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Polyhydroxybutyrate (PHB) Biosynthesis by an Engineered *Yarrowia lipolytica* Strain Using Co-Substrate Strategy

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Abstract: High production cost is one of the major factors that limit the market growth of *polyhydroxyalkanoates* (PHAs) as a biopolymer. Improving PHA synthesis performance and utilizing low-grade feedstocks are two logical strategies for reducing costs. As an oleaginous yeast, *Y. lipolytica* has a high carbon flux through acetyl-CoA (the main PHB precursor), which makes it a desired cell factory for PHB biosynthesis. In the current study, two different metabolic pathways (*NBC* and *ABC*) were introduced into *Y. lipolytica PO1f* for synthesizing PHB. Compared to the *ABC* pathway, the *NBC* pathway, which includes *NphT7* to redirect the lipogenesis pathway and catalyze acetoacetyl-CoA synthesis in a more energy-favored reaction, led to PHB accumulation of up to 11% of cell dry weight (CDW), whereas the *ABC* pathway resulted in non-detectable accumulations of PHB. Further modifications of the strain with the *NBC* pathway through peroxisomal compartmentalization and gene dose overexpression reached 41% PHB of CDW and a growth rate of 0.227 h^{-1}. A low growth rate was observed with acetate as the sole source of carbon and energy or glucose as the sole substrate at high concentrations. Using a co-substrate strategy helped overcoming the inhibitory and toxic effects of both substrates. Cultivating the engineered strain in the optimal co-substrate condition predicted by response surface methodology (*RSM*) led to 83.4 g/L of biomass concentration and 31.7 g/L of PHB. These results offer insight into a more cost-effective production of PHB with engineered *Y. lipolytica*.

Keywords: acetate; gene dosage effect; acetoacetyl-CoA synthase; compartmentalization; substrate inhibition

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1. Introduction

The increasing dependence on plastics has led to the generation of large amounts of plastic waste. This underscores the urgent need for biodegradable material as a sustainable replacement for petroleum-based plastics [1]. There are various alternatives to fossil-fuel-derived plastics, such as polylactic acid (PLA), bio-polyethylene, *polyhydroxyalkanoates* (PHAs), and protein-derived bioplastics [2]. PHAs are a promising option due to several advantages and special characteristics. For instance, these polymers have elastic and crystalline properties similar to petroleum-based plastics such as polystyrene and polyethylene. Their glass-to-rubber transition temperature (T_g) is commonly lower than 0 °C, and their melting point (T_m) is higher than 120 °C in most cases. PHAs' tensile strength is generally higher than 20 Mpa [3]. These characteristics qualify PHAs as a potential replacement for common plastics in the packaging industry.

Among different types of PHAs, polyhydroxybutyrate (PHB) is a naturally occurring polymer produced by many different bacterial and archaea species. The recognized advantages and special characteristics of PHB include sustainability, biocompatibility (non-toxic), biodegradability, versatility (using injection molding, extrusion, and film blowing as conventional techniques in plastic processing), customizability (possibility of modifying characteristics such as flexibility, tensile strength, and degradation rate), good resistance to

UV radiations, and insolubility in water. It is therefore considered to be one of the most promising alternatives for the current plastic market [4], with different applications ranging from food packaging to textiles, cosmetics, and biomedical implants.

Some microbial species that are widely studied as natural producers of PHB include *Cupriavidus necator* (formerly *Ralstonia eutropha*), *Azotobacter vinelandii*, *Aeromonas hydrophila*, and *Pseudomonas putida* [5]. To be a suitable microbial PHB cell factory, the selected species preferably has the following properties: first, it should be a generally recognized as safe (GRAS) microorganism; second, it should be a fast-growing organism with significant PHB accumulation; and third, it should be able to use different and low-cost substrates [6]. However, among the PHB natural producers, very few have all these characteristics.

These challenges relying on natural PHB producers have led to various attempts using the genetic modification of some robust GRAS microbial cell factories such as *Saccharomyces cerevisiae*, *Pichia pastoris*, *Corynebacterium glutamicum*, *Yarrowia lipolytica*, and *Zymomonas mobilis* [7]. Among these potential cell factories, *Y. lipolytica* is uniquely advantageous for PHB biosynthesis. In addition to being a GRAS microorganism, *Y. lipolytica* grows relatively fast with a doubling time of 1.7 h [8] and can be cultivated at various scales. Such a fast growth can avoid bacteriophage attacks at industrial levels. Moreover, *Y. lipolytica* inhibits tolerance to inhibitors and pH stresses, and forms filamentous cells at a late growth stage that facilitate gravity separation [9].

Y. lipolytica is an oleaginous yeast with considerable potential for producing different biochemicals on nutrient-limited media. *Y. lipolytica* has a high flux through acetyl-CoA. This makes it a desired cell factory for molecules that require acetyl-CoA as an intermediate. These products include TGs, carotenoids, fatty acid derivatives, organic acids, and PHAs [10]. To use its unique characteristics, some efforts were made to genetically modify *Y. lipolytica* to produce PHB. Generally, two strategies have been used to design PHB biosynthesis pathways in *Y. lipolytica*. First, the *ABC* pathway consists of three genes: *phaA*, *phaB*, and *phaC*. As a result of applying the *ABC* pathway from *Ralstonia eutropha* to *Y. lipolytica*, *Li* and colleagues successfully produced PHB at up to 10.2% of cell dry weight (CDW) when utilizing glucose as a substrate [11]. The second strategy, however, is to introduce only the *phaC* gene, because other PHB precursors, such as acetoacetyl-CoA, are naturally synthesized by *Y. lipolytica*. Using this strategy, in another study, the *phaC* gene from *Pseudomonas aeruginosa* contained a *PTS1* peroxisomal signal introduced to *Y. lipolytica*. It resulted in an mcl-PHA accumulation of up to 5% of CDW [12]. Similar results were observed by *Rigouin* and colleagues using the mutagenesis of the *phaC* enzyme (with E130D, S325T, S477R, and Q481M mutations), which led to an mcl-PHA accumulation of 25% of CDW (*w*/*w*). This study showed that specific mutations in *PhaC* can enhance the PHA titer and increase the length of the produced polymer, or a combination of both effects, when expressed in bacteria [13].

Despite all these efforts, the modified strains still have a low PHB titer, which is the major bottleneck in the scale-up process. A main reason for this is that *Y. lipolytica* has a robust flux toward the lipogenesis pathway, which leads to a high lipid accumulation rather than an accumulation of PHB [14]. Therefore, harnessing the lipogenesis pathway toward PHB precursors seems to be an effective strategy to increase the PHB titer in *Y. lipolytica*. Studies of redirecting malonyl-CoA (the main intermediate for fatty acid synthesis) into PHB biosynthesis showed a higher PHB titer in mesophyll cells compared with the *ABC* pathway in sugarcane. The *NBC* pathway (including the *NphT7*, *phaB*, and *phaC* genes) redirects the lipogenesis pathway to PHB biosynthesis by catalyzing the condensation reaction of malonyl-CoA to acetoacetyl-CoA. In contrast to *PhaA*, which conducts the condensation reaction reversibly, *NphT7* catalyzes the condensation reaction irreversibly, resulting in a higher accumulation of PHB [15].

The compartmentalization of enzymes into organelles is a promising strategy for limiting metabolic crosstalk and improving pathway efficiency. Indeed, the main concern in microbial metabolic engineering for optimizing production in modified strains is controlling such crosstalk between the high flux of the introduced pathway and the native

metabolic pathways in the host cell. This challenge could be addressed in eukaryotic cells by partitioning introduced pathways into membrane-bound subcellular organelles. An organelle should import heterologous enzymes in order to compartmentalize heterologous metabolic pathways. Peroxisome is primarily involved in the β-oxidation of long-chain fatty acids, which leads to a high acetyl-CoA flux in this organelle. As a result, peroxisomes may be a potential organelle for compartmentalizing the *NBC* pathway in order to redirect lipogenesis towards the accumulation of PHB. To import proteins from the cytosol, the peroxisomal matrix generally uses one of the PTS1 or PTS2 targeting signals recognized by its receptor proteins. Most proteins import into the peroxisome via the PTS1 tag, which consists of serine-lysine-leucine (*SKL*) amino acids at the carboxy-terminus [16].

Another bottleneck in producing PHB from modified *Y. lipolytica* is selecting a cost-effective and sustainable substrate for large-scale fermentation. Indeed, several efforts have been made to develop PHB biosynthesis using metabolically engineered *Y. lipolytica* strains. In most cases, glucose is used as a substrate. Also, several studies showed glycerol as another preferred substrate for producing biodiesel by *Y. lipolytica* [17].

However, there are many other substrates to consider as glucose and glycerol alternatives. These substrates should be considered not only due to their low cost but also for other benefits such as sustainability and better conversion to products of interest [18]. Acetic acid and other organic acids derived from waste material are among the promising candidates. Although acetic acid inhibits many microorganisms, including *E. coli* and *S. cerevisiae*, *Y. lipolytica*, on the other hand, can readily uptake it as a substrate. This ability of *Y. lipolytica* to tolerate relatively high concentrations of volatile fatty acids (VFAs) makes it possible to utilize a variety of wastes by converting these wastes into VFAs through acidogenic fermentation (the VFA route) and utilizing them as substrates for *Y. lipolytica* [19]. Incorporating waste as feedstock to produce biodegradable plastics would provide the most sustainable solution to the plastic production dilemma [20]. Using acetic acid (a major form of VFAs) as the only carbon source will, however, not satisfy the industrial demand for rapid PHA production. The main reason for this deficiency is the unbalanced reductive agents (as NADPH) and energy carriers (as ATP) produced during the metabolic pathway of acetic acid [21]. Compared to VFAs, glucose showed a noticeably higher growth rate and biomass productivity. Consequently, co-substrate utilization (glucose and VFAs) has been proposed as a reliable, efficient, and viable method of producing PHAs [22].

In the current study, the *ABC* and *NBC* pathways were introduced into *Y. lipolytica*. The resulting strains were compared for PHB biosynthesis. After assessing the efficiency of each pathway on PHB production, the gene dosage effect and compartmentalization strategies were implemented to further enhance the PHB titer. Also, a co-substrate strategy was implemented using acetate as a cost-effective substrate supplemented with glucose. The strategy was evaluated for the effect of each substrate and their mix on growth rate and biomass PHB content. These results led to an optimized co-substrate medium for PHB production using the engineered strain. It is expected that this study will provide significant insights into the metabolic engineering of *Y. lipolytica* for PHB biosynthesis.

2. Materials and Methods

2.1. Strain Construction

The auxotrophic strain *Y. lipolytica PO1f* (ATCC MYA-2613) was chosen as a microbial host for the introduced pathway. *E. coli DH5α* was employed for cloning the genes and amplifying the constructed shuttle vectors. To develop constructs, single genes were amplified using PCR and cloned by cloning vectors in *E. coli*. *R. eutropha* genomic DNA was used as a PCR template for the *phaA*, *phaB*, and *bktB* genes. *Pseudomonas* sp. and *Streptomyces* sp. genomic DNA were used for amplifying the *phaC1437* and *NphT7* genes, respectively. Table 1 shows the primers used for PCR reactions. Cloned genes were assembled in *E. coli*–*Y. lipolytica* shuttle vectors. Assembled cassettes included genes, biomarkers, and both well-characterized promoter (TEF1, GPM, and FBA) and terminator (XPR2, OCT1, and LIP1) elements. In the next step, the existence of the target genes within the expression

cassettes and the integration of the constructed expression cassettes into the *Y. lipolytica* genome were verified with colony PCR and markers.

Table 1. Oligonucleotides used as primers.

Primers	Sequences (5′–3′)	GC (%)	Length (Mer)	Molecular Mass (Da)
phaA-F	CCCGGGTTACTTTCTCTCGACGGCC	64	25	7560.9
phaA-R	AAGCTTATGACCGACGTGGTGATCGTGTCC	53	30	9238.1
phaB-F	CCCGGGAGAGTCGACCTGCAG-TTAGCCCATGTGC	65	34	10,444.8
phaB-R	AAGCTTATGACCCAGCGAATCGCCTAC-GTCACC	55	33	10,027.5
phaC1437-F	CCCGGGTTATCGCTCGTGGACGTAAGTGCC	63	30	9215.0
phaC1437-R	AAGCTTATGTCTAACAAGTCTAACGAC-GAGC	42	31	9512.3
NphT7-F	CCCGGGTTACCACTCAATCAGGG-CAAAGGAAGC	58	33	10,141.6
NphT7-R	AAGCTTACTGATGTGCGAT-TTCGAATTATTGGAAC	37	35	10,800.1
bktB-F	AAGCTTATGACCCGA-GAGGTGGTGGTCGTGTCCGG	60	35	10,884.1
bktB-R	CCCGGGTTAAATTCGTTCGAAGATGGC-GGCAATGCC	56	36	11,101.3
phaC1437-SKL-F	AAGCTTATGTCTAACAAGTCTAACGAC-GAGCTGAAGTACC	43	40	12,297.1
phaC1437-SKL-R	CCCGGGTTACAGCTTAGATCGCTCGTGG-ACGTAAGTGCCAGG	60	42	12,971.4

To cultivate and screen the transformed *E. coli* for constructed plasmids, *Luria Broth* (*LB*) medium (5 g/L yeast extract, 10 g/L *Bacto* tryptone, and 10 g/L NaCl) at 37 °C was used with ampicillin as a marker. Synthetic yeast nitrogen base (YNB) media consisting of 20 g/L glucose as the carbon source at 28 °C was used for screening the transformed *PO1f* host strain. Transformants were selected on a defined medium containing 6.7 g/L YNB without amino acids. In each step, the medium was supplemented with 0.77 g/L CSM-Ura (complete supplement mixture minus uracil, US-Biological, USA), 0.67 g/L CSM-leucine, and 0.3 g/L hygromycin [11,23,24] glucose, based on the introduced vector.

Y. lipolytica wild-type strains cannot synthesize PHB naturally. However, there are two common strategies for introducing the PHB biosynthesis pathway to non-natural PHB producer organisms. To evaluate the efficiency of these pathways in *Y. lipolytica*, two vectors were designed for expressing the *ABC* pathway and *NBC* pathway. These expression cassettes contained fragments (promoters, terminators, and other regulatory elements). The only difference was that the *ABC* cassette contained the *phaA* gene, which expressed β-ketothiolase (*pZX22*), while the *NBC* cassette contained the *NphT7* gene (*pZX30*). The list of constructed vectors used in this study is provided in Table 2, along with the promoter and terminator used for each gene and the copy number of heterologous genes in recombinant strains.

To obtain the final strain, *Y. lipolytica PO1f* was transformed by introducing the *NBC* pathway genes: *phaB* (from *R. eutropha*); *phaC1437*, which is a mutant of *phaC* encoded by *Pseudomonas* sp.; and the *NphT7* (acetoacetyl-CoA synthase) gene from *Streptomyces* sp. [11]. The modified strain was subjected to sequential transformations using integrative vectors. These modifications include increasing the copy number of *NBC* pathway genes [25]. Also, the *bktB* (β-ketothiolase) gene from *R. eutropha* was introduced to the modified strain to catalyze the condensation of acyl-CoA and acetyl-CoA into 3-ketoacyl-CoA molecules for copolymer synthesis in the modified strain [26].

Table 2. Development of gene constructs with shuttle vectors for biosynthesis of PHB.

Plasmids	Plasmid Backbone	Selective Marker	Genes Cassettes (Promoter–Gene–Terminator)	Recombinant Strains Gene: Copy Number		
				pZX22 (*NBC* Pathway)	*pZX30* (*ABC* Pathway)	PHB32
pCV35	*pJN34*	*Leucine*	P_{TEF}-*NphT7*-T_{XPR2}, P_{GMP}-*PhaC1437*-T_{OCT1}, P_{FBA}-*PhaB*-T_{LIP1}	*phaB*: 1, *phaC1437*: 1, *NphT7*: 1	*phaA*: 1, *phaB*: 1, *phaC1437*: 1	*phaB*: 3, *phaC1437*: 2, *NphT7*: 2, *phaC1437-SKL*: 1, *bktB*: 1
pCV88	*pJN34*	*Uracil*	P_{TEF}-*NphT7*-T_{XPR2}, P_{GMP}-*PhaC1437*-T_{OCT1}, P_{FBA}-*PhaB*-T_{LIP1}			
pCV342	*pJN44*	*Hygromycin*	P_{TEF1N}-*PhaB*-T_{XPR2}, P_{TEF1N}-*PhaC1437_SKL*-T_{XPR2}, P_{TEF1N}-*bktB*-T_{XPR2}			
pZX22	*pBR322*	*Leucine*	P_{TEF}-*NphT7*-T_{XPR2}, P_{GMP}-*PhaC1437*-T_{OCT1}, P_{FBA}-*PhaB*-T_{LIP1}			
pZX30	*pBR322*	*Leucine*	P_{TEF}-*PhaA*-T_{XPR2}, P_{GMP}-*PhaC1437*-T_{OCT1}, P_{FBA}-*PhaB*-T_{LIP1}			

To compartmentalize PHB synthesis in peroxisome organelles, PHB synthase was tagged with *SKL* tripeptide at its C-terminal [16]. Figure 1A shows the constructed pathway map. These vectors are named *pCV35*, *pCV88*, and *pCV342*, which are shown in Figure 1B–D, respectively [27,28]. During a sequential transformation, *Y. lipolytica PO1f* was integrated with *pCV35* (P_{TEF}-*NphT7*-T_{xpr2}, P_{GMP}-*PhaC1437*-T_{oct1}, P_{FBA}-*PhaB*-T_{lip1}, and *leu2* maker), and the resulting strain was transformed with the linearized plasmid, *pCV88* (P_{TEF}-*NphT7*-T_{XPR2}, P_{GMP}-*PhaC1437*-T_{OCT1}, P_{FBA}-*PhaB*-T_{LIP1}, and *ura3* maker). This strain was subjected to future transformation by the linearized plasmid *pCV342* (P_{TEF1N}-*PhaB*-T_{xpr2}, P_{TEF1N}-*PhaC1437_SKL*-T_{xpr2}, P_{TEF1N}-*bktB*-T_{xpr2}, and *hph* marker). Therefore, there were two copies of *PhaC1437*, one copy of *PhaC1437*_SKL, two copies of *NphT7*, two copies of *PhaB*, and one copy of *bktB* in *Y. lipolytica* PHB32 as the final strain.

2.2. Cultures for Y. lipolytica Cultivation and PHA Production

Inoculants were prepared from single colonies in 25 mL of YPD medium containing 10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose (dextrose) and incubated for 48 h at 28 °C with 150 rpm agitation. YNB medium was used for cell growth and PHB production in flask experiments. The YNB medium contained 1.7 g/L YNB without AA and 5 g/L NH_4Cl. Seven different levels of glucose (0, 5, 10, 20, 50, 100, and 150 g/L) and five different levels of sodium acetate (0, 10, 20, 50, and 100 g/L) were tested as substrates. Then, 250 mL flasks were inoculated with 0.5% (*v*/*v*) of prepared seed of the engineered strain and incubated at 28 °C for 96 h. To produce PHB in the fermenter, a 1 L bioreactor was employed. The base medium was YPD, and the bioreactor operated at optimum concentrations of glucose (50 g/L) and sodium acetate (20 g/L). The bioreactor was inoculated with 1% (*v*/*v*) prepared seeds, and fermentation was performed at 28 °C at a constant pH of 7. After 24 h of batch culture with increased cell concentration, the bioreactor was switched to fed-batch culture. YPD containing glucose and sodium acetate was continuously injected into the bioreactor during this phase. This fed-batch system was maintained with glucose and sodium acetate at their optimum concentrations for 48 h. Filtered air was sparged into the bioreactor at a flow rate of 2.5 L/min. To maintain the air saturation at 20%, flat blades were used with an adjusted agitation rate between 150 to 500 rpm.

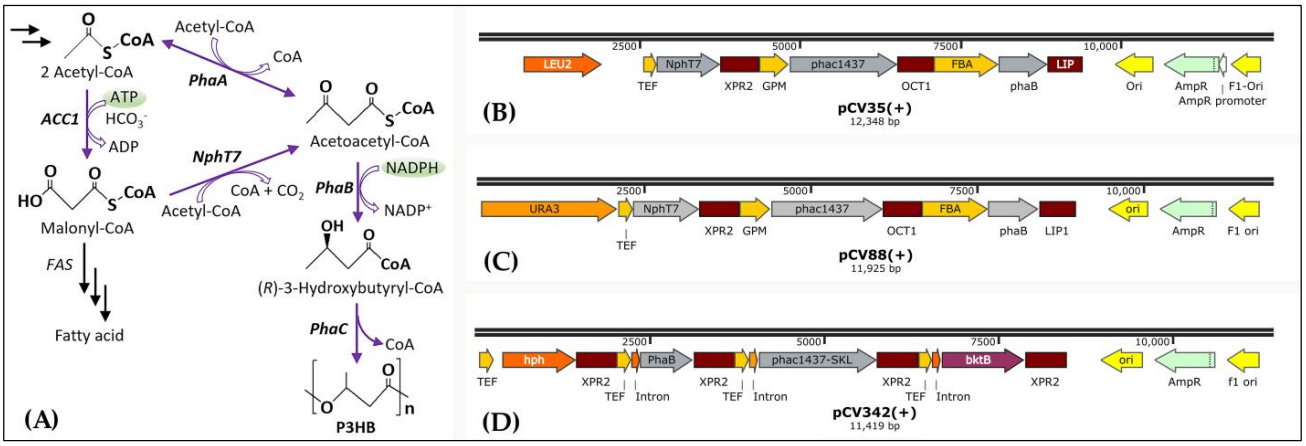

Figure 1. (**A**) Designed pathways (*ABC* and *NBC*) for producing PHB from acetyl-CoA as the main precursor. In *ABC* pathway, *PhaC* catalyzes a reversible reaction for condensing two acetyl-CoA molecules to produce acetoacetyl-CoA, while in *NBC* pathway, acetoacetyl-CoA is synthesized during an irreversible reaction catalyzed by *NphT7*. (**B**) Map of *pCV35* shuttle vector containing *NphT7*, *phaC1437*, and *phaB* genes; LEU as auxotrophic biomarker; TEF, GPM, and FBA promoters; and XPR2, OCT1, and LIP terminators. (**C**) Map of *pCV88* shuttle vector having similar genes, promoters, and terminators as *pCV35*, with URA3 as auxotrophic biomarker. (**D**) Map of *pCV342* shuttle vector containing *phaB*, *phaC1437*-SKL, and *bktB* genes; hygromycin as antibiotic biomarker; TEF promoter; and XPR2 terminator.

2.3. Growth Rate and PHB Content Analysis

To detect and quantify the growth rate of the engineered strains, the optical density (OD) of the culture at 600 nm was used to determine the cell dry weight (CDW). To measure the intracellular PHB content, 2 mL of culture was centrifuged at 5000× *g* for 5 min. Then, the pellets were washed with deionized water and dried at 55 °C overnight. The dried biomass was digested using a methanolysis solution containing 97% methanol and 3% H_2SO_4, 18M (*v*/*v*) to determine PHB content [11]. During the transesterification reaction, PHB polymers were monomerized to butanoic acid, 3hydroxy-, and methyl ester.

As a brief description, 1.5 mL of methanolysis solution and 3 mL of chloroform were added to each sample and then incubated at 95 °C for at least 6 h. After cooling the samples to room temperature, 1.5 mL deionized water was added to each sample, which created two layers. The upper layer contained water, methanol, and hydrophilic cell components, and the lower layer contained chloroform and hydrophobic cell components, including digested PHB (Figure 2). The separated chloroform layer was analyzed by an Agilent 7890A gas chromatograph (Santa Clara, CA, USA) equipped with an HP-5MS 30 m × 250 µm × 0.25 µm column. It was coupled with a 5975C mass detector. The temperature was held at 40 °C for 3 min, then ramped from 40 °C to 280 °C at 10 °C min^{-1} and held for 10 min. A standard curve was drawn using an external standard (which includes 20, 10, 5, 2, and 1 mg/mL of PHB in chloroform). By eliminating the drying step, the described method was used to prepare the external standards.

2.4. Statistical Analysis

For each designed experiment, data were collected from three biologically independent experiments. *SAS*® 9.4M7 software was applied to analyze the experimental data and calculate parameters such as mean value, standard deviation, and standard errors. Also, to evaluate the synergistic effects of both glucose and sodium acetate and the effects of their high concentrations on *Y. lipolytica* growth and PHB content, the response surface methodology (*RSM*) analysis was performed [29]. *RSM* analysis was conducted via the *SAS RSREG* procedure [30]. For statistical analysis, linear, second-degree polynomials and quadratic models were applied to describe the relationship between the input variables and

the responses. Two-factor ANOVA analysis was conducted to determine the interaction of two independent variables (glucose and sodium acetate) on the responses (growth rate and PHB content). Using the *SAS GML* procedure, the *F*-value was calculated to detect interactions between the variables. In this analysis, two factors were considered as cross factors, and a completely randomized design (*CRD*) was used for evaluating the interactions between these two factors.

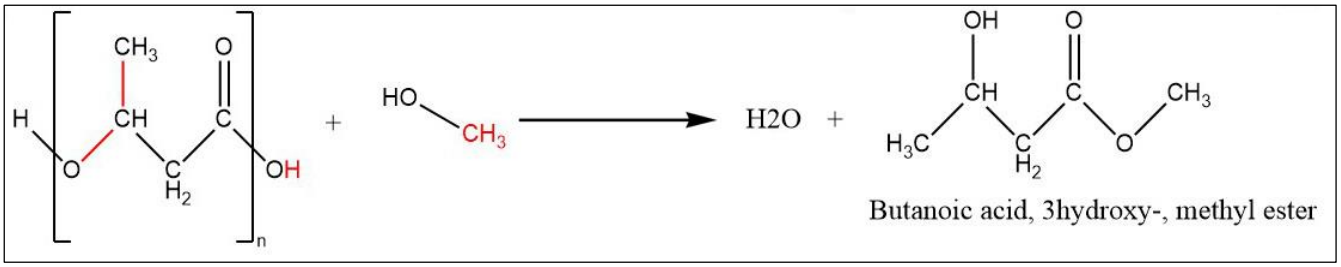

Figure 2. Transesterification of PHB polymer using methanolysis solution.

3. Results

3.1. Metabolic Pathway Design

A comparison of the results of the *ABC* pathway (Figure 3A) and *NBC* pathway (Figure 3B) showed that redirecting the lipogenesis pathway toward PHB production using the *NBC* pathway is a more effective strategy. As Figure 3C shows, cultivating the transformed *PO1f* strain with the *pZX30* vector (in YPD media with 30 g/L glucose) led to an accumulation of PHB up to 11% of CDW. However, there was no detectable PHB synthesis in the transformed strain with the *pZX22* plasmid. Therefore, the *NBC* pathway was selected as an efficient design for PHB biosynthesis in *Y. lipolytica*. One reason for the high efficiency of the *NBC* pathway in *Y. lipolytica* is the high carbon flux toward the lipogenesis pathway in this yeast. This characteristic resulted in the significant synthesis of malonyl-CoA as a main intermediate in fatty acid production [31]. Introducing *NphT7* redirects the *Y. lipolytica* metabolic pathway from lipogenesis to producing more acetoacetyl-CoA as a PHB precursor. However, no natural replicative vectors (episomes) have been observed in *Y. lipolytica*. Therefore, to make impermanent the expression of *ABC* and *NBC* pathways in this yeast, chromosomal centromere (cEN) fragments were added to the constructed plasmids (*pZX22* and *pZX30*) [32].

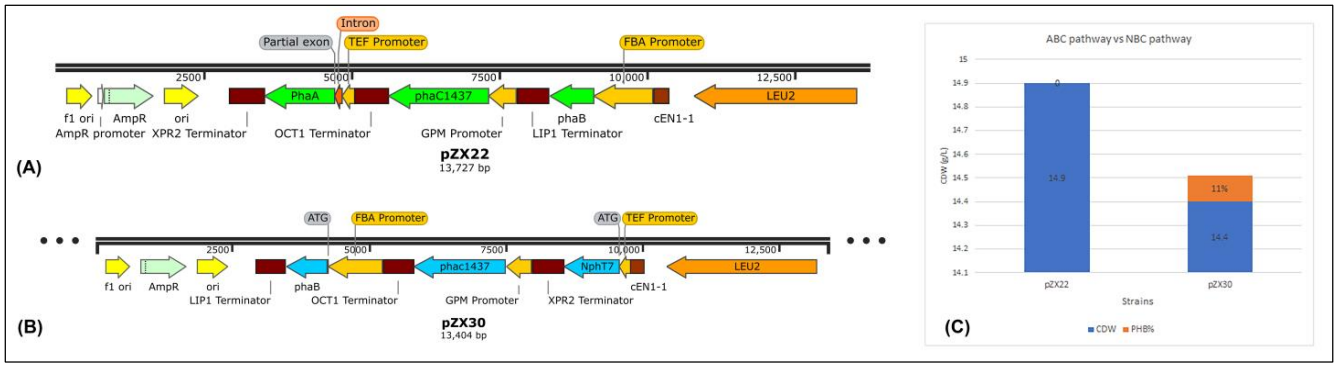

Figure 3. (**A**) Map of *pZX22* vector containing *ABC* pathway; (**B**) map of *pZX30* vector containing *NBC* pathway; (**C**) comparing biomass concentration and PHB accumulation in modified *Y. lipolytica* strains using *ABC* and *NBC* pathways.

The enzymatic reactions employed to catalyze acetoacetyl-CoA synthesis are the main difference between these pathways. Acetoacetyl-CoA synthesis is usually catalyzed by acetoacetyl-CoA thiolase via the condensation of two acetyl-CoA molecules [33]. In

the *ABC* pathway, *phaA*, *phaB*, and *phaC* are involved in PHB biosynthesis. *PhaA* (β-ketothiolase) catalyzes the acetyl-CoA condensation reaction to produce acetoacetyl-CoA as a precursor for PHB biosynthesis [11]. However, *McQualter* and colleagues [15] found that using the acetoacetyl-CoA synthase instead of a β-ketothiolase enhances PHB production in transgenic sugarcane. Their study showed that introducing the *NBC* PHB pathway (containing *NphT7*, *phaB*, and *phaC*) instead of the *ABC* PHB pathway into the sugarcane plant increased the PHB content in mesophyll cells by up to 11.8% of its CDW. It was more than twice the *ABC* pathway. Other studies showed that *PhaA* preferentially catalyzed thiolysis (breaking down acetoacetyl-CoA to acetyl-CoA) compared to the synthesis of acetoacetyl-CoA [34].

On the other hand, *NphT7* catalyzes acetoacetyl-CoA synthesis in an more energy-favored reaction [33]. Indeed, the high equilibrium constant value (Km) of β-ketothiolase to acetyl-CoA (up to 1.1 mM) compared to the low Km value of *NphT7* to acetyl-CoA (0.068 mM) and malonyl-CoA (0.028 mM) showed that *NphT7* was a much more effective enzyme for initiating the condensation reaction for producing acetoacetyl-CoA compared to *PhaA* [15]. On the other hand, *PhaA* catalytic behavior is highly reversible. This leads to the high tendency of this enzyme to catalyze the thiolysis reaction and break down the acetoacetyl-CoA into two acetyl-CoA molecules, while the *NphT7* enzyme exhibited more irreversible behavior by catalyzing the condensation reaction [35].

The resulting strain from sequential transformation (by *pCV35*, *pCV88*, and *pCV342* plasmids) showed a significant increase in PHB content compared to the strain modified by *pZX30*, without any detectable effect on the growth rate (Figure 4A). The sequential transformation led to a strain capable of accumulating PHB at up to 41% of CDW, which is remarkably higher than the 11% PHB titer in the *pZX30* strain (Figure 4B).

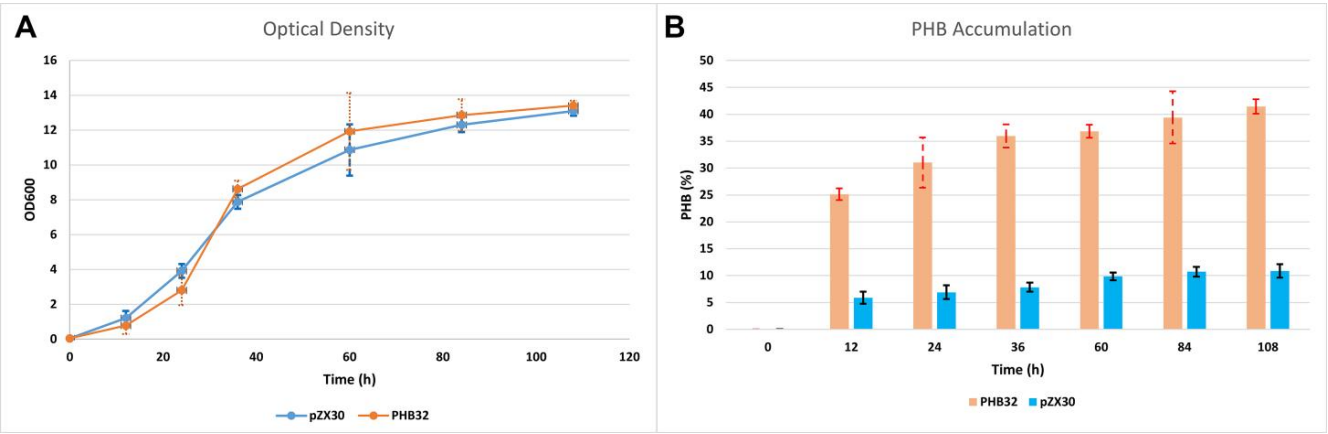

Figure 4. (**A**) Comparing the growth and (**B**) PHB content in strain *pZX30* (containing only *NBC* pathway) and strain PHB32 (result of sequential transformation) cultivated on YNB containing 50 g/L glucose.

Previous studies proved the gene dosage effect on desired gene expression in *Y. lipolytica* host cells. Gene dosage refers to the number of copies of a particular gene present in an organism's genome. The gene dosage effect relates to how changes in the number of copies of a gene influence an organism's phenotype. In a study on gene-dose-dependent overexpression of the isocitric lyase encoding gene (*ICL1*), multiple copies of *ICL1* were introduced to *Y. lipolytica*, which led to high levels of *ICL1* expression in the transformant. This overexpression resulted in a significant shift in the citric acid/isocitric acid ratio. It also resulted in a more than 30% reduction in isocitric acid proportions without affecting the total amount of produced acid [36]. In another study, a similar strategy was used for the same objective (increasing the citric acid/isocitric acid ratio), this time by boosting the aconitase encoding gene (*ACO1*) copy numbers. The recombinant *Y. lipolytica* strain showed a high level of aconitase expression, which increased the citric acid/isocitric acid

ratio from 41% to 68% [37]. Studies on the effect of gene dosage on the overproduction of β-farnesene and heterologous lipase showed the same results [38,39].

3.2. Co-Substrate Strategy for PHB Production

Using acetic acid as the only carbon source led to low growth rates and biomass productivity. This is a major issue in commercial PHA production. The efficient growth of *Y. lipolytica* requires a balanced supply of ATP, acetyl-CoA, and NADPH near a 1:1:2 ratio [40]. A single acetate substrate is very efficient at producing ATP and acetyl-CoA. Acetate conversion to NADPH through the oxidative pentose phosphate pathway (*Ox-PPP*), however, is an intensive ATP-consuming pathway. This is the reason that growing modified *Y. lipolytica* on acetate was much slower than glucose [40]. On the other hand, using glucose as the sole energy and carbon source led to a significantly higher growth rate and PHB content. Nevertheless, the cost of glucose seems to be a serious obstacle to the feasibility of using it as the sole substrate in a cost-effective bioprocess for PHB production [41]. As a result, a co-substrate strategy with a mix of glucose and sodium acetate was evaluated to determine the feasibility of this method as a solution to the efficiency and cost dilemma. These results showed that high concentrations of glucose and acetate inhibited the engineered strain's growth rate.

In the current study, 10 g/L of sodium acetate (7.2 g/L acetate) as the sole carbon source led to a growth rate of 0.148 h^{-1}. These results are similar to previous studies on the *Y. lipolytica* wild-type strain W29, which showed a growth rate of 0.16 h^{-1} when cultivated on 5 g/L of acetate [42], but are higher than SZYL004, a modified strain for using xylose as a substrate (by overexpressing the three native genes XR, XDH, and XK). SZYL004 could not tolerate 75 mM of acetate (6.15 g/L) [43]. However, in current research, increasing the sodium acetate concentration to 50 g/L caused a 12% decrease in the PHB32 strain growth rate. Increasing sodium acetate to 100 g/L (72 g/L acetate) significantly inhibited the engineered strain.

It was previously attempted to increase *Y. lipolytica*'s tolerance to high acetate concentrations. For instance, *Y. lipolytica* was modified to tolerate higher acetate concentrations by expressing acetyl-CoA synthase. It was attempted to adapt this modified strain to 50 g/L of acetate. The resulting ACS 5.0 strain (after genetic modification and culture adaptation) could grow effectively in 30 g/L of acetic acid, while 40 and 50 g/L of acetate showed high toxicity for this strain [44]. Considering that the current engineered strain (PHB32) could grow effectively on 36 g/L of acetate, with a growth rate of 0.131 h^{-1}, this strain could be comparable to other strains tolerating high acetate concentrations. In addition to acetate tolerance, the *PO1f Y. lipolytica* strain was modified in some studies to increase its growth rate using acetate as the sole source of carbon and energy. It was found that overexpressing the acetyl-CoA synthetase (*ACS*) and acetyl-CoA carboxylase (*ACC1*) resulted in a strain with a 5.27 times higher growth rate and a 5.39 times faster acetate consumption [45].

Using glucose as the sole carbon and energy source in our study showed a significantly higher growth rate. In a flask containing YNB and 10 g/L glucose, a growth rate of 0.204 h^{-1} was observed. This was 38% higher than the growth rate achieved with 10 g/L of sodium acetate. These results showed a slightly higher growth rate than a previous study on cultivating strain *Y. lipolytica* H222 using glucose for citric acid production. In that study, H222 reached 0.192 h^{-1} as its maximum specific growth rate [46]. In spite of this, PHB32 grows slower than *Y. lipolytica* IBT 446 (0.24 h^{-1} growth rate), with the same submerged cultivation system and glucose as the sole carbon source [47]. In a comparison between four different strains of *Y. lipolytica*, A-101, *Wratislavia* 1.31, *Wratislavia* AWG7, and *Wratislavia* K1, for citric acid production from glucose, growth rates of 0.19 h^{-1}, 0.23 h^{-1}, 0.36 h^{-1}, and 0.23 h^{-1} were observed, respectively. These results showed that strain *Wratislavia* AWG7 had a higher growth rate than our engineered strains [48].

The current study indicates that increasing glucose concentration from 10 g/L to 150 g/L results in a negative effect on growth rate. At 100 g/L of glucose, a growth rate of 0.163 h^{-1} was observed. This is 20% lower than the growth rate at 10 g/L of glucose. Such

substrate inhibition effects were observed in other studies, which showed that increasing the glucose concentration from 50 g/L to 200 g/L led to an intensive depletion in the growth rate from 0.17 h^{-1} to 0.055 h^{-1} in the H222 strain [46]. A similar inhibitory effect on the growth rate and lipid productivity in the *PO1f* strain was observed by increasing the glucose concentration to 160 g/L. This was suggested to be a result of osmotic stress [49].

In the current study, the co-substrate strategy with glucose and sodium acetate showed a synergistic effect on growth rate. The highest growth rate of 0.227 h^{-1} was observed at 10 g/L of both glucose and sodium acetate. Previous studies suggested a similar ameliorative effect of co-substrate medium on bio-oil production in *Y. lipolytica*. Pereira and colleagues [50] showed that the simultaneous application of glucose and acetate improved the growth rate of *Y. lipolytica* NCYC 2904. Similar studies were conducted using agricultural wastes containing stearin, glycerol, and glucose as co-substrates. In another study, when *Y. lipolytica ACA-DC 50109* was modified to produce a cocoa-butter-like lipid, the results demonstrated that co-substrate media could be an effective strategy for such value-added products [51]. Our results showed that in flasks with 100 g/L of sodium acetate as the sole substrate, there was no growth after 6 days. However, adding 5 g/L of glucose to such flasks led to a 0.033 h^{-1} growth rate. Similarly, in flasks with 50 g/L of sodium acetate as a mono-substrate, a 0.131 h^{-1} growth rate was observed, while using a co-substrate strategy with the same sodium acetate concentration and 5 g/L of glucose as a dopant resulted in a 40% increase in the growth rate up to 0.184 h^{-1}.

Metabolism, stress-signaling pathways, and pH homeostasis are the main factors affecting yeast's response to acetic acid stress [52]. Among other physiological features, cell membrane transporters play a crucial role in yeast tolerance to high acetate concentrations [53]. If the pH of the environment is below acetic acid's pKa (4.76), this acid is primarily undissociated and can be easily diffused into the cell membrane and the cytosol [54]. Increasing the extracellular concentrations of acetate may lead to high passive transport of acetate into the cytoplasm [55]. This causes intracellular acidification, inhibiting growth and metabolism and causing oxidative damage. In this condition, pH homeostasis stimulates the proton pumps to prevent further intracellular proton accumulation. The active transport process consumes up to 20% of the produced ATP [56], which is considered an energy-intensive process.

Our results showed a similar compensatory effect of acetate at high glucose concentrations. It was observed that adding 10 g/L of sodium acetate to all initial glucose concentrations significantly increased growth rates. For example, while 100 g/L of glucose had some inhibitory effect on the engineered strain, adding 10 g/L of sodium acetate increased its growth rate by up to 15%. Co-substrate strategies using multiple carbon and energy sources can balance key biosynthetic components. Synthetic pathways in cells are generally complex processes involving various types of molecules as their building blocks. Considering the nature of metabolism, each pathway shows different degrees of efficiency in converting the various substrates into products. In the co-substrate concept, glucose is commonly considered an energy-deficient substrate (high carbon-to-energy ratio). This deficiency could be compensated by the simultaneous use of a substrate (such as C1 substrates) with a lower carbon-to-energy ratio as a convenient energy donor [57].

For instance, a study on gluconate doping in *Y. lipolytica* revealed that co-utilizing this substrate led to accelerated acetate lipogenesis by providing NADPH through the pentose cycle [40]. The main reason for glucose toxicity in high concentrations was the resulting osmostress, which was compensated by acetate [58]. Previous studies showed that acetate could increase intracellular proline concentration, which is considered an effective cellular osmoprotectant agent [59]. Another study on the effect of acetate on *Lens culinaris* showed that acetate stimulated the cellular accumulation of proline by up to 24%. It also increased the ascorbic acid content more than 2 times and catalase activity up to 1.5 times, which are both considered antioxidant agents [60]. Such results suggest that the co-utilization of acetate has a compensatory effect on glucose toxicity at high concentrations because it

triggers cells to accumulate proline with an osmoprotective effect and enhances antioxidant concentrations and activity.

The Supplementary Material contains relevant data concerning the growth rate and growth curves of the final modified strain in the current study. The interaction between glucose and acetate may play a key role in the co-substrate strategy, as indicated in the primary analysis. To reveal the interaction between acetate and glucose on the modified strain's growth rate, a two-factor design using the General Linear Model (*GLM*) was employed. The resulting ANOVA analysis (Table 3) showed that acetate and glucose concentrations had significant interactive effects on the *Y. lipolytica* growth rate.

Table 3. ANOVA results for two-factor *GLM* between glucose and acetate on *Y. lipolytica PHB32* growth rate.

Source	*SS*	*df*	*MS*	*F*-Value	*p*-Value
Glucose	0.110	6	0.0183	591.4	<0.0001
Acetate	0.459	4	0.1148	3702	<0.0001
Glucose * Acetate	0.078	24	0.00325	105.0	<0.0001
Model	0.647	34	0.01904	614.0	<0.0001
Error	0.002	70	0.00003		
Corrected Total	0.6495	104			

As shown in Table 3, both glucose ($p < 0.0001$) and acetate ($p < 0.0001$) had significant effects on biomass concentration. Also, these results suggest that glucose and acetate had a significant interaction ($F_{\text{Glucose*Acetate}} = 105.0$, $p < 0.0001$) on the engineered *Y. lipolytica* strain's growth rate and biomass productivity.

The synthesis of PHB by engineered *Y. lipolytica* was found to be a growth-associated product, as it accumulated in the produced biomass simultaneously with the yeast's growth. To determine the optimal medium for PHB production in a fed-batch system, response surface methodology (*RSM*) was used. The 3D surface plot and 2D contour plot shown in Figure 5 are graphical representations of statistical regression and growth rate prediction. This statistical modeling and 2D contour plot suggested 50 g/L of glucose and 10 to 20 g/L of sodium acetate as the optimum concentrations of these substrates (Figure 5A). The 3D surface plot predicted that increasing the concentration of both glucose and acetate would have inhibitory effects on this strain's growth rate.

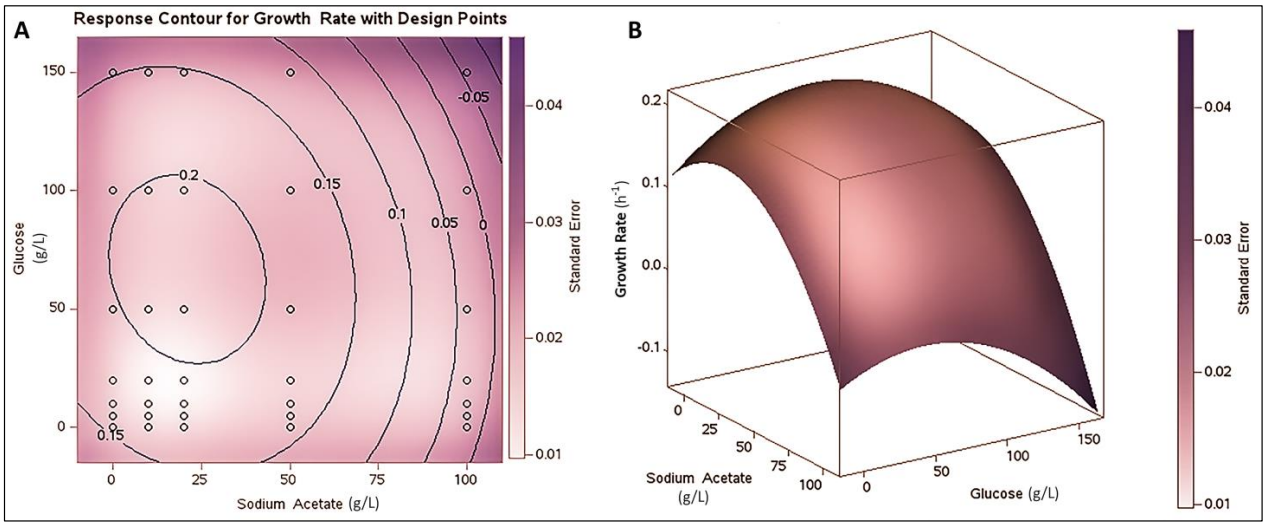

Figure 5. (**A**) A 2D contour plot indicating the optimum levels of substrates for maximum growth rate; (**B**) 3D surface plot shows the effect of glucose and sodium acetate on modified strain's growth rate.

3.3. Production of PHB in Bioreactor

In aerobic fermentation using *Y. lipolytica* PHB32 (in a pH-controlled and co-substrate medium), substrate concentrations were maintained optimally with a fed-batch system (Figure 6A). The initial concentrations of glucose and sodium acetate were set at 50 g/L and 20 g/L, respectively, based on *RSM* optimal prediction (Figure 6B). The CDW reached 83.4 g/L and the PHB titer reached 31.7 g/L (Figure 6C).

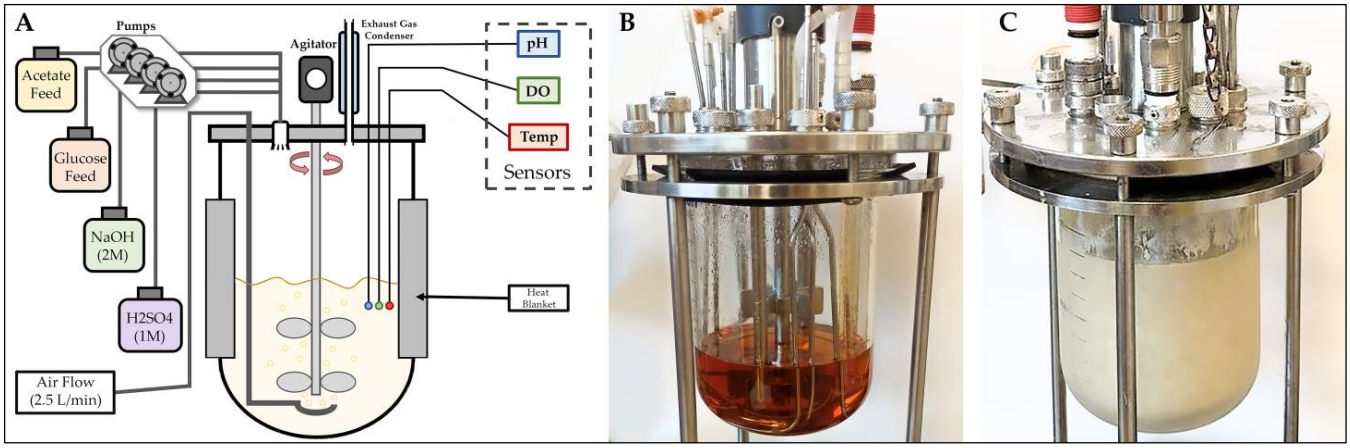

Figure 6. (**A**) Schematic diagram of the fed-batch bioreactor; (**B**) bioreactor before inoculation and fermentation; (**C**) bioreactor at the end of fermentation using *Y. lipolytica* PHB32.

The polymer content in the fed-batch culture and flask experiments was almost the same. This fact suggested that changing the pH and nutrient starvation during flask cultivation (as the main inhibitory parameters in batch culture) did not affect the expression of the introduced PHB pathway. Similar results in previous studies suggest that PHB accumulation capacity in engineered *Y. lipolytica* is mainly restricted by the expression level of the introduced heterologous PHB pathway [11]. In the current study, the PHB content reached 38% of CDW in the modified *Y. lipolytica* PHB32 stain in the bioreactor experiment using YPD medium. Compared with the previously engineered *Y. lipolytica* CAB strain using the *ABC* pathway [11], these results are significantly higher. *Y. lipolytica* PHB32 showed a noticeably high capacity for PHB production, not only for its high PHB titer but also for its significant biomass productivity even under minimal YNB medium.

A comparison between the current and previously reported studies suggested that the *NBC* pathway seems to be an efficient pathway for PHB biosynthesis in *Y. lipolytica*. *Haddouce* and colleagues [61] showed that between six acyl-CoA oxidase (*Aox*) isoenzymes, *Aox*3p has a key role in converting C9:0 and C13:0 fatty acids into PHA. In their study, PHA synthase (*phaC*) from *Pseudomonas aeruginosa* was introduced to *Y. lipolytica*, which led to 2% PHA accumulation in produced biomass [61]. In a similar study by Rigouin et al. [13], different variants of *phaC* were used as the single heterologous gene for synthesizing medium-chain-length (mcl) PHA in engineered *Y. lipolytica*. Up to 25% (*g*/*g*) of PHA accumulation was observed in some *PhaC* variants. According to their study, some modified strains synthesized mcl-PHA with an average molar mass of 316,000 g/mol, which could be an excellent packaging material [13]. In another study, Gao and colleagues [12] used multicopy integration of *PhaC*1 (*P. aeruginosa* PAO1) into *Y. lipolytica*, leading to an engineered strain that could accumulate up to 5% mcl-PHA in its biomass. Their study found that the highest biomass concentration reached 21.9 g/L of CDW on YNB medium [12]. Clearly, the current study achieved a higher PHB titer than the previous published studies on *Y. lipolytica*.

For *Y. lipolytica* to become an industrially favorable microbial factory for PHB production, however, several additional modifications must be made. To increase the acetate/glucose ratio, acetate uptake should be accelerated. Second, its substrate must be

expanded to include other sugars that are abundant in lignocellulosic biomass, such as pentose. Also, gene dosage and peroxisomal compartmentalization effects should be quantified separately. Furthermore, the formation of granular PHB in peroxisome organelles should be evaluated in the modified strain.

4. Conclusions

The *NBC* pathway was introduced into the *Y. lipolytica* genome. The resulting engineered strain was capable of accumulating PHB at up to 41% of CDW in a shake flask culture on glucose and acetate as substrates. Bioreactor cultivation led to an 83.4 g/L biomass and a 31.7 g/L PHB concentration. These results suggest that the *NBC* pathway is a more efficient strategy than the *ABC* pathway or introducing *PhaC* as a single gene pathway in *Y. lipolytica*. In high concentrations, acetate or glucose as the sole carbon and energy source showed a slow growth rate and inhibitory effects. The co-substrate strategy using both substrates simultaneously increased the modified strain's growth rate significantly. The simultaneous use of glucose and acetate alleviated substrate inhibition. These results showed the possibility of utilizing acetic acid derived from wastes for PHB production by modifying *Y. lipolytica*. Also, our results indicate significantly higher PHB productivity using *Y. lipolytica* PHB32 compared to previous studies.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9121003/s1, Figure S1: Effect of co-substrate culture on *Y. lipolytica* PHB32 growth rate; Figure S2: Growth curve of *Y. lipolytica* PHB32 using 10 g/L of sodium acetate as the only carbon and energy source; Figure S3: Growth curve of *Y. lipolytica* PHB32 using 20 g/L of sodium acetate as the only carbon and energy source; Figure S4: Growth curve of *Y. lipolytica* PHB32 using 50 g/L of sodium acetate as the only carbon and energy source; Figure S5: Growth curve of *Y. lipolytica* PHB32 using 100 g/L of sodium acetate as the only carbon and energy source; Figure S6: Growth curve of *Y. lipolytica* PHB32 using 5 g/L of glucose as the only carbon and energy source; Figure S7: Growth curve of *Y. lipolytica* PHB32 using co-substrate strategy by 10 g/L of sodium acetate and 5 g/L of glucose; Figure S8: Growth curve of *Y. lipolytica* PHB32 using co-substrate strategy by 20 g/L of sodium acetate and 5 g/L of glucose; Figure S9: Growth curve of *Y. lipolytica* PHB32 using co-substrate strategy by 50 g/L of sodium acetate and 5 g/L of glucose; Figure S10: Growth curve of *Y. lipolytica* PHB32 using co-substrate strategy by 100 g/L of sodium acetate and 5 g/L of glucose; Figure S11: Growth curve of *Y. lipolytica* PHB32 using 10 g/L of glucose as the only carbon and energy source; Figure S12: Growth curve of *Y. lipolytica* PHB32 using co-substrate strategy by 10 g/L of sodium acetate and 10 g/L of glucose; Figure S13: Growth curve of *Y. lipolytica* PHB32 using co-substrate strategy by 20 g/L of sodium acetate and 10 g/L of glucose; Figure S14: Growth curve of *Y. lipolytica* PHB32 using co-substrate strategy by 50 g/L of sodium acetate and 10 g/L of glucose; Figure S15: Growth curve of *Y. lipolytica* PHB32 using co-substrate strategy by 100 g/L of sodium acetate and 10 g/L of glucose; Figure S16: Growth curve of *Y. lipolytica* PHB32 using 20 g/L of glucose as the only carbon and energy source; Figure S17: Growth curve of *Y. lipolytica* PHB32 using co-substrate strategy by 10 g/L of sodium acetate and 20 g/L of glucose; Figure S18: Growth curve of *Y. lipolytica* PHB32 using co-substrate strategy by 20 g/L of sodium acetate and 20 g/L of glucose; Figure S19: Growth curve of *Y. lipolytica* PHB32 using co-substrate strategy by 50 g/L of sodium acetate and 20 g/L of glucose; Figure S20: Growth curve of *Y. lipolytica* PHB32 using co-substrate strategy by 100 g/L of sodium acetate and 20 g/L of glucose; Figure S21: Growth curve of *Y. lipolytica* PHB32 using 50 g/L of glucose as the sole energy and carbon source; Figure S22: Growth curve of *Y. lipolytica* PHB32 using co-substrate strategy by 10 g/L of sodium acetate and 50 g/L of glucose; Figure S23: Growth curve of *Y. lipolytica* PHB32 using co-substrate strategy by 20 g/L of sodium acetate and 50 g/L of glucose; Figure S24: Growth curve of *Y. lipolytica* PHB32 using co-substrate strategy by 50 g/L of sodium acetate and 50 g/L of glucose; Figure S25: Growth curve of *Y. lipolytica* PHB32 using co-substrate strategy by 100 g/L of sodium acetate and 50 g/L of glucose; Figure S26: Growth curve of *Y. lipolytica* PHB32 using 100 g/L of glucose as sole carbon and energy source; Figure S27: Growth curve of *Y. lipolytica* PHB32 using co-substrate strategy by 10 g/L of sodium acetate and 100 g/L of glucose; Figure S28: Growth curve of *Y. lipolytica* PHB32 using co-substrate strategy by 20 g/L of sodium acetate and 100 g/L of glucose; Figure S29: Growth curve of *Y. lipolytica* PHB32 using co-substrate strategy by 50 g/L of sodium acetate and 100 g/L of

glucose; Figure S30: Growth curve of *Y. lipolytica* PHB32 using 150 g/L of glucose as the sole energy and carbon source; Figure S31: Growth curve of *Y. lipolytica* PHB32 using co-substrate strategy by 10 g/L of sodium acetate and 150 g/L of glucose; Figure S32: Growth curve of *Y. lipolytica* PHB32 using co-substrate strategy by 20 g/L of sodium acetate and 150 g/L of glucose; Figure S33: Growth curve of *Y. lipolytica* PHB32 using co-substrate strategy by 50 g/L of sodium acetate and 150 g/L of glucose.

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