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Insertion of Three Genes Encoding *Polyhydroxybutyrate* into *Escherichia coli* BL21 as the First Host: A Preliminary Exploration of Plasmid Transformant

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Abstract. The use of biodegradable plastics presents a sustainable option as concerns about the environmental effects of petroleum-based plastics grow. PHB (*polyhydroxybutyrate*) is one of the biodegradable biopolymers, which can be produced by *Escherichia coli* encoded by the *PhaA, PhaB*, and *PhaC* genes. This research aims to determine and study the ability of *E. coli* BL21 as a host in transforming the *PhaA, PhaB*, and *PhaC* genes encoding PHB, and characterizing successful *E. coli* BL21 carrying each gene. Recombinant plasmids carrying *PhaA*, *PhaB*, and *PhaC* genes were constructed by cloning into a *pUC57* vector and transformed into *E. coli* BL21 by electroporation. The positive transformants were analyzed using colony-PCR, restriction digestion, and Sanger Sequencing to confirm the successful insertion of the target. Recombinant plasmids were isolated, purified, and verified, obtaining target fragments of 1195bp for *PhaA*, 752bp for *PhaB*, and 1786bp for *PhaC*. The results showed that *E. coli* BL21 bacteria could act as a transformation host for the recombinant plasmid carrying the *PhaA, PhaB*, and *PhaC* genes, which was confirmed through growth on ampicillin LB medium. The *PhaA, PhaB*, and *PhaC* genes were successfully transformed with recombinant *E. coli* BL21 and showed a gene size of 1195 bp, 752 bp, and 1786 bp, respectively. Based on sequences analyses showed a high percent identity and query cover against the reference gene database (KP681582, KP681583, and KP681584). The entire *PhaA, PhaB*, and *PhaC* gene sequence has been inserted into recombinant cells, with the result of cutting according to the gene band size and the *pUC57* plasmid size. This study provides a foundation for the optimal expression of PHB in recombinant bacteria and serves as a reference in designing genetic transformation strategies for E. coli BL21. The method is also of industrial relevance for replacing or reducing conventional plastics, thereby boosting the circular bioeconomy and directly contributing to the UN Sustainable Development Goals, especially in SDG 9 which focuses in industry, innovation, and infrastructure and SDG 12 which focuses in responsible consumption and production.

Keywords: biodegradable plastic; *E. coli* BL21; *polyhydroxybutyrate*; recombinant plasmid

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INTRODUCTION

Plastic waste is still a significant problem globally (Adeniran & Shakantu, 2022; Evode et al., 2021). The plastics synthesized from petroleum-based polymers remain a major

pollutant to the environment (Shen et al., 2020). Polymers such as polystyrene, polyethylene, polyvinyl chloride, and polypropylene are difficult for environmental organisms, including natural microbes, to degrade over 1200 years (Ghatge et al., 2020). It means that people must develop various techniques to address the issues to enhance the well-being of all living things on earth (Tiago et al., 2023).

Biodegradable plastic may be an excellent approach to solving environmental problems (Havstad, 2020). Some microorganisms can degrade the polymer (Nduko & Taguchi, 2021). It can be broken down into simpler molecules like (CO_2) or water (H_2O) (Moshood et al., 2022). Also, these plastics can be recycled and decompose faster than conventional ones (Báreková et al., 2021; Guliyev et al., 2023). The resulting molecules are environmentally non-toxic and relatively safe (Mahmoud et al., 2023).

PHB (*polyhydroxybutyrate*) is categorized under PHA (polyhydroxyalkanoate), one of the most prevalent types of bioplastics (Raju et al., 2024). This biopolymer has a shorter chain and is composed of 3-hydroxybutyrate units (Ushani et al., 2020). PHB can be produced within the cytoplasm of microorganisms (Aristya et al., 2022). PHB is produced as a result of the secondary microbial activities under nutrient-deficient or stressful environmental conditions (McAdam et al., 2020). This phenomenon is observed in both gram-positive and gram-negative bacteria (Chanprateep, 2010). PHB accumulates in the cells as a carbon and energy reserve under nutrient imbalance or starvation conditions (Mohanrasu et al., 2022).

PHB retains both biodegradable and biocompatible properties (Moreira et al., 2022; Wei et al., 2011). It is a biosynthetic thermoplastic (Olejnik et al., 2021). As people become more aware of the problems related to climate change, the topic is gaining importance (Sirohi et al., 2021). PHB may aid in solving the problem of plastic waste accumulation and microplastic environmental pollution (Manikandan et al., 2020). It also has the potential for application in various other fields, such as biomedicine, tissue engineering, food processing and coating, and waste management (Akhlaq et al., 2023).

The PHB synthesis is expressed through an essential metabolic pathway in bacteria (McAdam et al., 2020). This pathway is controlled by the expression of three genes. First, bacteria produce acetyl-CoA. After that, the 3-ketothiolase enzyme, regulated by the *PhaA* gene, combines two acetyl-CoA molecules to produce acetoacetyl-CoA (Batista et al., 2018). The *PhaB* gene regulates the expression of acetoacetyl-CoA reductase, which is responsible for reducing acetoacetyl-CoA from the previous reaction to 3-hydroxybutyryl-CoA (Peoples & Sinskey, 1989). The 3-hydroxybutyryl-CoA molecule is then synthesized by the PHB synthase enzyme, whose expression is regulated by the *PhaC* gene, to become PHB (Rehm, 2003). PHB production generally emerges when nutrients are limited (McAdam et al., 2020).

PHB synthesis has been successfully documented across various bacterial species, including *Ralstonia eutropha* (Rondľošová et al., 2022), *Escherichia coli* (Narayanan et al., 2020), *Shewanella marisflavi* (Lee et al., 2021), *Synechocystis* sp. (Rueda et al., 2022), and *Vibrio alginolyticus* (Li et al., 2023). Among these, *E. coli* stands out as a proficient PHB production. Jiang et al. (Jiang et al., 2023) executed PHB synthesis from *E. coli* DH5α through genetic manipulation using the pETDuet plasmid. However, there is a lack of research regarding PHB synthesis from *E. coli* BL21 transformants. This study aimed to assess the PHB synthesis capability of *E. coli* BL21 by transmitting the *PhaA, PhaB*, and *PhaC* genes.

No research has specifically focused on the PHB productivity of *E. coli* BL21. *E. coli* is a well-suited bacterium to be used as competent cells, which makes it likely to accept the transformation of the target gene that encodes PHB. Consequently, it is important to advance research related to PHB productivity to identify the most effective methods for addressing issues associated with plastic waste through genetic engineering.

METHODS

Microorganism and media

Plasmid *pUC57* was used for the *PhaA, PhaB*, and *PhaC* genes in *E. coli* BL21, which was cultured in LB broth. Single colonies were selected and subcultured from 1 mL to 4 mL and then to 50 mL of LB broth, incubating at 37°C for 12 hours at 200 rpm until the OD600 reached 0.6-0.8. Cultures were harvested by centrifugation at 5,000 rpm for 5 minutes at 4°C (Tuttle et al., 2021).

Plasmid construction and primer design

The design of recombinant plasmids was referred to in research by Aristya et al., (2022) with several adjustments. The DNA sequences of the *PhaA*, *PhaB*, and *PhaC* genes were inserted

into the MCS of the *pUC57* plasmid. Forward and reverse primers were designed to identify the nearest restriction site to the target gene on the *pKLAC2* plasmid. Additionally, the selected restriction site had to have only one cutting site on the plasmid. The forward primer was designed by selecting 20 bases upstream of the target gene's starting base on the plasmid. The same approach was used for designing the reverse primer.

Bacterial transformation

The *pUC57* recombinant plasmid containing the *PhaA*, *PhaB*, and *PhaC* genes was introduced into *E. coli* BL21 competent cells through electroporation using the method described by Aristya (2022). 50 µL of competent bacterial cell stock was combined with 1 µL of the plasmid carrying the genes and transferred into a 1 mm electroporation cuvette. The electroporation process was conducted using an electroporator set to an electrical voltage of 2500 volts, resistance 100 Ω, and capacity 25 µF. The transformed cells were cultured in 1 mL of LB medium and incubated for 40 minutes at 37°C with agitation at 200 rpm. Afterward, the cultures were harvested by centrifugation, and the resulting pellets were plated on LB agar medium supplemented with 10% ampicillin using a spread plate technique. The cultures were incubated for 12 hours at 37°C to allow for growth.

Transformant screening

Colony PCR was used to ascertain the existence of *PhaA, PhaB*, and *PhaC* genes in the transformant colonies. A total of 30 selected colonies of the successfully grown transformant were used as a template. The amplification process utilized three pairs of primers for each target gene to amplify specific segments (Table 1). The PCR reaction was performed according to Aristya et al. (2022) with adjustments. The products were subjected to horizontal electrophoresis in a 2% agarose gel for visualization.

Furthermore, the successful insertion of each gene into the host *E. coli* BL21 was also analyzed by Sanger Sequencing. Purification of PCR products was carried out using 2 µL of ExoSAP-ITTM reagent. The supernatant was then collected in an amount of 10-20 µL and placed in vials, which were subsequently sealed. Sequencing data analysis was performed using SeqA software and BLAST NCBI.

***E. coli* BL21 recombinant plasmid isolation**

A single *E. coli* BL 21 colony containing the recombinant plasmid was grown in 7 mL of LB broth enriched with 10% ampicillin. The incubation occurred at 37°C, with agitation at 200 rpm, for 12 hours. After that, the recombinant *E. coli* BL21 culture was isolated using the plasmid isolation kit with the original protocol (Geneaid, PrestoTM Mini Plasmid Kit). The isolate was incubated at room temperature for 2 minutes and subjected to a final centrifugation at 16,000 g for 2 minutes. DNA concentration and purity were then calculated (Allen et al., 2017).

Restriction enzyme on *PhaA*, *PhaB*, and *PhaC* from the plasmid

Restriction enzymes were used to break down the plasmid's target gene to confirm the transformation. The gene's location within the plasmid determined the enzyme selection (Table 2). A mixture that consists of 5 µL of 10x rCutSmart Buffer, 1 µL of restriction enzyme for both the upstream and downstream regions, 1 µg of DNA template, and nuclease-free water up to a total volume of 50 µL was prepared. The mixture was incubated for 15 minutes at 37°C. After the incubation, the mixture was heat-inactivated for 20 minutes at 80°C. Gel electrophoresis was used to analyze the digestion products. These steps were used for *PhaA* and *PhaC* restriction preparation (Loenen et al., 2014).

Table 1. Oligonucleotides were used in this study.

Oligonucleotide	Sequences (5′ – 3′)	References
PhaA-F	GGCAAGAAAATATGTCAGCCGCACCTCATG	Aristya *et al*., 2022 (Aristya et al., 2022)
PhaA-R	GGCATAGGACTTTATGGTGGCTAGCGGTGT	Aristya *et al*., 2022 (Aristya et al., 2022)
PhaB-F-new	TCGTCGACATGACGCAAAGG	This study
PhaB-F-new	CCTGCAGGTTAACCCATGTG	This study
PhaC-F-new	GTCGACGGATATGGCCACTGGTAA	This study
PhaC-F-new	CTGCAGGGAATTCGGTTATGCCTT	This study

Table 2. Restriction enzyme and restriction site

Gene	Restriction enzyme	Restriction site
PhaA	*NcoI; SbfI-HF*	C/CATGG; CCTGCA/GG
PhaB	*SalI; SbfI-HF*	G/TCGAC; CCTGCA/GG
PhaC	*NcoI; SbfI-HF*	C/CATGG; CCTGCA/GG

Difference for the PhaB gene, the digestion was performed using a premix of 5 µL of 10x rCutSmart Buffer, 1 µL of SbfI-hf restriction enzyme, 1µg of DNA template, and up to 50 µL of nuclease-free water. The premix was then incubated at 37°C for 15 minutes. The results were then purified according to GenepHlowTM Gel/PCR Kit Quick Protocol with only 10µL of elution buffer. The second reaction used a premix of 5 µL of 10x r3.1 Buffer, 1 µL of SalI restriction enzyme, 10µl of purified DNA template from the previous reaction, and up to 33 µL of nuclease-free water. The premix was then incubated at 37°C for 15 minutes, followed by heat inactivation at 65°C for 20 minutes.

Gel extraction

The gel extraction procedure followed the GenepHlowTM Gel/PCR Kit Quick ProtocolDNA concentration and purity could be analyzed using a nanodrop spectrophotometer. The validation of result were showed the size of PhaA, PhaB, and PhaC for 1195 bp, 752 bp, and 1786 bp, respectively, from the total size recombinant plasmid PhaA, PhaB, and PhaC for 3908, 3465 bp, and 4496 bp, respectively.

RESULTS AND DISCUSSION

E. coli BL21 was chosen as a host of competent cells due to its compatibility with genetic engineering, ability to express recombinant proteins, rapid proliferation, and quick harvestability (Zhang et al., 2022). Internal and external factors can influence bacterial growth (Qiu et al., 2022). The cultivation system is crucial for bacterial culturing, particularly the optimal temperature and aeration (Qiu et al., 2022). *E. coli* thrives at 37°C and requires several procedural considerations (Tuttle et al., 2021).

OD_{600} values from the random culture were 0.818, 0.741, and 0.746 (Table 3). These OD results are stable and show no significant differences, indicating that the culture remained in the logarithmic to stationary division phase at the analysis time. This OD value suggests that the bacterial cells are still in the logarithmic to the stationary growth phase (Kobayashi et al., 2006). The OD_{600} range typically used for producing competent *E. coli* cells is between 0.4 and 0.8 (Hans et al., 2018).The three genes involved in the PHB synthesis pathway were inserted into the MCS plasmid *pKLAC2*, as illustrated in Figure 1. The lengths of the *PhaA*, *PhaB*, and *PhaC* genes are 1195 bp, 752 bp, and 1786 bp, respectively. The design for the *PhaA* gene was obtained from GenBank data under accession number KP681582, the *PhaB* gene from accession number KP681583, and the *PhaC* gene from accession number KP681584.

Table 3. Competent cell culture's OD_{600} value.

Samples	OD600
1	0.818
2	0.741
3	0.746

Storing competent cells in 50% cold glycerol is intended to provide long-lasting conditions for the cells, allowing them to be stored for extended periods and enhancing their electrocompetence (Wu et al., 2010). Glycerol serves as a cryoprotectant, preventing the formation of ice crystals during freezing and thus protecting the cells from damage (Cabrera et al., 2020). Aliquoting the competent cell stock into several microliter-sized portions in each microtube reduces the number of freeze-thaw cycles when using the stock (Cabrera et al., 2020).

The *PhaA*, *PhaB*, and *PhaC* genes with lengths of 1195 bp, 752 bp, and 1786 bp were inserted into the MCS of *pKLAC2*. The plasmid backbone is 9107 bp, which increases to 12800 bp after the target genes are inserted. The *pKLAC2* plasmid operates as the expression vector that can replicate in *E. coli* and has been engineered to promote the expression of the inserted target gene (Aristya et al., 2022c).

The *pKLAC2* plasmid (Figure 1a) maintains components such as the *ADH1* promoter, *LAC4* promoter, *LAC4* transcriptional terminator, *amdS* selectable marker, and *ORI* (Zhan et al., 2014). Further, it also includes an ampicillin marker (Indriawati et al., 2018). This plasmid serves as an expression vector for gene expression (Gomes et al., 2019). The MCS site was chosen as the insertion site for the target gene due to its compatibility and ability to amplify the target gene

specifically (Figure 1a) (Crook et al., 2011). Besides, the MCS has several restriction sites that can be selected and adjusted per the needs of gene cloning. The *LAC4* promoter enables expression, which can be multiplied in *E. coli* prior to yeast cloning (Colussi & Taron, 2005). The *amdS* marker prompts non-antibiotic selection (Domínguez et al., 2016).

The transformation results in competent cells containing the target gene (Figure 2). Transformants are grown on LB agar plates supplemented with ampicillin antibiotics. The recombinant *E. coli* strain carries a plasmid with a marker for the ampicillin antibiotic. This procedure is conducted to confirm the success of the transformation. When the *pUC57* recombinant plasmid successfully enters the host cell, the *E. coli* BL21 will transform, resulting in resistance to the antibiotic ampicillin.

Thirty single colonies from the transformation were randomly selected and cultivated on a fresh LB medium plate supplemented with ampicillin (Figure 2). The transformant strain has an ampicillin resistance marker on the plasmid. Control samples (without the recombinant plasmid) were included to verify experimental conditions. No colonies were observed on the control medium, confirming that the bacterial cells did not possess the ampicillin resistance gene.

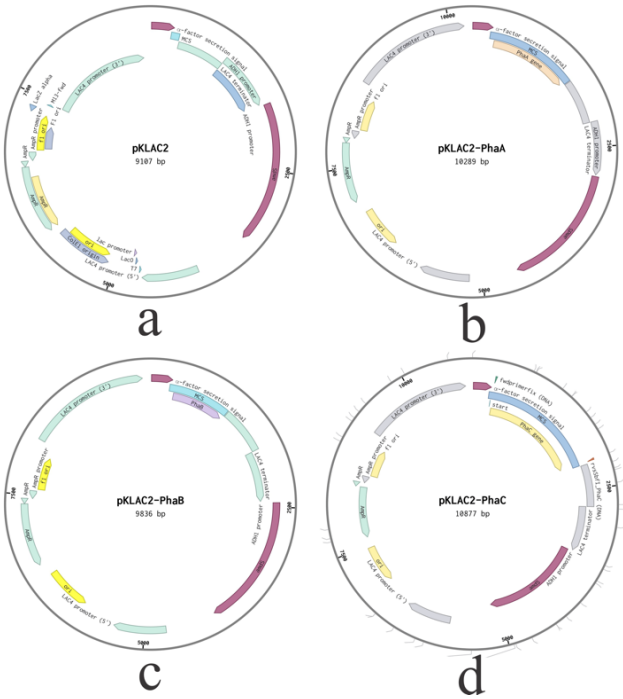

Figure 1. Construction of *pKLAC2* plasmid recombinant (a) Plasmid of *pKLAC2*, (b) *pKLAC2-PhaA* plasmid, (c) *pKLAC2-PhaB* plasmid, and (d) *pKLAC2-PhaC* plasmid.

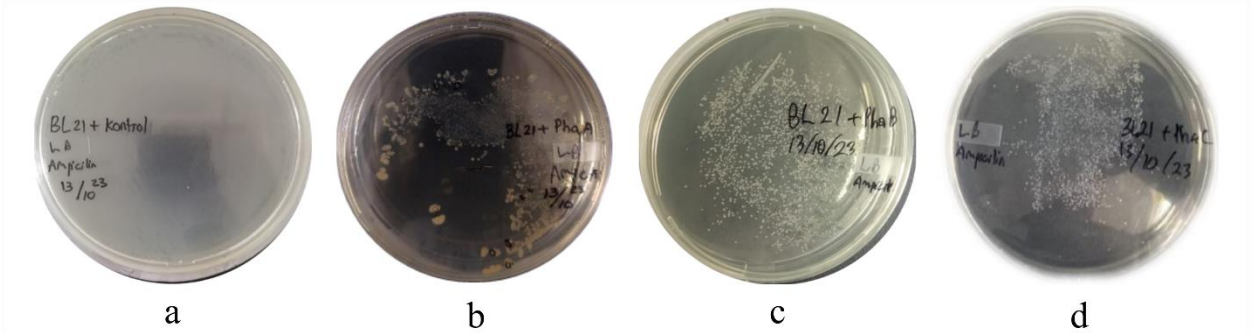

Figure 2. Transformed BL21 culture, (a) control + ampicillin, (b) culture + *PhaA* gene, (c) culture + *PhaB* gene, and (d) culture + *PhaC* gene.

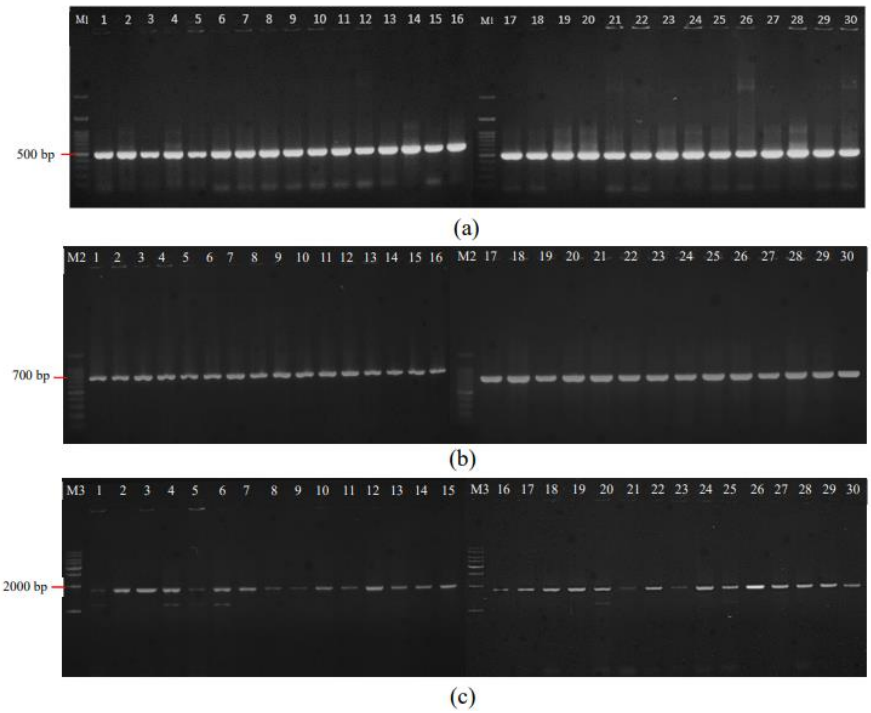

Figure 3. The electrophoresis results of the (a) *PhaA*, (b) *PhaB*, and (c) *PhaC* genes in 30 random colonies that were successfully transformed. M: Ladder; Number 1-30: representative sample number; (a) *PhaA* gene with 500 bp; (b) *PhaB* gene with 762 bp; (c) *PhaC* gene with 1770 bp. Note: Gels are cropped from various sections to the sizes that align with the target of the study. The image brightness and contrast are increased to make it easier for readers to observe.

The antibiotic resistance test method evaluates a bacterium's sensitivity to specific antibiotics (Kasagaki et al., 2022). Completing the entry of the *pUC57* recombinant plasmid into the host cell leads to the transformation of *E. coli* BL21 bacteria, generating resistance to ampicillin. As a result, the *E. coli* BL21 bacteria, which were initially unable to survive on media containing ampicillin, can grow on the media.

Recombinant cultures with successfully amplified genes were electrophoresed using an agarose gel (Figure 3). Out of 30 randomly selected cultures, the amplification band was observed, indicating the presence of a DNA band corresponding to the expected target size. This confirms that the 30 tested colonies were transformant *E. coli* colonies carrying the *PhaA*, *PhaB*, and *PhaC* genes.

The amplification and visualization were conducted using reference primers to ensure successful transformation detection in recombinant *E. coli* (Figure 3). The PCR results with these primers revealed bright bands at approximately 500 bp, 700 bp, and 1700 bp, indicating the specific amplification of the *PhaA*, *PhaB*, and genes (Aristya et al., 2022c). The target was also amplified using primers designed in this study, but the results were non-specific, making it ineffective for detection. Electrophoresis results confirmed that the *E. coli* BL21 bacteria effectively harbored the recombinant plasmids encoding the *PhaA*, *PhaB*, and *PhaC* genes.

The amplification results from the modified culture were subsequently confirmed by Sanger Sequencing for enhanced accuracy. The amplification findings of both primer sets were evaluated. The sequencing results were then compared with NCBI data to identify each amplicon. The alignment results indicate that the whole sequence is highly identical to each reference. These findings imply that the recombinant *E. coli* BL21 strain holds the *PhaA*, *PhaB*, and PhaC target genes. The query cover and percent identity values are close to 100% (Table 4).

The query cover value represents the extent to which the nucleotide lengths within the query sequence correspond to those found in the database sequences (Boratyn et al., 2013; Janaki et al., 2021). The percent identity value denotes the degree of nucleotide similarity between the query sequence and the sequences within the database (Nestor et al., 2023).A more favorable degree of sequence similarity with the database is characterized by an elevated percentage of query cover and percent identity (Nestor et al., 2023).

Table 4. BLAST results of recombinant *E. coli* BL21 from amplification of the *PhaA*, *PhaB*, and *PhaC* genes.

Sample	Primer used	Accession numbers	BLAST results Query cover (%)	Identity (%)
E. coli BL21 recombinant	*PhaA*-F & *PhaA*-R	KP681582	99	100
	PhaB-F & *PhaB*-R	KP681583	100	99.85
	PhaC-F & *PhaC*-R	KP681584	97	98.90

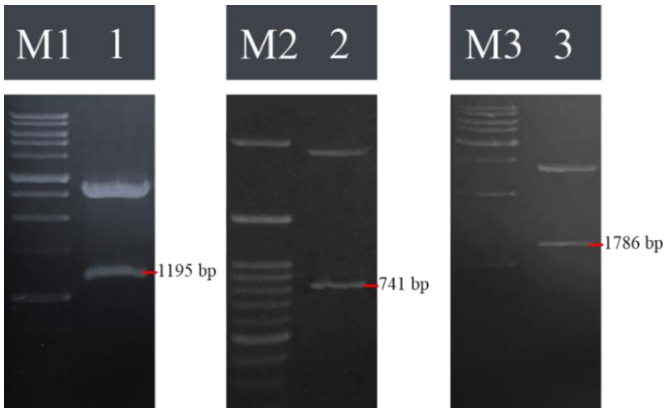

Figure 4. Visualization of plasmid digestion results by the specific restriction enzyme used in this study. M: Ladder, 1: *PhaA* (1195 bp), 2: *PhaB* (741 bp). 3: *PhaC* (1786 bp). Note: Gels are cropped from various sections to the sizes that align with the target of the study. The brightness and contrast are increased to make it easier for readers to observe.

All plasmids were digested by specific two restriction enzymes to allow the appearance of two bands of different sizes. The *pUC57* plasmid with the *PhaA* gene has a total size of 3908 bp, the one with the *PhaB* gene has 1191 bp, and the one with the PhaC gene has 2710 bp. The *pUC57-PhaA* plasmid was digested with *NcoI* and *SbfI-HF* enzymes, and the obtained *PhaA* band length was 1195 bp (Figure 4). The *pUC57-PhaB* plasmid was digested with *SalI* and *SbfI-HF* restriction enzymes to release the *PhaB* fragment (762 bp) (Figure 4). The digestion of *pUC57-PhaC* with *NcoI* and *SbfI-HF* resulted in a band of 1786 bp (Figure 4). These results corroborated that each target DNA band could be cleaved as predicted by in-silico analysis.

Sanger sequencing and restriction enzyme digestion were employed to validate the amplification outcomes and enhance the probability of success. The BLASTn analysis revealed that all sequenced samples exhibited identity values surpassing 98% (Table 4). Additionally, restriction enzyme digestion effectively segregated the gene integrated into the *pKLAC2* plasmid according to the anticipated target size (Figure 4). These findings confirm the successful integration of the target gene into *E. coli* BL21.

The study offers insights into the possibility of improving recombinant plasmid construction and genetic transformation strategies for optimal PHB (*polyhydroxybutyrate)* production. This study's approaches help improve gene expression efficiency by utilizing bacteria. The findings are important to the practical implementation of sustainable solutions. It offers a foundation that future researchers can expand to other industries.

This research integrates the achievement of SDG 9 and SDG 12 in a sustainable manner. Genetically engineered *E. coli* that produces PHB biopolymers is a form of cutting-edge biotechnology innovation that lays the foundation for a sustainable, bio-based manufacturing industry, which is correlated with SDG 9 (Industry, Innovation, and Infrastructure). This innovation also serves as the key instrument for achieving SDG 12 (Responsible Consumption and Production). By providing a biodegradable plastic alternative from renewable sources, the use of PHB offers a concrete solution to substantially reduce waste accumulation at the end of its useful life.

CONCLUSION

E. coli BL21 successfully enables as a host for the transformation of recombinant plasmids carrying every three genes, confirmed through

growth on LB ampicillin media. Each *PhaA, PhaB,* and *PhaC* gene was successfully transformed into recombinant *E. coli* BL21, showing a gene size of 1195 bp, 752 bp, and 1786 bp, respectively, and high percent identity and query cover against the reference gene database (KP681582, KP681583, and KP681584, for *PhaA, PhaB,* and *PhaC,* respectively). The entire sequence of the PHB gene has been inserted into the recombinant cells, resulting in each *PhaA, PhaB,* and *PhaC* gene band size of 1195 bp, 752 bp, and 1786 bp, respectively. The study enhances methods for gene expression in bacteria, which is a key step toward finding sustainable solutions. It offers a foundation for future researchers to build upon and expand. Future studies need to focus on the fermentation stage to quantitatively analyze the overexpression of PHB products from *PhaA, PhaB,* and *PhaC* genes. This will increase awareness of how recombinant plasmids are constructed and their use in biological processes.

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AUTHOR CONTRIBUTION STATEMENT

GRA: Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing. ATN: Data curation, Formal analysis, Visualization. LASM: Data curation, Formal analysis, Visualization. BAD: Data curation, Methodology, and visualization. AM: Methodology, Resources. APJ: Formal analysis, Investigation, Software. MFA: Methodology, Visualization, Writing – original draft. ARS: Conceptualization, Formal analysis, Writing – review & editing. IBN: Data curation, Methodology, Validation. HWY: Conceptualization, Supervision, Writing – review & editing

CONFLICT OF INTEREST STATEMENT

All authors agree to publish this article. There are no competing interests in this research.

USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that no artificial intelligence (AI) tools were used in the generation, analysis, or writing of this manuscript. All aspects of the research, including data collection, interpretation, and manuscript preparation, were carried out entirely by the authors without the assistance of AI-based technologies.

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