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Development of a Molasses-Based Medium for *Agrobacterium tumefaciens* Fermentation for Application in Plant-Based Recombinant Protein Production

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Abstract: The *Agrobacterium*-mediated transient gene expression system is a rapid and efficient method for heterologous recombinant protein expression in plants. The fermentation of genetically modified *Agrobacterium tumefaciens* is an important step in increasing the efficiency of recombinant protein production in plants. However, the limitation of this system that makes it economically non-competitive for industrial-scale applications is the *Agrobacterium* suspension production cost. In this study, the utilization of sugarcane molasses as an alternative low-cost source of carbon at a concentration of 8.7 g/L and nitrogen at a concentration of 2.4 g/L for *Agrobacterium* cultivation was investigated. Molasses pretreatment using sulfuric acid (SA) was applied before fermentation, and it resulted in a maximum specific growth rate of $0.232 \pm 0.0063\ h^{-1}$ in the *A. tumefaciens* EHA105 culture. The supplementation of antibiotics in the molasses-based medium was shown to be unnecessary for plasmid maintenance during fermentation in both *Agrobacterium* strains, which helped to reduce the production cost. We evaluated recombinant protein production using an *Agrobacterium* culture without antibiotic supplementation in the growth media by demonstrating green fluorescent protein expression in wild-type *Nicotiana benthamiana* leaves. In the evaluation of the culture medium cost, the molasses-based medium cost was 6.1 times lower than that of LB. Finally, this study demonstrated that the newly developed molasses-based medium for *Agrobacterium* fermentation is a feasible and effective medium for transient recombinant protein production in plant tissues.

Keywords: sugarcane molasses; molasses pretreatment; fermentation; *Agrobacterium tumefaciens*; transient protein expression

Citation: Watthanasakphuban, N.; Nguyen, L.V.; Cheng, Y.-S.; Show, P.-L.; Sriariyanun, M.; Koffas, M.; Rattanaporn, K. Development of a Molasses-Based Medium for *Agrobacterium tumefaciens* Fermentation for Application in Plant-Based Recombinant Protein Production. *Fermentation* **2023**, *9*, 149. https://doi.org/10.3390/fermentation9020149

Academic Editor: Johannes Wöstemeyer

Received: 9 December 2022
Revised: 26 January 2023
Accepted: 31 January 2023
Published: 2 February 2023

1. Introduction

A transient expression system is an attractive production system for transgenic expression that has been successfully used to investigate gene expression [1], virus-induced gene silencing [2] and gene-for-gene interactions between host resistance and pathogen virulence genes [3]. However, the high cost of the culture medium restricts the economic value of this system. In general, *Agrobacterium* is grown on Luria Broth (LB) or Yeast Extract Peptone (YEP) medium, which are complex and expensive media and therefore are not

appropriate for industrial-scale manufacturing. The development of sugar-based defined media has an undesirable influence on *Agrobacterium* growth, with a lower cell growth rate than in typical LB media cultivation. However, the final biomass concentration is higher, and a sugar-based defined medium provides a reproducible composition and is more economical based on the cost of materials per liter [4].

Cane molasses is a major byproduct of the sugar manufacturing process. Generally, cane molasses contains 48–60% (*w*/*w*) total sugars (sucrose, glucose, fructose, raffinose) [5], nitrogen compounds, metal ions, vitamins, solid sludge and ash [6], which makes it an attractive alternative growth medium for microorganisms for industrial-scale fermentation. However, due to the presence of trace elements in soils, fertilizers and irrigation water, each batch of molasses has a varied component concentration [7]. Some components, such as metal ions, are undesirable for microbial fermentation due to their effects on microbial growth [7]. The metal ions in molasses have been reported as the major growth-inhibiting substances for some microorganisms [7–9], and it has been suggested that metal ions taken up by microorganisms affect cell metabolism or are toxic to the cells [10].

Metal ions were eliminated using a pretreatment approach when molasses was used as the fermentation medium. Pretreated cane molasses has been utilized to produce a variety of useful compounds, including Coenzyme Q10 from *Paracoccus denitrificans ATCC 19367 Fusant* strain PF-P1 [11], Probiotic *Lactobacillus paracasei ssp. paracasei* F19 [12] and ethanol from *Saccharomyces cerevisiae* [13]. However, to date, there have not been any studies demonstrating the use of cane molasses as a carbon source in the culture medium for *Agrobacterium tumefaciens* cultivation for recombinant heterologous protein production in plants. Recently, molasses has been used as a cost-effective carbon source for culture medium formulation in industrial-scale fermentations. The results revealed that the cost reduction achieved compared to nutrient-rich commercial media was approximately 30–68.7% [14]. Furthermore, the addition of antibiotics to culture media for engineered microbes could enhance antibiotic resistance and may cause the morphological transformation of genetically engineered bacteria [15]. Therefore, the objective of this study was to investigate a low-cost medium for *Agrobacterium tumefaciens* using molasses as an alternative cost-effective carbon source. The reduction in antibiotic supplementation was also studied to further minimize the cost of the culture medium. To reduce the toxicity of byproducts in molasses, three different pretreatment methods were evaluated based on the growth of *Agrobacterium tumefaciens*. The cost evaluation of culture media was also calculated to provide the information and potential of the modified molasses medium. The results from this study would be beneficial for producing vaccines or human therapeutic proteins, with the advantage of using a reproducible medium that is cost-effective and free of animal-derived components.

2. Materials and Methods

2.1. Bacterial Strains, Plasmids and Culture Conditions

Agrobacterium tumefaciens strains EHA105 and GV3301 (courtesy of Prof. Karen McDonald, University of California Davis, Davis, CA, USA) were grown in LB medium at 28 °C for 24 h with agitation in a shaking incubator (model VS-8480SFN, Vision Scientific Co., Ltd., Daejeon, Korea) at 200 rpm and were used to carry the pEAQ vector, the recombinant binary plasmid containing the *gfp* reporter gene. Antibiotics were used for *A. tumefaciens* EHA105-pEAQ at final concentrations of 50 mg/L kanamycin, 10 mg/L rifampicin and 5 mg/L tetracycline. For *A. tumefaciens* GV3301-pEAQ, the antibiotic concentrations were 50 mg/L kanamycin, 10 mg/L rifampicin and 50 mg/L gentamicin.

2.2. Cane Molasses Pretreatment

Cane molasses obtained from the sugar industry in the north-eastern part of Thailand was prepared by diluting 1.5 times (*w*/*v*) with deionized water before three pretreatment methods were applied, as follows:

1. Sulfuric acid pretreatment (SA): The diluted cane molasses pH was adjusted to 3.0 using concentrated H_2SO_4 (95–97%) solution and incubated for 1 h in a water bath at 70 °C.
2. Tricalcium phosphate pretreatment (TC): The diluted cane molasses was pretreated with 2% (*w*/*v*) tricalcium phosphate and autoclaved at 105 °C for 5 min.
3. Tricalcium phosphate and sulfuric acid pretreatment (TCSA): The tricalcium phosphate-pretreated sample (TC) was adjusted to pH 3.0 using concentrated H_2SO_4 and incubated at 70 °C for 1 h.

All pretreated cane molasses samples were cooled down to room temperature, and the sediment was removed using centrifugation at 5000× *g* for 10 min (model MX-305, TOMY SEIKO Co., Ltd., Tokyo, Japan). The pretreated cane molasses samples were then adjusted to pH 7.0 using 2N NaOH before measuring total carbohydrates, total nitrogen and heavy metal content using the phenol-sulfuric method [16], Kjeldahl method and heavy metal determination method (AOAC, 2016), respectively. The pretreated samples were stored at −20 °C or diluted with distilled water to obtain the desired concentration and autoclaved at 121 °C for 20 min prior to use.

2.3. Effect of Molasses-Based Medium Formulation on the Growth of Engineered Agrobacterium tumefaciens

The effect of sucrose and cane molasses on the growth of *A. tumefaciens* strains and the medium composition are summarized in Table 1. The growth of *A. tumefaciens* in LB medium was compared to a sucrose-based defined medium (SBM) using sucrose as a sole carbon source to study the effect of sucrose. Meanwhile, a low-cost medium, a simplified sugarcane molasses-based medium (MBM), was also studied and compared to LB medium for supporting cell growth. Sugarcane molasses processed using three different pretreatment methods was applied to molasses-based media to study the effects of the pretreatment methods.

Table 1. Culture media used in this study.

Culture Medium	Compositions		pH	References
	Main Carbon Source	Medium Composition		
Lysogeny Broth **(LB)**	Casein tryptone type I (10 g/L)	Yeast extract (5 g/L) NaCl (10 g/L)	7.0	[12]
Initial basal medium **(IBM)**	Sucrose (20 g/L)	Yeast extract (5 g/L), K_2HPO_4 (6 g/L), NaH_2PO_4 (7 g/L), NH_4Cl (0.7 g/L), $MgSO_4$ (0.5 g/L)	7.0	[17]
Sucrose-based defined media **(SBM)**	Sucrose (4 g/L)	$(NH_4)_2SO_4$ (2.5 g/L), $MgSO_4{\cdot}7H_2O$ (0.6 g/L), $CaCl_2{\cdot}2H_2O$ (0.066 g/L), $FeSO_4{\cdot}7H_2O$ (10 mg/L), $MnSO_4{\cdot}H2O$ (0.6 mg/L), $ZnSO_4{\cdot}7H_2O$ (0.6 mg/L), Phosphate buffer 0.05 M	7.0	[4]
Molasses-based media **(MBM)**	Molasses (X g/L) *	$(NH_4)_2SO_4$ (2.5 g/L) $MgSO_4{\cdot}7H_2O$ (~0.25 g/L) ** $CaCl_2{\cdot}2H_2O$ (~0.031 g/L) ** Phosphate buffer 0.05 M	7.0	This study

Note: * Molasses concentration was adjusted to have an equal concentration of sucrose of 4 g/L. ** The concentrations of $MgSO_4$. and $CaCl_2$ depend on magnesium and calcium concentrations after pretreatment with various pretreatment methods and were adjusted to have final concentrations of 0.066 g/L $CaCl_2{\cdot}2H_2O$ and 0.6 g/L $MgSO_4{\cdot}7H_2O$.

The culture medium (50 mL) was prepared in 250 mL Erlenmeyer flasks in triplicate. Each medium was inoculated with 2% (*v*/*v*) overnight seed culture and incubated at 28 °C for 24 h with 200 rpm agitation in a shaking incubator (model VS-8480SFN, Vision Scientific Co., Ltd., Daejeon, Korea). At 4 h intervals, cell growth was measured via viable plate

count on LB agar supplemented with appropriate antibiotics and incubated at 28 °C for 48 h.

2.4. Effect of Minerals and Heavy Metals on Growth of Engineered Agrobacterium tumefaciens

The molasses-based medium was prepared in 250 mL Erlenmeyer flasks in triplicate using the medium components presented in Table 1. Cane molasses pretreated with 0.65%, 0.6% and 0.65% (*v*/*v*) SA, TC and TCSA was added to obtain a final sucrose concentration of 4 g/L and then autoclaved at 121 °C for 60 min. After autoclaving, the molasses-based medium was supplemented with exogenous filter-sterilized $FeSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$ and $ZnSO_4 \cdot 7H_2O$ at final concentrations of 10 mg/L, 0.6 mg/L and 0.6 mg/L, respectively. The 2% seed culture was inoculated and incubated at 28 °C for 24 h with 200 rpm agitation. After 24 h incubation, the samples were taken and checked for viable cell growth on the LB plate.

2.5. Effects of Antibiotics on Cell Growth and Genetic Instability

A. tumefaciens EHA105-pEAQ and *A. tumefaciens* GV3301-pEAQ were first cultivated in SA, TC or TCSA molasses-based medium and LB medium with and without antibiotic supplementation. The cultures were incubated at 28 °C with shaking at 200 rpm for 24 h, and every 4 h, samples were taken and checked for cell growth using the viable plate count. The collected samples were plated onto media with antibiotics to check the plasmid instability of the two *Agrobacterium* strains in the modified medium.

2.6. Plant Preparation, Agrobacterium Infiltration and Expression of Gfp in Nicotiana benthamiana

Seedlings of a *Nicotiana benthamiana* transplant (2 to 3 weeks old after germination) were transplanted to individual 6 × 8 cm pots and were grown at 28 °C under artificial light with a 16 h photoperiod and photon flux density of 150 μmol/m^2/s. Liquid fertilizer (20-8-20 (N-P-K)) was provided weekly until the plants were 5 to 7 weeks old.

Volumes of 50 mL of *A. tumefaciens* EHA105-pEAQ and *A. tumefaciens* GV3301-pEAQ were grown in LB medium, SA-molasses-based medium and TCSA-molasses-based medium in 200 mL flasks and incubated at 28 OC for 16–20 h with 200 rpm agitation. The cells were harvested using centrifugation (5000× *g*, 15 min) at room temperature and resuspended to an OD_{600} of 0.5 with an infiltration buffer (500 mM MES buffer at pH 5.6, 20 mM $MgCl_2$, 1 M acetosyringone and 50 mg D-glucose) and incubated at 28 °C for 3 h before infiltration.

Infiltration was performed using a 5 mL syringe, and 1 mL of the *A. tumefaciens* suspension (OD_{600} = 0.5) was infiltrated to the underside of 5–7-week-old *N. benthamiana* leaves. The expression of *gfp* was used to identify the presence and spread of transgenic cells around infiltration zones after 2, 4, 6 and 8 days under UV light.2.7. Cost Evaluation of Molasses Culture Media

The culture costs of LB medium and the molasses-based medium were analyzed for the laboratory. The price of the media composition was based on the online pricing of each component, with quantities ranging from 500 g to 5 kg. The data were collected and used to calculate the culture medium cost per liter.

3. Statistical Analysis

All experiments in this study were undertaken in triplicate unless otherwise stated. The mean values are reported with standard deviations. Statistical analysis was performed using SPSS software (SPSS Statistics version 28.0.0, IBM Corporation, Armonk, New York, NY, USA), and $p < 0.05$ was considered statistically significant.

4. Results and Discussion

4.1. Effects of Sucrose on Engineered A. tumefaciens Growth

To study the effect of sucrose, the sucrose-based defined medium (SBM) was used as the culture medium for *A. tumefaciens* compared to LB medium to investigate the

growth of engineered *A. tumefaciens*. The results showed that *A. tumefaciens* EHA105-pEAQ could grow in the SBM medium, and the growth was slightly lower than the growth of *A. tumefaciens* EHA105-pEAQ in LB medium. The maximum viable cell growth of *A. tumefaciens* EHA105-pEAQ was obtained in SBM at 24 h with a concentration of 8.968 ± 0.003 log CFU/mL. Interestingly, the cultivation of *A. tumefaciens* GV3101-pEAQ in SBM resulted in maximum cell growth with sucrose as a sole C source. Cell concentrations in colony-forming units at 24 h in both media were comparable to the growth of *Agrobacterium* in LB medium, with a maximum cell concentration of 9.459 ± 0.003 log CFU/mL (Figure 1B). Moreover, the maximum cell growth of *A. tumefaciens* GV3101-pEAQ was higher than that of *A. tumefaciens* EHA105-pEAQ when both strains were cultivated in SBM.

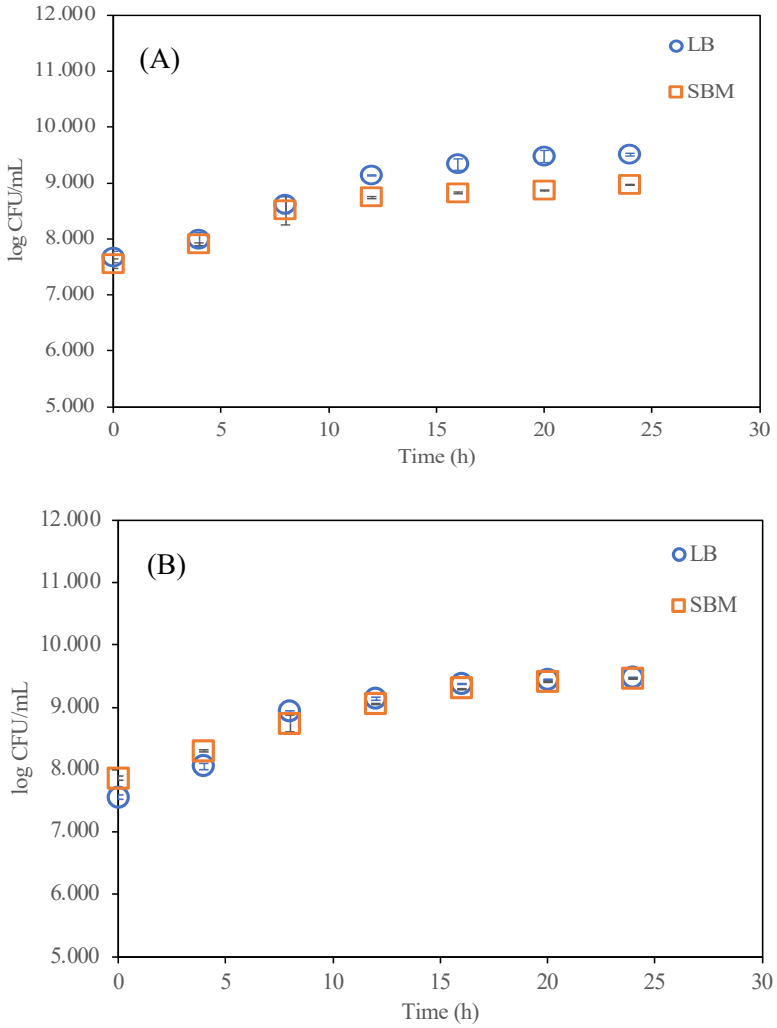

Figure 1. The cell growth of EHA105-pEAQ (**A**) and GV3101-pEAQ (**B**) cultivated in LB (LB medium) and SBM (sucrose-based defined medium). Error bars represent one standard deviation of six replicates.

From this study, we found that engineered *A. tumefaciens* EHA105-pEAQ and *A. tumefaciens* GV3101-pEAQ utilized sucrose and used it as a C source for growth, but *A. tumefaciens* EHA105-pEAQ could not grow as well as *A. tumefaciens* GV3101-pEAQ. Both *A. tumefaciens* GV3101 and *A. tumefaciens* EHA105 are genetically derived from *A. tumefaciens* C58, a common strain for gene transformation; however, the two strains carry different antibiotic resistance genes and a plasmid containing a vir gene [15]. The differential preference for carbon sources of *A. tumefaciens* GV3101 and *A. tumefaciens* EHA105 was previously demonstrated when glucose and acetosyringone were supplied during the GFP and HPT transformation of Dunaliella [18]. The main difference between the

sucrose-based defined medium and the initial basal medium is the presence of yeast extract in the initial basal medium. Meanwhile, the sucrose-based defined medium has a lower price and is safe for vaccine or human therapeutic protein production. Because the sucrose-based defined medium is devoid of any animal-derived components [4], it was selected for further studies using sugarcane molasses as a low-cost carbon source, and this could be an alternative strategy to reduce the production cost of the cultivation of engineered *A. tumefaciens* for recombinant protein production in plants. However, some cane molasses components are unfavorable for microbial fermentation due to their microbial growth inhibitory effects, especially heavy metal ions [7]. As shown in Figure 2, this inhibitor could directly limit the growth of *Agrobacterium*, which is in accordance with previous reports [7–9]. Therefore, the pretreatment of cane molasses is necessary before it can be used, and three selected pretreatment methods were studied in the following experiments.

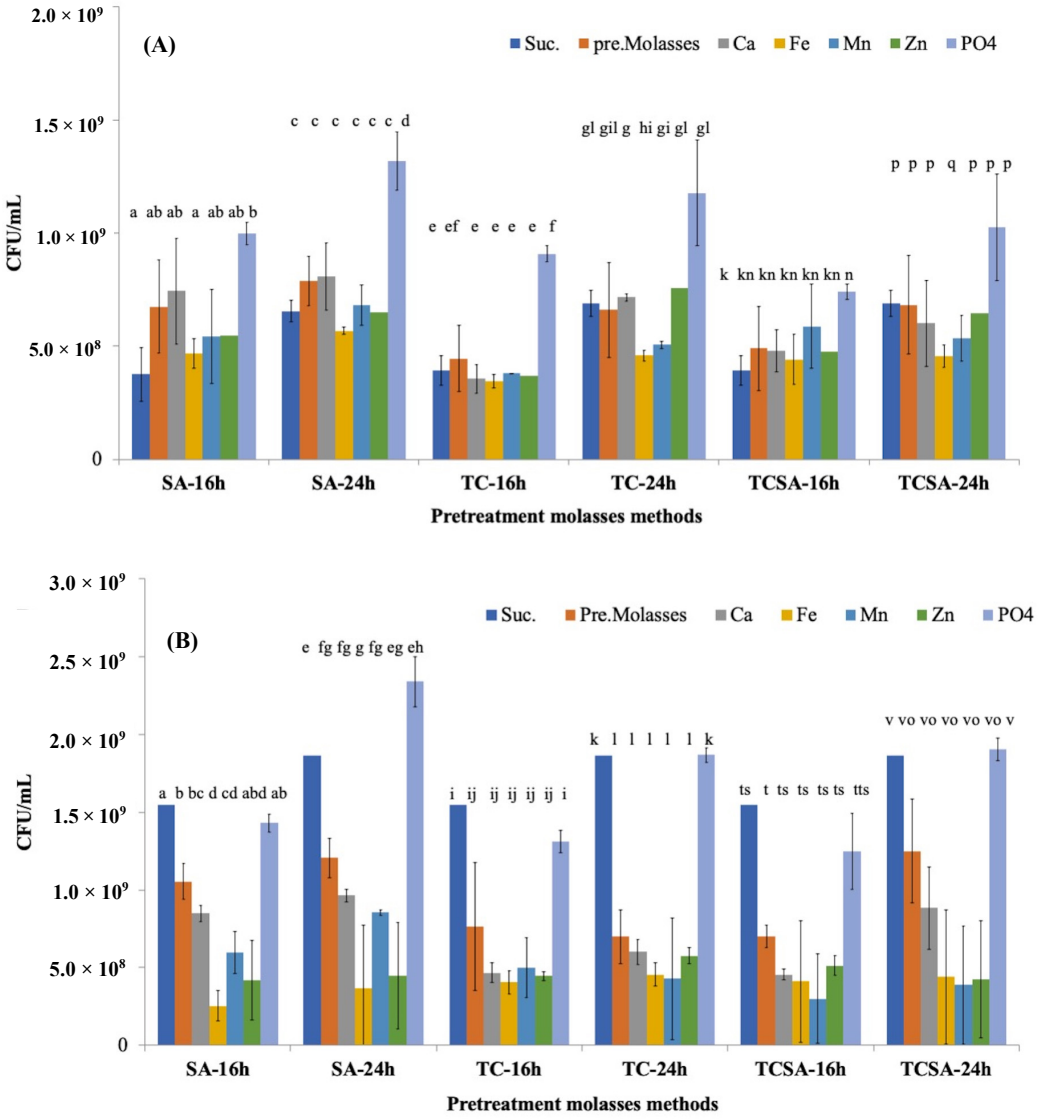

Figure 2. Effects of calcium (Ca), iron (Fe), manganese (Mn), zinc (Zn) and phosphate (PO_4) supplementation and pretreated molasses (pre.molasses) on growth of (**A**) EHA105-pEAQ strain and (**B**) GV3101-pEAQ strain compared to sucrose (Suc). Three molasses pretreatments (SA: sulfuric acid treatment; TC: tricalcium phosphate treatment; TCSA: tricalcium phosphate treatment) were used. Error bars represent one standard deviation of three replicates. Letters indicate mean separation results. Same letters: no significant difference ($p > 0.05$).

4.2. Effect of Pretreatment Methods on Molasses and Growth of Agrobacterium

Untreated cane molasses was used as a carbon source in the molasses-based medium, but cell growth was not detected. Because it was untreated, cane molasses contained high concentrations of metal ions, suspended colloids and ash, which were inevitably generated during cane molasses refining, resulting in the inhibition of cell growth. Therefore, various pretreatment methods for reducing the concentrations of these hazardous components in molasses were studied to enhance microbial growth [12].

In this study, three cane molasses pretreatment methods (SA, TC, TCSA) were applied for cane molasses preparation, and the important components were analyzed before and after each pretreatment. It was found that about 30% of magnesium (Mg) was removed in all samples, and a 90% reduction in calcium (Ca) was observed in molasses after SA and TCSA pretreatments; meanwhile, 58% of calcium was removed after the TC pretreatment, as shown in Table 2.

Table 2. The concentrations of mineral ions and total sugar in cane molasses before and after various pretreatment methods.

Minerals	Units	Molasses	SA	TC	TCSA	Reference Methods
Total sugar	g/100 g	57.5	45.2	53.4	50.8	Estimated as total carbohydrates by phenol-sulfuric acid method [19]
Ash	g/100 g	7.7	3.8	4.3	3.8	AOAC (2016) 920.153
Sulfate ash	g/100 g	9.0	4.9	5.9	4.1	AOAC (2016) 900.02
Calcium (Ca)	mg/kg	7173.5	589.5	3029.8	589.4	In-house method based on AOAC (2012) 984.27
Magnesium (Mg)	mg/kg	2949.2	2121.8	2163.6	2087.6	In-house method based on AOAC (2012) 984.27
Potassium (K)	mg/kg	9530.5	9449.4	9692.3	9665.5	In-house method based on AOAC (2012) 984.27
Nitrogen (N)	g/kg	0.085	0.016	0.011	0.036	Determination of Kjeldahl Nitrogen Horizontal, 2003

Note: SA = sulfuric acid treatment; TC = tricalcium phosphate treatment; TCSA = tricalcium phosphate treatment combined with sulfuric acid treatment.

The sulfuric acid pretreatment is the most frequently used pretreatment technique for cane molasses and could efficiently reduce the heavy metal ions in cane molasses [7–9,13]. The results showed that the calcium content in TC-pretreated molasses was approximately 5-fold higher than in other pretreatment methods (Table 2) due to the effect of tricalcium phosphate. The tricalcium phosphate pretreatment could reduce zinc and iron concentrations in molasses by causing iron and zinc to enter the apatite crystal structure of tricalcium phosphate ($Ca_3(PO_4)_2$) and replacing the calcium atoms, resulting in the distortion of the tricalcium phosphate crystal structure [20,21]. This not only reduces the heavy metal concentration, but the liberated calcium atoms lead to an increase in the calcium concentration in the cane molasses solution, which is clearly seen in Table 2.

Moreover, the pretreatment methods used in this research barely affected the total sugar concentration, as shown in Table 2. The total sugar concentrations of molasses pretreated with SA, TA and TCSA pretreatments were 45.21, 53.36 and 50.70 g/100 g molasses, respectively. These sugar contents were used to calculate the amount of molasses to be added to the molasses-based media to obtain a final sugar concentration of 4 g/L. Furthermore, the cane molasses analysis results showed a low nitrogen content in all cane molasses samples, which was in the range of 0.010–0.040 g/kg after pretreatment (Table 2). So, for the molasses-based medium (MBM), $(NH_4)_2SO_4$ needed to be supplemented as an inorganic N source for *Agrobacterium* growth.

4.3. Effect of Minerals and Heavy Metals in Molasses on Growth of Agrobacterium

Sugarcane molasses subjected to three different pretreatment methods was applied to prepare the molasses-based medium and used to study the effect of the pretreatment meth-

ods on the growth of *Agrobacterium* compared to its growth in the sucrose-based defined medium after 24 h incubation. The molasses-based medium with SA-pretreated molasses (SA-molasses-based medium) enhanced the growth of *A. tumefaciens* EHA105-pEAQ compared with the sucrose-based defined medium (Figure 2A). However, *A. tumefaciens* GV3101-pEAQ preferred the sucrose-based defined medium for growth over the SA-molasses-based medium (Figure 2B). Meanwhile, TC- and TCSA-molasses-based media showed similar results. The growth of *A. tumefaciens* EHA105-pEAQ in TC- and TCSA-molasses-based media was as good as *Agrobacterium* growth in the sucrose-based defined medium (Figure 2A) but had a significantly lower growth rate than *A. tumefaciens* GV3101-pEAQ cultivated in both molasses-containing media (Figure 2B).

Moreover, minerals (calcium, phosphate) and heavy metals (Fe^{2+}, Mn^{2+}, Zn^{2+}) were also added to the molasses-based medium to study the effects of minerals and heavy metals. Supplementation with phosphate in SA-, TC- and TCSA-molasses-based media significantly enhanced the growth of both *A. tumefaciens* EHA105-pEAQ and *A. tumefaciens* GV3101-pEAQ, which was higher than their growth in the sucrose-based defined medium. The highest cell growth was observed in *A. tumefaciens* GV3101-pEAQ with the SA-molasses-based medium supplemented with phosphate (Figure 2B) with a viable count of 2.34×10^9 CFU/mL at 24 h cultivation.

Supplementation with Fe^{2+} slightly affected the growth of *A. tumefaciens* EHA105-pEAQ, but calcium and manganese supplementation had no effect (Figure 2A). In contrast, supplementation with Fe^{2+}, Mn^{2+} and Zn^{2+} in the molasses-based medium resulted in the growth inhibition of *A. tumefaciens* GV3101-pEAQ (Figure 2B).

4.4. Effects of Antibiotics on Cell Growth and Genetic Instability

Antibiotic supplementation in culture media for genetically modified microorganism fermentation is used to minimize plasmid loss, prevent contamination or induce the expression of the target recombinant protein [22,23]. However, for the transient expression of heterologous proteins in plants with *Agrobacterium*-mediated gene transformation, antibiotic supplementation is used only to maintain plasmid stability [22,23] in the cloning host. The protein of interest is produced in a later step after agroinfiltration in the target plant. The high cost of the antibiotic directly impacts production costs, especially in industrial-scale fermentation. Therefore, the effect of antibiotic supplementation on the growth of the two *A. tumefaciens* strains was investigated in this research. According to the growth curve, the two engineered *Agrobacterium* strains (*A. tumefaciens* EHA105-pEAQ and *A. tumefaciens* GV3101-pEAQ) exhibited similar growth profiles in all culture media with and without antibiotic selection, IBM and molasses-based media, as shown in Figure 3. The viable cell counts of EHA105-pEAQ and GV3101-pEAQ strains in the IBM medium with and without antibiotic supplementation were about 3.0×10^9 CFU/mL after 24 h incubation (Figure 3), and this bacterial growth profile was similar in all molasses-based media. Among the molasses-based media, the SA-molasses-based medium showed the highest cell growth; the viable cell counts were greater than 2.0×10^9 CFU/mL (Figure 3) for both strains, and no obvious growth differences were observed between culturing with and without antibiotic supplementation. This result confirmed that the cultivation of *A. tumefaciens* EHA105-pEAQ and *A. tumefaciens* GV3101-pEAQ in all molasses-based media did not affect plasmid instability, so antibiotic supplementation during the fermentation process may not be essential, which could also reduce the production cost. The stabilization of the plasmid without antibiotic supplementation in this study might be due to the following reasons. pEAQ, the plasmid used in this study, is a high-copy-number plasmid [24,25], so the chance of segregation loss is low when culturing without antibiotic supplementation. Other reasons could be that the *Agrobacterium* inocula used in this study were freshly prepared, and *Agrobacterium* cells were harvested after 16–20 h of cultivation, which was the beginning of the stationary phase of growth. So, this could maintain the high copy numbers of plasmids and reduce the plasmid loss during cultivation without antibiotic supplementation.

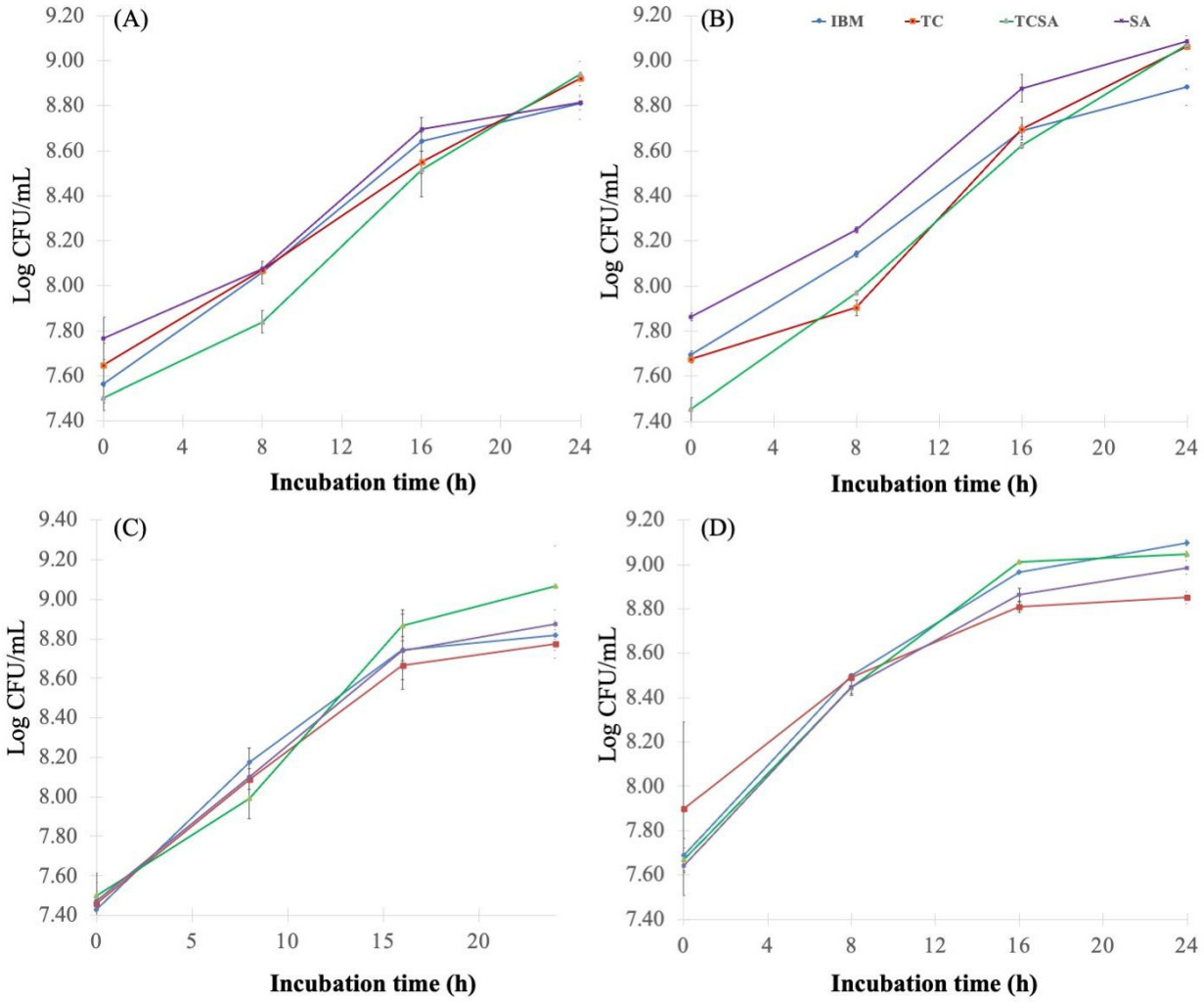

Figure 3. The viable cell count of EHA105-pEAQ cultivated in molasses-based media with antibiotic (**A**) and without antibiotic (**B**) supplementation and the colony-forming units of GV3101-pEAQ cultivated in molasses basal media with antibiotic (**C**) and without antibiotic (**D**) supplementation. SA: sulfuric acid pretreatment; TC: tricalcium phosphate pretreatment; TCSA: tricalcium phosphate and sulfuric acid pretreatment; IBM: initial basal medium. Error bars represent one standard deviation of six replicates.

In this study, the transient production of the target protein in *N. benthamiana* leaf tissue obtained from *A. tumefaciens* EHA105-pEAQ and *A. tumefaciens* GV3101-pEAQ cultivated in SA-, TC- and TCSA-molasses-based media without antibiotic supplementation was studied and compared to that in LB medium. The expression of green fluorescent protein in all *Agrobacterium* strains prepared from various molasses-based media was clearly seen in *N. benthamiana* leaves under UV illumination, as shown in Figure 4. Additionally, the expression of GFP in *N. benthamiana* leaves similarly appeared under UV illumination when infiltrated with engineered *Agrobacterium* harboring the pEAQ vector cultured in molasses-based media with and without antibiotic supplementation (Figure 4). These results confirm that the fermentation of *A. tumefaciens* EHA105-pEAQ and *A. tumefaciens* GV3101-pEAQ in the molasses-based medium used for recombinant heterologous protein production in plants via agrobacterium-mediated gene transformation does not require antibiotic supplementation to maintain plasmid stability during the fermentation period.

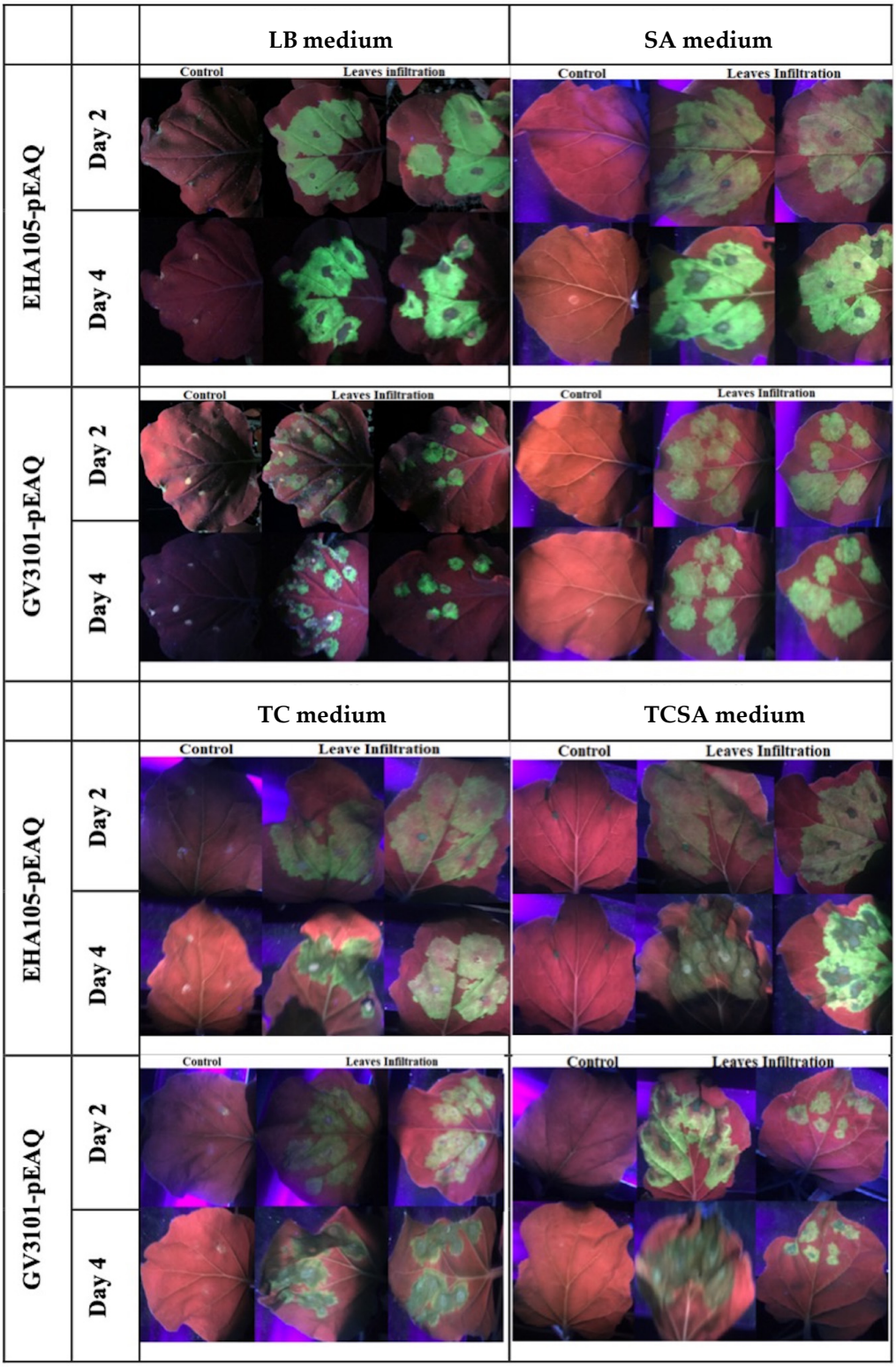

Figure 4. Recombinant green fluorescent protein (GFP) expression in *Nicotiana benthamiana* leaves using *A. tumefaciens* EHA105-pEAQ and GV3101-pEAQ strains cultured in LB and molasses-based media for agrobacterium-mediated gene transformation.

4.5. Cost Evaluations of Molasses Culture Media

The results above demonstrated that LB as the culture medium results in the maximum growth for both *A. tumefaciens* EHA105-pEAQ and *A. tumefaciens* GV3101-pEAQ.

Nevertheless, from the economic viewpoint, LB medium, which contains yeast extracts, tryptone and NaCl, costs 1.52 USD/L, and therefore, it is not the best choice for *A. tumefaciens* fermentation (Table 3), especially when compared to the molasses-based medium, which is much cheaper. The cost of the molasses-based medium components ($(NH_4)_2SO_4$, $MgSO_4 \cdot 7H_2O$, Na_2HPO_4, KH_2PO_4 and molasses) was calculated, and the total cost was only 0.25 USD/L, which is about 6.1 times lower than that of LB medium. Another advantage of the molasses-based medium is that it is free of animal-derived ingredients. Therefore, it is safe for the production of human therapeutic proteins. Furthermore, this study also indicated that antibiotic supplementation in molasses-based media is not essential for maintaining recombinant plasmids, which is a benefit for *Agrobacterium* fermentation at a commercial scale.

Table 3. Comparative cost estimation of materials for molasses-based medium and LB medium.

	Composition	Size	Bulk Cost (USD)	Source	Amount Used (g/L)	Price (USD/L)
LB medium						
1	Yeast Extract	500 g/bot	35.18	Hi-media	5	0.3518
2	Tryptone type I	500 g/bot	55.06	Hi-media	10	1.1012
3	NaCl	1 kg	6.42	Kemaus	10	0.0642
					Total	1.52
Molasses-based medium						
1	$(NH_4)_2SO_4$	500 g/bot	11.32	Kemaus	2.4	0.0543
2	$MgSO_4 \cdot 7H_2O$	500 g/bot	11.32	Kemaus	0.25	0.0056
3	Na_2HPO_4	500 g/bot	14.07	Kemaus	4.543	0.1278
4	KH_2PO_4	500 g/bot	11.93	Kemaus	2.448	0.0584
5	Molasses	1 kg/bot	0.81		8.7	0.0070
					Total	0.25

(January 2023) Thailand, market price according to presentation (1 USD = 32.64 THB; THB: Thai baht).

5. Conclusions

To develop alternative, low-cost culture media for recombinant *A. tumefaciens* EHA105-pEAQ and GV3101-pEAQ, three types of carbon sources, including oligopeptides in tryptone (LB media), sucrose and cane molasses, were formulated in this study. The maximum cell concentrations (based on logCFU/mL) of *A. tumefaciens* EHA105-pEAQ and GV3101-pEAQ cultured in the sucrose-based medium (SBM) were 8.968 ± 0.003 and 9.459 ± 0.003, respectively. To prepare molasses-based media (MBMs), cane molasses was pretreated by using three pretreatment methods (SA, TC, TCSA). The SA pretreatment results revealed that 45.55% of sulfate ash contamination in molasses was removed, while 78.6% of total sugar contents still remained, which subsequently enabled the highest cell growth of *A. tumefaciens* GV3101-pEAQ at 2.34×10^9 CFU/mL at 24 h cultivation. Furthermore, to reduce the cost of the formulated media, the addition of antibiotics to maintain the genetic stability of recombinant *A. tumefaciens* was tested to determine whether it is necessary. Similarly, cell growth was not compromised without the addition of antibiotics when culturing both *Agrobacterium* strains in the SA-molasses-based media. Then, the obtained recombinant *A. tumefaciens* strains were tested for their efficiency in transferring the recombinant GFP gene to the host plant (*N. benthamiana*) leaf tissue by using an in vitro infiltration method. The success of GFP expression in infiltrated *N. benthamiana* without antibiotic supplementation suggests that recombinant *Agrobacterium* strains grown in SA-molasses-based media are genetically stable and effectively transfer the targeted recombinant gene to the host plant. To confirm the benefit of using the MBM from an economic perspective, the raw material cost of each medium formula was calculated, and the cost of the MBM is 6.1 times cheaper than that of LB. Altogether, the SA-molasses-based medium formulated in this study could be used as an alternative option to nutrient-rich

media for the preparation of recombinant *Agrobacterium* used in the production process of recombinant proteins.

Author Contributions: Conceptualization, K.R.; methodology, K.R., N.W. and L.V.N.; validation, K.R., N.W. and L.V.N.; formal analysis, K.R., N.W. and L.V.N.; investigation, K.R.; resources, M.S., N.W. and K.R.; writing—original draft preparation, K.R., N.W. and L.V.N.; writing—review and editing, M.S., Y.-S.C., M.K., P.-L.S. and K.R.; project administration, K.R.; funding acquisition, K.R. and M.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by an Agro-Industry (AI) Scholarship for International Graduate Students, Faculty of Agro-industry, Kasetsart University, and partly supported by the Graduate Program Scholarship from the Graduate School, Kasetsart University. This research was also funded by King Mongkut's University of Technology North Bangkok, grant number KMUTNB-66-BASIC-16.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data generated or analyzed during this study are included in this published article.

Acknowledgments: The authors thank the Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Bangkok, Thailand, for an Agro-Industry (AI) Scholarship for International Graduate Students and also funding from King Mongkut's University of Technology North Bangkok to Malinee Sriariyanun (No. KMUTNB-66-BASIC-16). This research was also partly supported by the Graduate Program Scholarship from the Graduate School, Kasetsart University. The authors are grateful for the support from the Fermentation Technology Research Center, Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Thailand.

Conflicts of Interest: The authors declare no conflict of interest.

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