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Polyhydroxybutyrate (PHB) Biosynthesis by an Engineered *Yarrowia Lipolytica* 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 feedstock are two logical strategies for reducing the cost.   As an oleaginous yeast, *Y. lipolytica* has a high carbon flux through acetyl-CoA (main PHB precursor) which makes it a desired cell factory for PHB biosynthesis. Two different metabolic pathways introduced into *Y. lipolytica PO1f* in this study for enhancing PHB biosynthesis, along with glucose and acetate as co-substrates. Results showed that *NBC* pathway led to more than 11% PHB accumulation, while there was non-detectable PHB accumulations using *ABC* pathway. Further modifications were done using peroxisomal compartmentalization and gene-dose overexpression. Final strain showed up to 41% PHB of CDW with a growth rate of 0.227 h^{-1}. Low growth rate was observed using acetate or glucose as sole carbon and energy source. The co-substrate strategy showed compensatory effect on overcoming inhibitory and toxic effects of both substrates at high concentrations. Cultivating the engineered strain in optimal co-substrate condition predicted by *RSM* led to 83.4 g/L biomass concentration and 31.7 g/L PHB.

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

1. Introduction

The increasingly dependence on plastics has led to the generation of a large amount of plastic wastes, underscoring a critical need for biodegradable material as a sustainable replacement of the petroleum-based plastics [1]. There are several alternatives for fossil fuel-based plastics including: poly lactic acid (PLA), bio-polyethylene, *polyhydroxyalkanoates* (PHAs), and protein-based bioplastics [2]. Among different types of PHAs, polyhydroxybutyrate (PHB) is a naturally occurring polymer which is 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 characteristics such as flexibility, tensile strength, and degradation rate), good resistance to UV radiations and insolubility in water. PHB is therefore considered as one of the alternatives that has most potential for the current plastic market [3] with different applications from food packaging to textiles, cosmetics, and biomedical implants.

Some of the microbial species are widely studied as natural producers of PHB, such as *Cupriavidus necator* (formerly *Ralstonia eutropha*), *Azotobacter vinelandii*, *Aeromonas hydrophila*, *Pseudomonas putida*, and *Alcaligenes eutrophus* [4]. To be a suitable microbial cell factory for producing PHB the selected species should have 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, to make the scale-up process feasible; and third, it should be able to use different and low cost substrates to make an affordable bioprocess [5]. However, among the PHB natural producers, very few have all these characteristics.

These challenges relying on the natural PHB producers have led to various attempts using genetic modification of some most robust GRAS microbial cell factories such as *Saccharomyces cerevisiae, Pichia pastoris, Corynebacterium glutamicum, Yarrowia lipolytica,* and *Zymomonas mobilis* [6]. Among these potential cell factories, *Y. lipolytica* is uniquely advantageous for PHB biosynthesis. *Y. lipolytica* historical uses in food, feed, lipid production, and several other industrial applications proved that *Y. lipolytica* was generally nonpathogenic and does not pose significant health risks to humans or animals. Indeed the U.S. Food and Drug Administration (FDA) declare *Y. lipolytica* as a GARS microorganism which indicates the reason of such high interest toward this yeast in industrial biotechnology [7].

Y. lipolytica grows relatively fast with a doubling time of 1.7 h^{-1} [8] and can be cultivated at various scales, which makes it suitable for both research and large-scale bioproduct applications. Additional benefits of this yeast for PHB production include: 1) the avoidance of bacteriophage attacks at industrial levels; 2) tolerant to inhibitors and pH stresses; and 3) forming filamentous cells under late growth stage that facilitate gravity separation [9].

Y. lipolytica is an oleaginous yeast with a great potential for producing different biochemicals using nutrient limited media and its metabolism is well-established for biosynthesizing triglycerides (TGs) [10]. Therefore, *Y. lipolytica* has a high flux through acetyl-CoA which makes it a desired cell factory for those molecular that require acetyl-CoA as intermediate. These products include TGs, carotenoids, fatty acid derivatives, organic acids, and PHAs [11]. Some efforts have been made to genetically modify this cell factory to produce PHB to using its unique characteristics. 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 up to 10.2% of cell dry weight (CDW) by using glucose as a substrate [12]. 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*. Its result was mcl-PHA accumulation up to 5% of CDW [13]. Similar results were observed by *Rigouin* and colleagues, using the mutagenesis of *phaC* which led to 25% mcl-PHA of CDW (w/w) [14].

Despite all these efforts the modified strains still have a low PHB titer which is the main bottleneck for the scale-up process. A main reason is that *Y. lipolytica* has a robust flux toward the lipogenesis pathway which leads to high lipid accumulation rather than PHB [15]. Therefore, harnessing the lipogenesis pathway toward PHB precursors seems to be an effective strategy to increase PHB titer in *Y. lipolytica*. Indeed, previous studies on redirecting malonyl-CoA (main intermediate for fatty acid synthesis) in sugarcane toward PHB biosynthesis showed higher PHB titer in mesophyll cells than *ABC* pathway. The *NBC* pathway (including *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 catalyzes the condensation reaction reversibly, *NphT7* catalyzes the condensation reaction irreversibly, resulting in a higher accumulation of PHB [16].

Another bottleneck for producing PHB using 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 the substrate. Also, several studies showed glycerol as another preferred substrate for producing biodiesel using *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 show inhibitory effects to many microorganisms including *E. coli* and *S. cerevisiae*. *Y. lipolytica* on the other hand, can readily uptake and convert acetic acid. This making it possible to utilize a variety of wastes through the VFA route that are less efficiently utilized by other organisms [19]. Using wastes as feedstock to produce biodegradable plastics would provide the most

sustainable solution for the plastic production dilemma [20]. Despite this, using acetic acid (generally VFAs) as only carbon source cannot satisfy the industrial demand for fast growth media with optimized PHAs production. The main reason for this deficiency is unbalanced reductive agents (as NADPH) and energy carriers (as ATP) produced during the metabolic pathway of acetic acid [21]. On the other hand, glucose showed a noticeable higher growth rate and biomass productivity than acetic acid. Therefore, co-substrate utilization is a reliable strategy for low-cost and efficient PHA production [22].

In the current study, two PHB pathways, *ABC* pathway and *NBC* pathway, were introduced into *Y. lipolytica* and compared for PHB biosynthesis. In order to further enhance the PHB titer, gene dosage effect and compartmentalization strategies were used to reach the final modified strain. Also, a co-substrate strategy was implemented using acetate as a cost-effective substrate and glucose as dopant. 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. Furthermore, this study provides significant insights into metabolic engineering of *Y. lipolytica* for the biosynthesis of PHB.

2. Materials and Methods

2.1. Strain Construction

The auxotrophic strain *Y. lipolytica PO1f* (ATCC MYA-2613) was used as a microbial host for the introduced pathway. *E. coli DH5α* was used for cloning the genes and amplifying the constructed shuttle vectors. To develop constructs single genes are amplified using PCR and cloned using a cloning vector in *E. coli*. The list of primers used for PCR reaction shows in Table 1. Cloned genes are assembled in *E. coli-Y. lipolytica* shuttle vectors. Assembled cassettes include genes, biomarkers, both well-characterized promoter (TEF1, GPM, and FBA) and terminator (XPR2, OCT1, and LIP1) elements. Next, existing the target genes in expression cassettes, and integration of the assembled expression cassettes into *Y. lipolytica* genome were verified using colony PCR and markers.

To cultivate and screen transformed *E. coli* for constructed plasmids, *Luria-Bertani* (*LB*) medium (5 g/l yeast extract, 10 g/l *Bacto* tryptone, and 10 g/l NaCl) at 37 °C with ampicillin as a marker was used. Synthetic yeast nitrogen base (YNB) media consisting of 20 g/L glucose as carbon source, at 28 °C was used for screening the transformed *PO1f* host. Transformants were selected on a defined medium containing 6.7 g/l YNB without amino acids. In each step medium was supplemented with 0.77g/L CSM-Ura (Complete supplement mixture minus uracil, US-Biological, USA), 0.67 g/L CSM-leucine, and 0.3 g/L hygromycin [12,23,24] glucose based on introduced vector.

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	CCCGGGAGAGTCGACCTGCAGTTAGCCCATGTGC	65	34	10444.8
phaB-R	AAGCTTATGACCCAGCGAATCGCCTACGTCACC	55	33	10027.5
phaC1437-F	CCCGGGTTATCGCTCGTGGACGTAAGTGCC	63	30	9215.0
phaC1437-R	AAGCTTATGTCTAACAAGTCTAACGACGAGC	42	31	9512.3
NphT7-F	CCCGGGTTACCACTCAATCAGGGCAAAGGAAGC	58	33	10141.6
NphT7-R	AAGCTTACTGATGTGCGATTTCGAATTATTGGAAC	37	35	10800.1
bktB-F	AAGCTTATGACCCGAGAGGTGGTGGTCGTGTCCGG	60	35	10884.1
bktB-R	CCCGGGTTAAATTCGTTCGAAGATGGCGGCAATGCC	56	36	11101.3
phaC1437-SKL-F	AAGCTTATGTCTAACAAGTCTAACGACGAGCTGAAGTACC	43	40	12297.1
phaC1437-SKL-R	CCCGGGTTACAGCTTAGATCGCTCGTGGACGTAAGTGCCAGG	60	42	12971.4

Y. lipolytica wild-type strains are unable to synthesize PHB naturally. However, there are two common strategies for introducing the PHB biosynthesis pathway to non-natural PHB producer organisms. To evaluating the efficiency of these pathways in *Y. lipolytica* two vectors designed for expressing the *ABC* PHB biosynthesis pathway and *NBC* pathway. These expression cassettes contained fragments (promoters, terminators, and other regulatory elements). The only difference was that the *ABC* cassette contained *phaA* gene which expressed β-ketothiolase (*pZX22*), while the *NBC* cassette contained *NphT7* gene (*pZX30*).

To reach to the final strain, *Y. lipolytica PO1f* modified by introducing the *NBC* pathway contained following genes: *phaB* (from *R. eutropha*), *phaC1437* which encoding a mutant *Pseudomonas sp*, and *NphT7* (acetoacetyl-CoA synthase) gene from *Streptomyces sp.* [12]. Then modified strain subjected to sequential transformations using integrative vectors. These modifications include increase the copy number of *NBC* PHB pathway genes [25]. Also *bktb* (β-ketothiolase) gene from *R. eutropha* was introduced to modified strain to catalyze the condensation of acyl-CoA and acetyl-CoA into 3-ketoacyl-CoA molecules to copolymer synthesis in new strain [26]. PHB synthase tagged with carboxyl-terminal amino acid sequence serine-lysine-leucine (*SKL*) to peroxisomal compartmentalization of synthesized PHB [27]. The map of constructed designed pathway shown in Figure 1A. These vectors are named *pCV35*, *pCV88*, and *pCV342* which are shown in Figure 1B, Figure 1C, and Figure 1D respectively [28,29]. 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}, *leu2* maker), and the result strain was transformed with linearized plasmid, *pCV88* (P_{TEF}-*NphT7*-T_{XPR2}, P_{GMP}-*PhaC1437*-T_{OCT1}, P_{FBA}-*PhaB*-T_{LIP1}, *ura3* maker). This current strain subjected to future transformation by linearized plasmid *pCV342* (P_{TEF1N}-*PhaB*-T_{xpr2}, P_{TEF1N}- *PhaC1437_SKL*-T_{xpr2}, P_{TEF1N}-*BktB*-T_{xpr2}, *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 the *Y. lipolytica* PHB32 as the final strain.
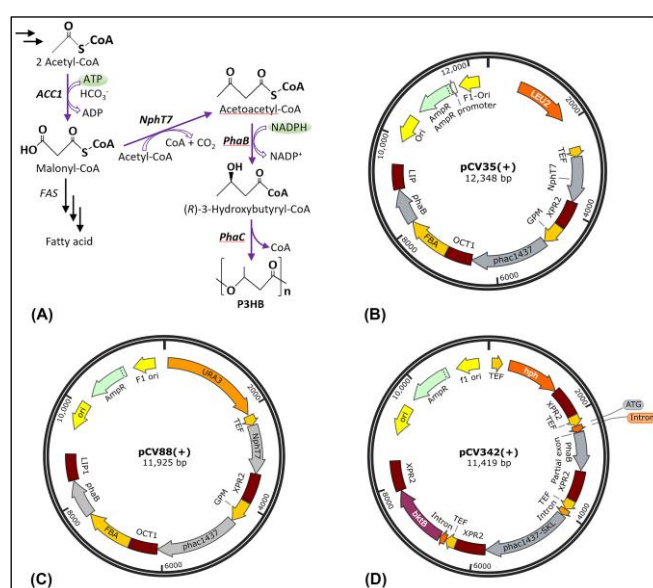
Figure 1. A) Designed pathway for producing PHB from acetyl-CoA as the main precursor. B) Map of *pCV35* shuttle vector containing *NphT7*, *phaC1437*, and *phaB* genes; LEU as auxotrophic biomarker; TEF, GPM, and FBA promoters; XPR2, OCT1, and LIP terminators. C) Map of *pCV88* shuttle vector containing similar genes, promoters, and terminators to *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.

## *2.2. 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, 20g/L peptone and 20g/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 w/o AA and 5 g/L NH$_4$CL. 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 substrate. 250 mL flasks were inoculated by 0.5% (v/v) of prepared seed of the engineered strain and incubated at 28 ℃ 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 and sodium acetate. The bioreactor was inoculated with 1% (v/v) prepared seeds and fermentation was done at 28 ℃ at a constant pH of 7. After 24 hours of batch culture and achieving an increased cell concentration, the bioreactor was switched to fed-batch culture. During this phase a constant flow of YPD containing carbon sources were injected into bioreactor. This fed-batch system was maintained with glucose and sodium acetate at their optimum concentration for an additional 48 hours. Filtered air was sparged into bioreactor with a flow rate of 2.5 L/min. To maintain the air saturation at 20%, flat blade used with adjusted agitation rate between 150 to 500 rpm.

2.3. Growth Rate and PHB Content Analysis

To detect and quantify the growth rate of the engineered strain, optical density (OD) of the culture at 600 nm was used for determining the cell dry weight (CDW). To measure the intracellular PHB content, 2 mL of culture were centrifuged at 5000 g for 5 min. Then, settled cell pellets were washed with deionized water and dried at 55 ℃ overnight. The dried biomass was digested by using a methanolysis solution containing 97% methanol and 3% H$_2$SO$_4$, 18M (v/v) for determined the PHB content [12]. During the transesterification reaction, PHB polymers were monomerized to butanoic acid, 3hydroxy-, 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 ℃ 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 lower layer contained chloroform and hydrophobic cell components including digested PHB (Figure 2). The separated chloroform layer was analyzed by *Agilent* 7890A gas chromatograph, equipped with HP-5MS 30m X 250μm X 0.25μm column, and coupled by 5975C mass detector. The temperature was held at 40 ℃ for 3 min, then ramped from 40 ℃ to 280 ℃ at 10 ℃ min^{-1} and held for 10 min.
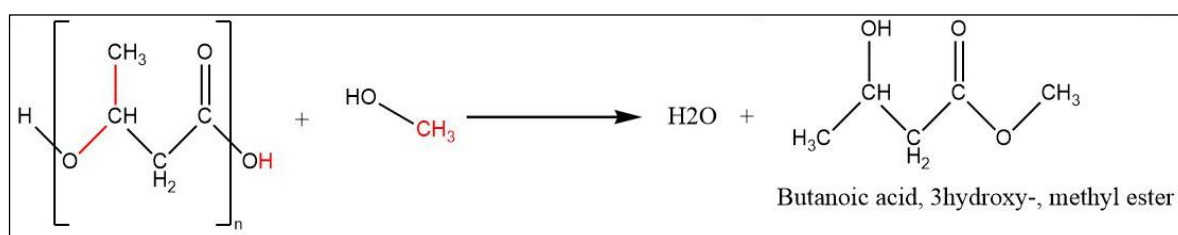
Figure 2. Transesterification of PHB polymer using methanolysis solution. .

2.4. Statistical Analysis

For each designed experiment, data were collected from three biologically independent experiments. *SAS® 9.4M7* software was used for analyzing experimental data and calculating parameters such as mean value, standard deviation, and standard errors. Also, in order to evaluate the synergistic effects of both glucose, sodium acetate and the effects of their high concentration on *Y. lipolytica* growth and PHB content, response surface methodology *RSM* analysis was used [30]. *RSM* analysis was done using *SAS RSREG* procedure [31]. For the statistical analysis, linear, second-degree polynomials, and quadratic models were used to describe the relationship between the input variables and the responses. Two-factor *ANOVA* analysis was used for evaluating the interaction of two independent variables (glucose and sodium acetate) on the responses (growth rate and PHB content). Using *SAS GML* procedure, the *F-value* was calculated for detecting the 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.

3. Results

3.1. Metabolic Pathway Design

Comparing the results of *ABC* PHB biosynthesis pathway (Figure 3A) and *NBC* pathway (Figure 3B) showed that redirecting the lipogenesis pathway toward PHB production using *NBC* pathway is a more effective strategy for PHB biosynthesis in *Y. lipolytica*. As it could be observed in Figure 3C, cultivating the transformed *PO1f* host strain by *pZX30* vector (in YPD media with 30 g/L glucose) led to accumulation of PHB up to 11% of CDW. However, there was no detectable PHB synthesis in transformed strain with *pZX22* plasmid. Therefore, the *NBC* pathway was selected as an efficient design for PHB biosynthesis in *Y. lipolytica*. One reason for high efficiency of *NBC* pathway in *Y. lipolytica* is high carbon flux toward the lipogenesis pathway in this yeast. This characteristic results a significant synthesis of malonyl-CoA as a main intermediate for fatty acids production [32]. Introducing the *NphT7* redirects the *Y. lipolytica* metabolic pathway from lipogenesis to producing more acetoacetyl-CoA as PHB precursor. Considering that no natural replicative vectors (episome) have ever been observed in *Y. lipolytica*, designed vectors contain chromosomal centromere (cEN) fragments to transient expression of these heterologous genes [33].

The enzymatic reactions which are employed to catalyze the acetoacetyl-CoA synthesis were the main difference between these pathways. Synthesis of acetoacetyl-CoA is usually catalyzed by acetoacetyl-CoA thiolase via the condensation of two molecules of acetyl-CoA [34]. In the first pathway *phaA*, *phaB*, and *phaC* were used as basic PHB production pathway which is called *ABC* pathway. *PhaA* (β-ketothiolase) is used for catalyzing the acetyl-CoA condensation reaction to produce acetoacetyl-CoA as precursor for PHB biosynthesis [12]. However, in a study *McQualter* and colleagues [16] found that using the acetoacetyl-CoA synthase in place of a β-ketothiolase enhances PHB production in transgenic sugarcane. This study showed that introducing the *NBC* PHB pathway (containing *NphT7*, *phaB*, and *phaC*) instead of *ABC* PHB pathway into the sugarcane plant increased PHB content in mesophyll cells up to 11.8% of its CDW. It was more than twice the *ABC* pathway. Studies showed that *PhaA* preferentially catalysis thiolysis (breaking down the acetoacetyl-CoA to acetyl-CoA) compared to synthesis of acetoacetyl-CoA [35]. On the other hand, *NphT7* catalysis acetoacetyl-CoA synthesis in an more energy-favored reaction [34]. Indeed, high equilibrium constant value (Km) of β-ketothiolase to acetyl-CoA (up to 1.1mM) compared to low Km value of *NphT7* to acetyl-CoA (0.068mM) and malonyl-CoA (0.028mM) shows that *NphT7* is a much more effective enzyme for catalyzing the condensation reaction for producing acetoacetyl-CoA compared to *PhaA* [16]. On the other hand, *PhaA* catalytic behavior is highly reversible which lead to high tendency of this enzyme catalyzing the thiolysis reaction and breaking down the acetoacetyl-CoA into two acetyl-CoA molecules, while *NphT7* more shows irreversible behavior on catalyzing the condensation reaction [36].
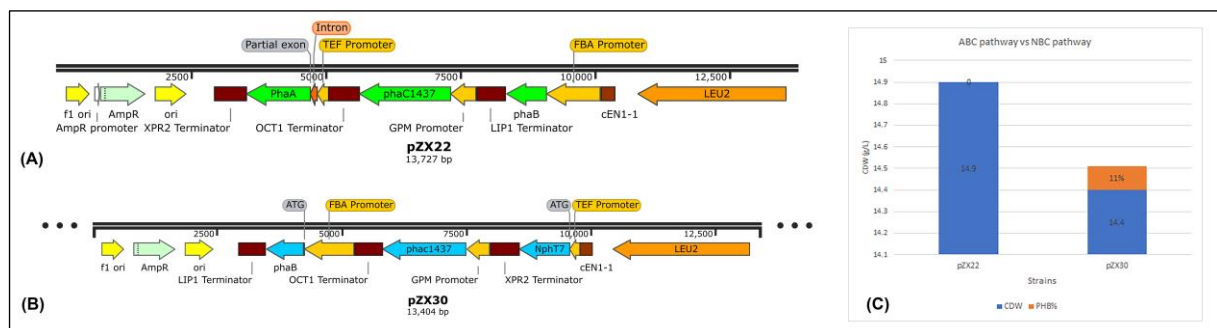
Figure 3. A) Map of *pZX22* vector containing *ABC* pathway, B) Map of *pZX30* vector containing *NBC* pathway, C) Comparing Biomass concentration and PHB accumulation in modified *Y. lipolytica* strains using *ABC* and *NBC* pathways.

The resulted strain from the sequential transformation showed a significant increase in the PHB content compared to strain modified by *pZX30*, without any detectable effect on growth rate (Figure

4A). Sequential transformation led to a strain capable of accumulating PHB up to 41% of CDW which is remarkably higher than 11% PHB titer in strain contains the basic PHB biosynthesis pathway (Figure 4B). Indeed, previous studies prove the gene dosage effect on the expression of desired genes in *Y. lipolytica* host cells. In a study on gene-dose-dependent overexpression of isocitric lyase encoding gene (*ICL1*), multiple copies of the *ICL1* were introduced to *Y. lipolytica* which led to high levels of *ICL1* expression in transformant. This over expression resulted in a significant shift of citric acid/isocitric acid ratio, more than 30% reduction in isocitric acid proportions without affecting the total amount of produced acid [37]. In another study a similar strategy was used for the same objective (increasing the citric acid/isocitric acid ratio), this time by increasing the aconitase encoding gene (*ACO1*) copy numbers. The recombinant *Y. lipolytica* strain showed a high-level of aconitase expression which resulted in increasing the citric acid/isocitric acid ratio from 41% to 68% [38]. Studies on the effect of gene dosage for overproduction of β-farnesene and heterologous lipase showed the same results [39,40].
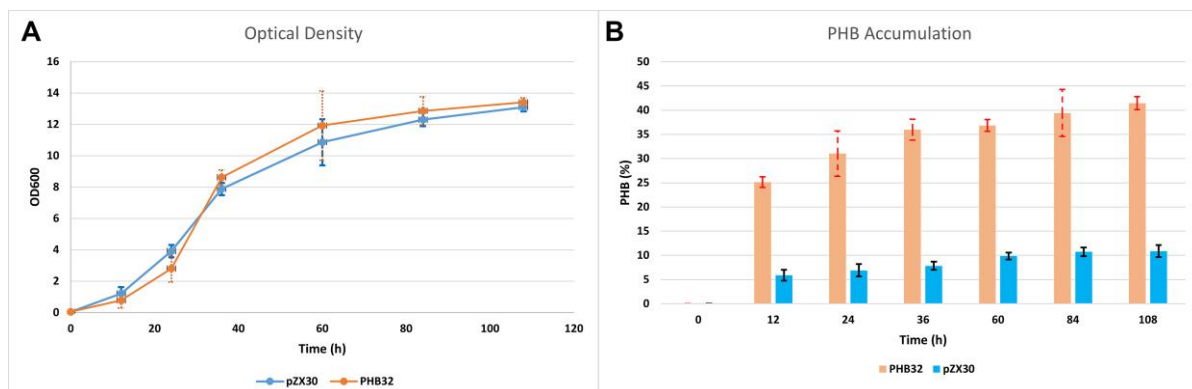
Figure 4. A) Comparing the growth rate, and B) PHB content; in strain *pZX30* (containing only *NBC* pathway) and strain PHB32 (result of sequential transformation) cultivated on YNB containing 50g/L glucose.

3.2. Co-substrate Strategy for PHB Production

Using acetic acid as the only carbon source led to low growth rate and low biomass productivity which is a serious issue on industrial production of PHAs. Efficient growth of *Y. lipolytica* requires a balanced supply of ATP, acetyl-CoA and NADPH near to 1:1:2 ratio [41]. Single acetate substrate is very efficient in producing ATP and acetyl-CoA. However, it is an intensive ATP consumer process through oxidative pentose phosphate pathway (*Ox-PPP*) to produce NADPH from acetate. It is the reason that growing the modified *Y. lipolytica* on acetate was much slower than glucose [41]. On the other hand, using glucose as the sole energy and carbon source led to significantly higher growth rate and PHB content. Nevertheless, the cost of glucose seems to be a serious obstacle to feasibility of using it as the sole substrate in a cost-effective bioprocess for PHB production [42]. As a result, a co-substrate strategy with a mix of glucose and sodium acetate was evaluated to find the feasibility of this method as a solution for efficiency and cost dilemma. These results showed that high concentrations of both glucose and acetate have inhibitory effects on engineered strain's growth rate. 10 g/L of sodium acetate (7.2 g/L acetate) as sole carbon source led to growth rate of 0.148 h^{-1}. These results are similar to previous studies on W29 strain *Y. lipolytica* wild type that showed 0.16 h^{-1} growth rate cultivated on 5 g/L acetate [43]; but pretty higher than SZYL004, a modified strain for using xylose as substrate (by overexpressing the three native genes XR, XDH, and XK). SZYL004 couldn't tolerate 75 mM of acetate (6.15 g/L) [44]. However, increasing the sodium acetate concentration to 50 g/L caused a 12% decrease in PHB32 strain growth rate. Further increasing of sodium acetate to 100 g/L (72 g/L acetate) significantly inhibited the engineered strain to grow. In an effort to modify *Y. lipolytica* to tolerate higher acetate concentration, recombinant strain (expressed acetyl-CoA synthase) tried to be adapted to 50 g/L 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 acetate yet

showed highly toxicity for this strain [45]. Considering that the current engineered strain (PHB32) is able to grow effectively on 36 g/L 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 high acetate tolerance, some studies had done to modifying *PO1f Y. lipolytica* strain to increase its growth rate using acetate as sole carbon and energy source. In one study overexpressing the two key enzymes in acetate metabolism (acetyl-CoA synthetase (*ACS*) and acetyl-CoA carboxylase (*ACC1*)), led to a strain showed 5.27 times more growth rate on acetate, and 5.39 times faster acetate consumption [46].

Using glucose as the sole carbon and energy source showed significantly higher growth rate. In a flask containing YNB and 10 g/L glucose a growth rate of 0.204 h^{-1} observed which was 38% higher than the growth rate using 10 g/L of sodium acetate. These results showed a little higher growth rate compared to previous study on cultivating strain *Y. lipolytica* H222 using glucose for citric acid production which is reached to 0.192 h^{-1} as the maximum specific growth rate [47]. However, PHB32 grow slower than *Y. lipolytica* IBT 446 with 0.24 h^{-1} of growth rate, using same submerged cultivation system with glucose as the sole carbon source [48]. In a comparison between four different strains of *Y. lipolytica* including A-101, *Wratislavia* 1.31, *Wratislavia* AWG7, and *Wratislavia* K1 for citric acid production from glucose, results showed 0.19 h^{-1}, 0.23 h^{-1}, 0.36 h^{-1}, and 0.23 h^{-1} of growth rate respectively. These results showed that strain *Wratislavia* AWG7 had a   higher growth rate compared to our engineered strain [49]. Increasing the glucose concentration from 10 g/L to 150 g/L showed a negative effect on growth rate. In 100 g/L of glucose, a growth rate of 0.163 h^{-1} was observed which is 20% lower than the growth rate at 10 g/L of glucose. Such substrate inhibition effects observed in other studies which showed that increasing the glucose concentration from 50 g/L to 200 g/L lead to an intensive depletion in growth rate from 0.17 h^{-1} to 0.055 h^{-1} in H222 strain [47]. Similar inhibitory effect on growth rate and lipid productivity in *PO1f* strain observed by increasing the glucose concentration to 160 g/L which suggested as a result of osmotic stress [50].

Using co-substrate strategy with glucose and sodium acetate showed a synergistic effect on growth rate. In 10 g/L of both glucose and sodium acetate, highest growth rate of 0.227 h^{-1} was observed. Such an ameliorative effect on bio-oil production using these two substrates simultaneously observed in *Y. lipolytica* NCYC 2904. However, this study showed that increasing 5 g/L glucose to media didn't lead to biomass productivity improvement [51]. Similar study was conducted on using agricultural wastes containing a mixture of stearin, glycerol, and glucose as co-substrates. In the study *Y. lipolytica ACA-DC 50109* was modified for producing of a cocoa-butter-like lipid.   *The* results demonstrated that co-substrate media could be an effective strategy for such value added products [52]. Our results showed that in flasks with 100 g/L of sodium acetate as sole substrate, there was not any growth after 6 days, while adding 5 g/L glucose to such flasks led to 0.033 h^{-1} of growth rate. Similarly, in flasks with 50 g/L sodium acetate as mono-substrate a 0.131 h^{-1} growth rate observed while using co-substrate strategy with same sodium acetate concentration and 5 g/L glucose as dopant caused a 40% increase in growth rate up to 0.184 h^{-1}.

Metabolism, stress-signaling pathways, and pH homeostasis are main factors affecting how yeast respond to acetic acid stress [53]. Among other physiological features, cell membrane transporters play an important role in yeast tolerance to high acetate concentrations [54]. Indeed, if the environmental pH is lower than acetic acid pKa (4.76), acetic acid exists mainly in the undissociated form which could be easily diffused into cell membrane and cytosol consequently [55]. Increasing the extracellular concentrations of acetate also could lead to high passive transport of acetate into cytoplasm [56]. It results intracellular acidification and lead to growth and metabolic activity inhibition, and oxidative damages. In this condition pH homeostasis stimulates the proton pumps to prevent further intracellular proton accumulation. Such active transport is a high energy-consuming process, which consume up to 20% of the produced ATP [57].

Our results showed a similar compensatory effect of acetate in high concentrations of glucose. It was observed that adding 10 g/L of sodium acetate in all initial concentrations of glucose significantly increased the growth rate. For example, while 100 g/L of glucose had some inhibitory effect on engineered strain, adding 10 g/L sodium acetate increased its growth rate up to 15%. Co-substrate strategy using multiple carbon and energy sources can balance key biosynthetic components.

Synthetic pathways in cells are generally complex processes which are involving various types of molecules as their building blocks. Considering the nature of metabolism, each pathway showed different degrees of efficiency on converting the various substrates to the products. In the co-substrate concept, glucose is commonly considered as an energy deficient substrate (high carbon-to-energy ratio). This deficiency could be compensated by simultaneous use of a substrate (such as C1 substrates) which has a lower carbon-to-energy ratio as convenient energy donors [59]. For instance, in a study on gluconate doping in *Y. lipolytica* results showed that co-utilizing of this substrate caused an accelerated acetate lipogenesis by providing NADPH through pentose cycle [41]. Main reason for toxicity of glucose in high concentrations is the resulting osmostress which seems that acetate has a compensatory effect on it [60]. Previous studies showed that acetate could increase the intracellular proline concentration which is considered as an effective cellular osmoprotectant agent [61]. Another study on the effect of acetate on *Lens culinaris* showed that acetate increases the cellular accumulation of proline up to 24% and also it led to increasing the ascorbic acid content more than 2 times and catalase activity up to 1.5 times which both considered antioxidant agents [62]. Such results suggest that co-utilization of acetate has a compensatory effect for glucose toxicity in high concentrations because it triggers cells to accumulate proline with osmoprotective effect and increase the antioxidants' concentrations and activity.

The interaction between glucose and acetate may play a key role in a co-substrate strategy as indicated in primary analysis. In order to reveal such interaction between acetate and glucose on modified strain's growth rate, a *two-factor design* using General Linear Model (*GLM*) was employed. The resulting *ANOVA* analysis (Table 2) showed that acetate and glucose concentration had significant interactive effects on *Y. lipolytica* growth rate.

Table 2. *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 2, 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 ($F_{Glucose*Acetate}=105.0$, $p<0.0001$) on engineered *Y. lipolytica* strain growth rate and biomass productivity.

PHB synthesized by engineered *Y. lipolytica* showed to be a growth associated product which was accumulated in produced biomass simultaneously with the yeast growth. Therefore, in order to find the optimal medium for the 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 glucose and 10 to 20 g/L sodium acetate as the optimum concentrations of these substrates using in a co-substrate strategy (Figure 5A). 3D surface plot predicted that increasing the concentration of both glucose and acetate have inhibitory effects on this strain's growth rate.
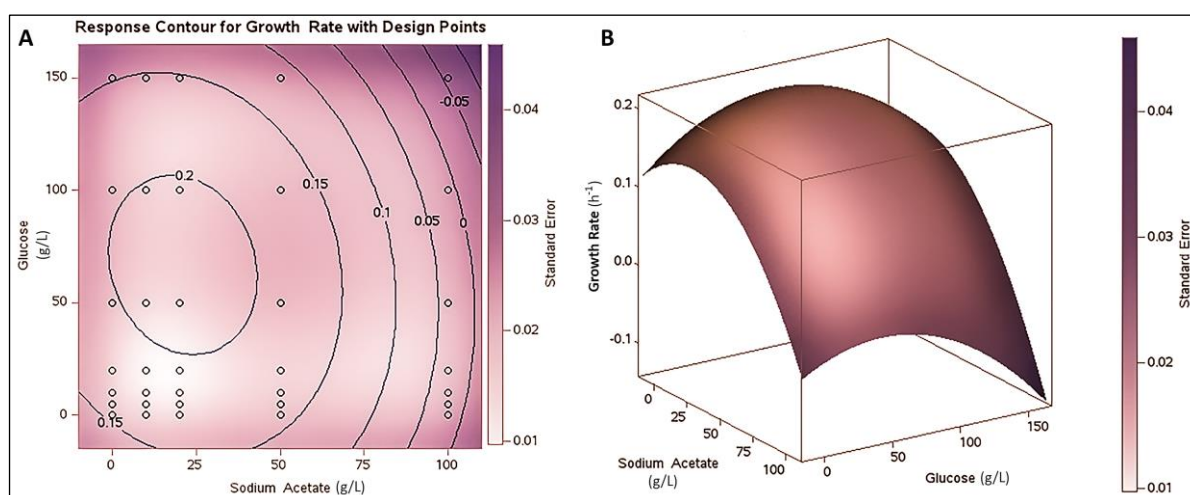
Figure 5. A) 2D contour plot indicating the optimum levels of substrates for maximum growth rate, B) 3D surface plot shows the effect of glucose and sodium acetate on modified strain's growth rate.

3.3. Bioreactor Production of PHB

During the aerobic fermentation using *Y. lipolytica* PHB32 strain (in a controlled pH and co-substrate medium), fed-batch system used for maintaining the substrates concentration on their optimal level (Figure 6A). Initial concentration of glucose and sodium acetate set at 50 g/L and 20 g/L based on *RSM* optimal prediction (Figure 6B). Using this system, the CDW reached to 83.4 g/L with a noticeable high PHB titer of 31.7 g/L (Figure 6C).
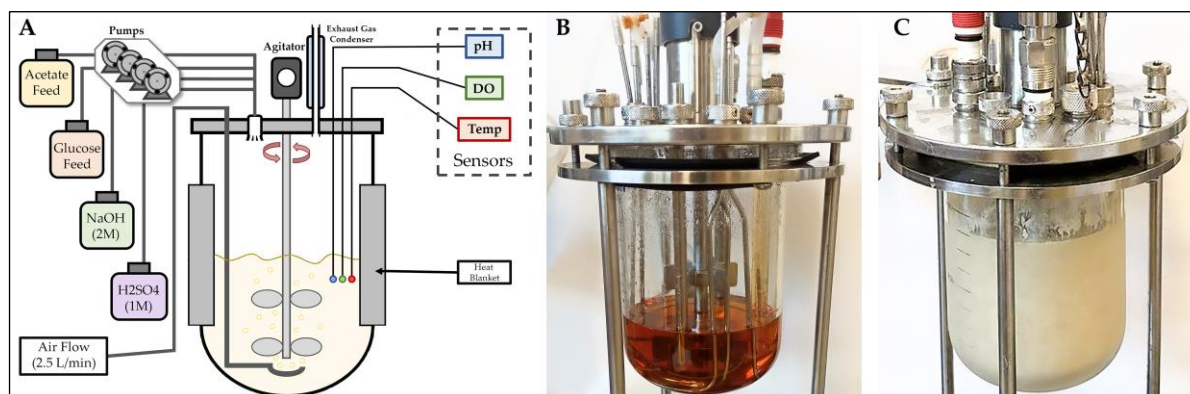
Figure 6. A) Schematic diagram of the fed-batch bioreactor, B) Bioreactor before inoculation and fermentation, C) Bioreactor at the end of fermentation using Y. lipolytica PHB32.

Results showed that the polymer content in fed-batch culture and flask experiment was almost same. This fact suggested that changing the pH and also nutrient starvation during flask cultivation (as 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 expression level of introduced heterologous PHB pathway [12]. In our study, PHB content in the bioreactor experiment using YPD medium reached to 38% of CDW in modified *Y. lipolytica* PHB32. These results are significantly higher than that previously engineered *Y. lipolytica* CAB strain using *ABC* pathway [12]. *Y. lipolytica* PHB32 showed noticeably high capacity for PHB production not only for its high PHB titer, but also high biomass productivity even using minimal YNB medium. In an attempt, instead of using either *ABC* or *NBC* pathway, just PHA synthase (*phaC*) of *Pseudomonas aeruginosa* was introduced to this yeast which led to 2% PHA accumulation in produced biomass. Result of *Haddouce* and colleagues study showed that between six acyl-CoA oxidase (*Aox*) isoenzymes, *Aox*3p has a key role on converting C9:0 and C13:0 fatty acids into PHA [63]. Similar study used variant *PhaC* as the single heterologous gene for synthesizing

medium-chain-length (mcl) PHA in engineered *Y. lipolytica*. Up to 25% (g/g) of PHA accumulation observed in some of these *PhaC* variants. In this study some of the modified strains were capable to synthesis mcl-PHA with an average molar mass of 316,000 g/mol which has a high potential in packaging applications [14]. Multicopy integration *PhaC*1 (*P. aeruginosa* PAO1) into *Y. lipolytica* led to an engineered strain which could accumulate up to 5% mcl-PHA in its biomass. In this study the highest biomass concentration reached to 21.9 g/L of CDW using YNB medium [13]. Among these strategies *NBC* pathway seems to be a more efficient pathway for PHB biosynthesis in *Y. lipolytica*.

4. Conclusion

NBC PHB pathway was introduced into the *Y. lipolytica* genome. The resulting engineered strain showed to be capable of accumulating PHB up to 41 % of CDW in shake flask culture using glucose and acetate as substrate. These results suggest that the *NBC* PHB pathway is a more efficient strategy compared to the *ABC* PHB pathway or introducing *PhaC* as a single gene pathway in *Y. lipolytica*. Using acetate or glucose as sole carbon and energy source showed slow growth rate and inhibitory effects in high concentrations. Co-substrate strategy using both substrates simultaneously increased the modified strain's growth rate significantly. Simultaneous use of glucose and acetate showed an alleviative effect for substrate inhibition which suggests co-substrate strategy as a reasonable method for cultivating *Y. lipolytica* in high concentrations of substrate. For bench-scale production of PHB co-substrate strategy with a constant pH in a fed-batch fermentation system was used. During the fermentation, substrates' concentrations maintained at optimal condition (based on *RSM* results). Bioreactor cultivation led to 83.4 g/L biomass and 31.7 g/L PHB concentration which is shows noticeably higher performance in PHB productivity using *Y. lipolytica* compared to previous studies.

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