ for 10min. 3min, denaturation 95℃ for 10s, annealing 55℃ for 15s, and extension 72℃ for 10min, in which the denaturation to extension stage needs to be repeated for 30-35 cycles.

(2) Agarose nucleic acid gel electrophoresis: weigh the appropriate amount of agarose, add 1×TAE buffer in the ratio of 1:100, and mix in a conical flask. Put it in microwave oven to heat and fully dissolve, add appropriate amount of nucleic acid stain, shake to make it uniform, and pour it into the gel tank with inserted comb. After cooling and solidifying at room temperature, add the nucleic acid samples in the spotting wells, add Marker as a reference to determine the length of different bands. After the completion of the above placed in the electrophoresis apparatus electrophoresis, waiting for 40 minutes, the gel will be placed in the gel imaging system for observation, to verify whether the sample bands are correct.

(3) One-step seamless cloning: mix the PCR-amplified target gene with the vector and add one-step cloning enzyme proportionally, react at 50℃ for 15min, and ice bath for 5min to complete the directed cloning.

(4) Plasmid extraction: *E. coli* containing the target plasmid was streaked on the plate of LB solid medium containing antibiotic Amp and incubated at 37℃ for 12 h. Single colonies were picked into the LB liquid medium containing Amp and incubated in a shaker at 37℃ for 12 h. The *E. coli* bacterial fluid was centrifuged at 8000×g for 2 min, and the supernatant was removed, and then the procedure of the plasmid extraction kit of Novozymes was strictly followed. Plasmid Extraction Kit to obtain the plasmid with high purity.

(5) Preparation of *E. coli* sensory state: Pick *E. coli* 1mL culture medium, ice bath for 25min, 4℃, centrifuge at 4000×g for 5min, remove the supernatant. Add 400μL of pre-cooled 0.1 $mol \cdot L^{-1}$ $CaCl_2$ solution, re-suspend the *E. coli* bacterial precipitate, 4°C, centrifuge at 4000×g for 5 min. remove the supernatant and add 200μL volume of pre-cooled 0.1 $mol \cdot L^{-1}$ $CaCl_2$ solution, re-suspend the *E. coli* bacterial Precipitate, centrifuge at 4000×g for 5 min at 4°C and remove the supernatant. *E. coli* receptor cells were obtained by resuspending the bacterium with 50 μL volume of pre-cooled 0.1 $mol \cdot L^{-1}$ $CaCl_2$ solution and ice bath for 12h.

(6) *S. cerevisiae* sensory preparation: *S. cerevisiae* fermentation broth with OD_{600} up to 1.5 was dispensed into sterile 2 mL EP tubes and centrifuged at 4℃, 4000×g for 5 min. yeast bodies were retained, and the bodies were washed twice with 1 mL of pre-cooled sterile water, and then re-suspended with 0.1$mol \cdot L^{-1}$ pre-cooled LiAc to blow and resuspend the bacteriophage, the made S. cerevisiae sensory state.

II. D.Transformation of Saccharomyces cerevisiae

Saccharomyces cerevisiae transformation was performed using the lithium acetate transformation method:

(1) 30 μL of 10% salmon sperm ssDNA was denatured in boiling water for 5 min and then quickly inserted into ice for use.

(2) Add to Saccharomyces cerevisiae receptor cells placed on ice in the following order:

a) 25 μL of the co-transformed fragment mixture.

b) 30 μL of denatured salmon sperm 10% single-stranded ssDNA.

c) 50% w/v PEG3350 460 μL.

d) 1 M LiAc 70μL.

(3) Centrifuge tubes were vortexed at high speed for 1 min and mixed thoroughly.

(4) Place the transformation system in a 30°C incubator for 30 min.

(5) Add 70 μL of dimethyl sulfoxide (DMSO) and gently vortex to mix well.

(6) The centrifuge tube was placed in a 42°C water bath and heat-excited for 30 min, then centrifuged at 4000 rpm for 2 min, and the supernatant was discarded. Add 400 μL of 5 mMCaCl2, gently blow to mix, let stand for 5 min, then centrifuged at 4000 rpm for 1 min, discard the supernatant. 100 μL was coated on the corresponding solid plate.

(7) Invert the culture in 30℃ constant temperature incubator for 2-4 days.

II. E. Construction of engineering strains

Multi-copy rDNA was selected as an integration site for biofuel metabolic pathway genes. A biofuel gene-related expression cassette was cloned from it using primers. Two non-transcriptomic spacer regions were then cloned from Saccharomyces cerevisiae genomic DNA using primer pairs, and the biofuel production-related gene expression cassette fragments were assembled by overlap extension PCR. The plasmids or purified DNA fragment mixtures were transferred into S. cerevisiae strains using the LiAc method.

II. F. Fermentation product extraction

The cell cultures were mixed with an equal volume of methanol (100% v/v), and the biofuel and its metabolites were extracted by high-speed vortexing. 14000 rpm centrifugation was performed for 10 min, and the samples were filtered through 0.22 μm needle filters, and the sample processing was completed and ready for use. The liquid chromatograph used was a Shimadzu LC-20A, the column was an InertSustain ODS-3 C18 (4.6×250 mm, 5 μm) column, the detector was a SPD-20A UV detector, and the gradient elution was carried out with the mobile phases of A-phase (water-1‰ trifluoroacetic acid) and B-phase (acetonitrile-1‰ trifluoroacetic acid). 0-15min for 10%-40% of B phase, 15-20min for 40%-60% of B phase and kept for 5min. 25-30min for 60%-100% of phase B, hold for 3min. volumetric flow rate: 0.5mL/min, injection volume: 10μL, column temperature: 37℃.

III. Strategies for optimizing the yield of engineered strains of biofuels

As the enormous application value of biofuels is being explored, higher demands are being placed on their yield. Its yield is generally increased in three ways, first, by optimizing the expression of enzymes related to the synthetic pathway. Second, by optimizing the supply of key precursor substances. Third, by exploring the optimal fermentation conditions.

III. A. Optimization of expression of key enzymes of the synthetic pathway

The production of biofuels using cell factories requires the synergistic action of multiple enzymes. The expression of related key enzymes will directly affect the biofuel yield. By optimizing the expression of key enzymes of the synthetic pathway, biofuel yield can be significantly increased.

III. B. Optimization of precursor substance supply

The synthesis of biofuels requires a variety of precursors, such as phosphoenolpyruvic acid and N-acetylglucosamine, and the supply of these precursors has a direct impact on the yield of biofuels. The supply of phosphoenolpyruvic acid and N-acetylglucosamine was enhanced by utilizing the glucose and malic acid synergistic utilization pathway. By knocking out the bypass metabolism genes and increasing the supply of pyruvate, the yield of biofuel was significantly improved. Optimization of precursor supply is also an important strategy to increase biofuel yield.

III. C. Optimization of fermentation conditions

The use of genetic engineering to modify the biofuel synthesis pathway is an important aspect of achieving high yields of the target products from within the strain, and optimizing the culture and fermentation conditions from the outside is also an important aspect of improving product yields. Different carbon and nitrogen sources, different dosing ratios, different dissolved oxygen levels, and different substrate addition strategies can affect product generation.

IV. Analysis of results of biofuel metabolism regulation and optimization

IV. A. Fermentation assay of S. cerevisiae strains in different environments

In order to screen the optimal fermentation conditions of S. cerevisiae strains, in this section, YDP medium containing different concentration gradients of glucose was configured according to the composition of YDP medium to optimize the fermentation conditions of S. cerevisiae strains.The volume of YDP liquid medium was 30 mL, and

the concentration gradients of glucose were 0g/L, 10g/L, 20g/L, 30g/L , 40g/L, and the concentration of other components in the medium was strictly consistent to exclude the influence of other substances on the fermentation of S. cerevisiae strains. The fermentation duration was 48h, and samples were taken every 6h.

The OD_{600} measured values of S. cerevisiae strains under different glucose concentration gradients are shown in Table 1. As can be seen from the table, the fermentation of S. cerevisiae strains increased with the increase of glucose concentration when the glucose concentration in the medium was from 0 to 30 g/L. The fermentation of S. cerevisiae strains at 30 g/L glucose concentration was the best, with the highest OD_{600} value reaching 2.897. The OD_{600} values of S. cerevisiae strains at the concentrations of 0 and 40 g/L were the best, with the highest OD_{600} value reaching 2.897. The OD_{600} values of S. cerevisiae strains at the concentrations of 0 and 40 g/L were the best. The fermentation of S. cerevisiae strains at 0g/L and 40g/L concentrations were close to each other, showing lower OD_{600} values. This is due to the fact that the growth of S. cerevisiae strain lacked the main carbon source at 0g/L concentration, and it was difficult to maintain a large amount of bacterial growth by the trace carbon source provided by other substances. The 40g/L concentration resulted in a high osmotic pressure of the medium, which would inhibit the growth of the bacteria and even lead to death.

Table 1: OD_{600} of different glucose concentration gradient

Time (h)	OD_{600}				
	0 g/L	10 g/L	20 g/L	30 g/L	40 g/L
0	0.000	0.000	0.000	0.000	0.000
6	0.139	0.216	0.354	0.486	0.268
12	0.351	0.492	0.643	0.902	0.357
18	0.573	0.714	1.136	1.430	0.456
24	0.767	0.931	1.524	1.806	0.538
30	0.867	1.130	1.858	2.098	0.755
36	0.949	1.283	2.076	2.368	0.943
42	1.043	1.371	2.210	2.591	1.074
48	1.102	1.448	2.333	2.897	1.166

Neutral lipids are the main feedstock for the production of biofuels, and in order to study the lipid composition and biofuel production and biomass of S. cerevisiae strains at different glucose concentrations, total lipids in the fermentation broths of S. cerevisiae strains were extracted in this section, and the lipid composition was calculated using total ion flow chromatography. Where lipid composition is the percentage of lipids in the biofuel produced by the strain.

Figure 1 shows the results of changes in biomass, lipid production, and lipid composition of the strain at different glucose concentrations. From the figure, it can be seen that within a certain range, high concentrations of glucose had a very significant enhancement of cell growth and lipid accumulation, as well as a substantial reduction in the growth cycle. Among them, 30 g/L glucose culture yielded the highest biomass of 3670.81 mg/L and more than 55% lipid content as well as 2366.52 mg/L biofuel yield. This experiment further verified that the optimization of fermentation conditions has a promoting effect on the metabolic regulation of biofuels.

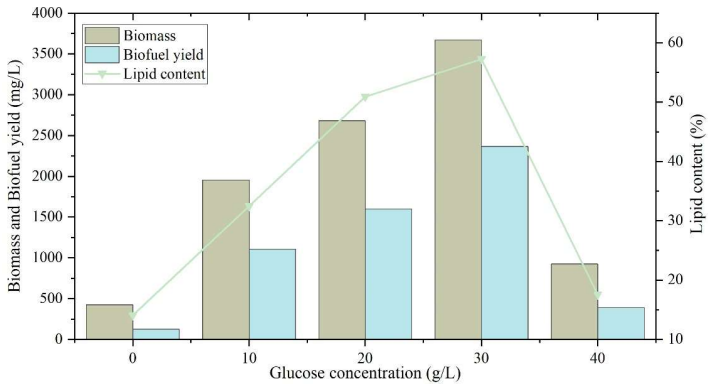

Figure 1: The results of biomass, lipid production and lipid composition

IV. B. Biofuel accumulation under precursor supply

In this section, different biofuel prerequisite substances, i.e., 50 mM phosphoenolpyruvic acid (PEP) and N-acetylglucosaminoglucose (NAG), were added to the YDP medium to analyze the biofuel conversion rate in the fermentation of S. cerevisiae strains. The biofuel yield in the fermentation broth was examined by gas chromatography.

The biofuel production of S. cerevisiae strain under the prerequisite substances of phosphoenolpyruvic acid and N-acetylglucosamine is shown in Table 2. As can be seen from the table, without the addition of precursor substances, S. cerevisiae strain showed the lowest biofuel accumulation and the highest biofuel production was only 47.851 mg/L. With the addition of phosphoenolpyruvic acid S. cerevisiae strain showed the highest biofuel production of 202.545 mg/L. While with the addition of N-acetylglucosamine the maximum biofuel yield of S. cerevisiae strain was also increased to 186.595 mg/L. Thus, it can be seen that the precursor supply increased the maximum biofuel yield of the strain by 2.8-3.3 times, which can greatly improve the conversion of biofuel.

Table 2: Results of biofuel production

Time (h)	Biofuel production (mg/L)		
	No precursor	Add PEP	Add NAG
0	0.000	0.000	0.000
6	21.833	48.303	38.235
12	27.715	63.801	50.113
18	30.939	84.785	69.287
24	33.654	115.385	95.305
30	37.783	129.072	118.552
36	40.045	150.509	139.140
42	44.174	179.299	160.577
48	47.851	202.545	186.595

IV. C. Effect of synthesizing key enzymes on biofuel production performance

The ultimate goal of constructing recombinant strains for biofuels is biofuel production, and the main biofuel fermentation methods are stepwise saccharification co-fermentation (SHCF) and synchronized saccharification co-fermentation (SSCF). In this section, we utilized the simultaneous saccharification co-fermentation method and constructed recombinant strains of S. cerevisiae through the optimized metabolic regulation of key enzymes, and compared the fermentation performances of the original S. cerevisiae strain and the recombinant strains in the production of biofuels in the fermentation feedstocks, such as wheat stover, maize stover, withered grass, ogi and rice straw.

Table 3: Biofuel fermentation performance under different raw materials

Strain	Raw material	Biofuel production (g/L)	Yield (%)
Recombination S. cerevisiae	Wheat stalk	52.55	73.42
	Straw stalk	42.31	84.19
	Dead grass	55.72	82.89
	Gidge	63.12	75.81
	Straw	46.26	86.91
Primordial S. cerevisiae	Wheat stalk	46.51	64.98
	Straw stalk	35.38	70.40
	Dead grass	50.48	75.09
	Gidge	54.51	65.47
	Straw	36.64	68.84

Table 3 shows the biofuel fermentation performance of the strains under different feedstocks. As can be seen from the table, the recombinant S. cerevisiae strain, which was metabolically regulated and optimized, had higher biofuel production and yield than the original S. cerevisiae strain under different feedstocks. The reconstituted strain obtained 52.55 g/L of biofuel from SSCF using wheat straw hydrolysis products as feedstock and the biofuel yield exceeded 70% of the theoretical yield. The biofuel yield was as high as 86.91% when rice straw hydrolysate was utilized for SSCF. Under the same conditions, the strains with metabolic regulation and optimization had stronger

fermentation performance, and the SSCF process realized that the material hydrolysis and biofuel fermentation processes were carried out in the same reactor, which could improve the intensity of biofuel production to a certain extent.

V. Conclusion

Metabolic regulation and optimal design are effective ways to improve biofuel yield. Saccharomyces cerevisiae, as a host cell, significantly increased the biofuel yield by optimizing the fermentation conditions, precursor material supply and synthetic key enzyme expression. The optimization experiments of fermentation conditions showed that the best growth state of Saccharomyces cerevisiae was achieved at a glucose concentration of 30 g/L, when the biomass reached 3670.81 mg/L, the lipid content was more than 55%, and the biofuel yield reached 2366.52 mg/L. The optimization experiments of precursor supply confirmed that the addition of phosphoenolpyruvic acid (PEP) and N-acetylglucosamine (NAG) could promote the biofuel accumulation, in which the addition of PEP made the biofuel production more efficient and the biofuel yield was higher than that of NAG. Fuel accumulation, in which the addition of PEP increased the biofuel yield to 202.545 mg/L, which was 3.3 times higher than that of the group without precursor addition. The optimization of key enzymes of the synthetic pathway showed that the recombinant strain with optimized metabolic regulation had stronger fermentation performance than the original strain, and the biofuel yield could reach 52.55g/L with 73.42% when wheat straw was used as the feedstock, and the yield was as high as 86.91% when rice straw was used as the feedstock. These results provide an important reference for the construction of efficient biofuel production process, and also provide new ideas and methods for the development of bioenergy field.

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