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Potential of salted egg-white hydrolysate as an alternative nitrogen source and salt supplement in bacterial culture media

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Abstract

Salted egg-white (SEW) is a common waste product from Chinese-mooncake manufacturing. Due to its abundance of proteins and minerals, breaking down SEW into small building blocks was shown to be an appealing approach for making basic ingredients of culture media. To verify this assumption, SEW was subject to alkali hydrolysis and further analyses for its potential to replace tryptone and peptone, standard protein hydrolysates used in culture media preparation. As expected, the results indicated that autoclave-assisted alkali hydrolysis of SEW yielded small peptides and amino acids, salted egg-white hydrolysate (SEWH). Furthermore, alkali treatment equivalent to 0.5 M NaOH and typical autoclaving times (15-20 minutes) were shown to deliver the optimal hydrolysis. To test whether SEWH was able to support the cultivation of *Escherichia coli*, modified LB medium was formulated using SEWH as a tryptone and sodium chloride substitute. However, this modified LB-SEWH medium could only support *E. coli* growth up to 87% relative to the regular LB medium when 20% SEWH (v/v) was used, suggesting the presence of some recalcitrant molecules created by nonspecific alkali treatment. To extend its application to other aspects of microbiology and biotechnology, biochemical identification of coliform bacteria and plasmid DNA amplification in *E. coli* using culture media made from SEWH were tested. SEWH performed well as a substitute of peptone and tryptone for each medium though its performance was slightly hindered yet not significant compared to those of the original media. This implies that SEWH was as efficient as other protein hydrolysates in providing a nitrogen source and salt supplementation for facilitating bacterial cell growth. This was introduces a novel and simple approach to convert salted egg-white, a waste from the food industry in to economical protein hydrolysate. Therefore, large scale production of SEWH will provide an alternative nitrogen source at a competitive price for several biotech industries.

Keywords**:** *alternative nitrogen source, culture media, egg-white hydrolysate, Escherichia coli, protein hydrolysate, salted egg-white*

1. Introduction

Salted egg is common food in Southeast Asia. It can be made by submerging duck eggs in brine for several weeks. After the salting processes, the salted egg yolk is separated and inserted into mooncakes or dim-sums, leaving the salted egg-white (SEW) unused. Typically, ovalbumin and ovomucoid are major proteins in egg-white (Huang, & Lin, 2011). It has been reported that SEW is as nutritious as the fresh egg-white, however, its protein content decrease as the salting time is increased (Kaewmanee, Benjakul, & Visessanguan, 2009). In microbiology, peptides, amino acids and salts are essential key elements in nutrient-rich media such as Lysogeny broth (LB). Peptides and amino acids provide nitrogen sources for cellular metabolisms. On the other hand, salts help stabilize osmotic pressure and modulate enzymatic activity (Buchanan & Klawitter, 1992).

In general, protein hydrolysates from various sources have been used for culture media preparation. For example, commercial peptone and tryptone, mixtures of small peptides and amino acids, are enzymatically hydrolyzed from beef and casein (milk protein) by pepsin and trypsin, respectively. It was shown that greater degree of protein hydrolysis in the culture media led to better growth of *Escherichia coli* (Paliy & Gunasekera, 2007; Hortsch & Weuster-Botz, 2011). Due to the relatively high cost of peptone and tryptone, recent studies have suggested that hydrolysates from animal waste (chicken feather, ram horn, and fish waste) could also support bacterial cell growth (Kurbanoglu & Kurbanoglu, 2002; Vázquez, González, & Murado, 2004; Taskin & Kurbanoglu, 2011; Orak, Caglar, Ortucu, Ozkan, & Taskin, 2018). Hydrolysis of egg-white protein can be achieved through chemical, heat and enzymatic treatments. Alkali hydrolysis was reported to effectively break down

the disulfide bound in ovalbumin and other proteins in egg-white, resulting in an increase in the solubility of the egg-white (Chen et al., 2015). In addition, high temperature was also shown to promote the fragmentation of the waste protein during alkaline hydrolysis yielding amino acids and peptides (Song, Shi, Li, Wang, & Ren, 2019; Gao, Wang, Yan, Li, & Chen, 2020). Even though egg-white hydrolysates were shown to have a wide variety of bioactivities as bioactive-peptides, including antioxidant and protease-inhibitor (Yu et al., 2011; Chen, Chi, Zhao, & Lv, 2012; Garcés-Rimón, López-Expósito, López-Fandiño, & Miguel, 2016; Garcés-Rimón et al., 2019), supplementation using salted egg-white hydrolysate (SEWH) in bacterial culture media has never been reported. In this study, we provided strong evidence suggesting that SEWH could be another simple and low-cost nutrient-rich supplement for further application in microbiology and biotechnology.

2. Objectives

The objective of this study is to demonstrate that SEWH is a potential substitute for protein hydrolysates and salts in bacterial culture media. Optimization of SEWH was achieved using alkali hydrolysis. The growth and plasmid production of *E. coli* grown in culture media supplemented with SEWH were analyzed and compared to those of regular culture media containing tryptone or peptone.

3. Materials and methods

3.1 Bacterial strain

Stock cultures of *Escherichia coli* DH5α were aliquotted and stored in the freezer at -80°C. Before each experiment, vials were thawed at room temperature for 5 minutes prior to streaking on LB agars. Fresh overnight grown *E. coli* DH5α was used for all experiment unless otherwise indicated.

3.2 Salted egg-white hydrolysate preparation

Bulk salted egg-white was obtained from a local bakery and stored at 4°C. Salted egg-white (SEW) was mixed with various concentrations of NaOH and autoclaved for 10 to 40 minutes. Sated egg-white hydrolysates (SEWH) were left at room temperature to cool and the pH was adjusted to 7.2 using 6 N HCl.

3.3 Chemical analysis

The degree of hydrolysis (availability of amine groups in SEWH) was determined by Trinitrobenzene sulfonic acid (TNBSA) which reacts with free amino acids to form a complex showing an absorbance maximum at 348 nm (Goodwin & Choi, 1970). SDS-PAGE was then preformed to verify hydrolysis of SEW proteins as described previously (Laemmli, 1970). The amino acid composition of SEWH was analyzed by thin layer chromatography. Samples were spotted on silica gel TLC plates, dried and elutes in butanol-acetic acid-water solution (3:1:1). Plates were sprayed with ninhydrin solution and dried until colored spots were developed (Pachuski, Fried, & Sherma, 2002).

Sodium ion concentration was measured by flame atomic absorption spectrophotometry technique. In brief, 500 µl of SEW and SEWH samples were incinerated in a furnace (Carbolite/Contral 201) at 400°C for 4 hours. The samples were dissolved in deionized water and injected to atomic absorption spectrophotometer (Perkin Elmer/PinAAcle 900F). An absorbance at 589 nm was selected to measure sodium ion concentration in the samples using 0-10 ppm NaCl as reference standards.

3.4 Bacterial growth analysis

A single colony of *E. coli* was inoculated in 50 ml of LB medium (10 g/l tryptone, 10 g/l NaCl and 5 g/l yeast extract, pH 7.2) and cultured at 37°C, 150 rpm overnight. Then, 1 ml of inoculum was added to 50 ml of culture media containing various concentration of SEWH ranging from 10%-50% (v/v/) and cultured at 37°C, 150 rpm. Tryptone broth (10 g/l tryptone and 10 g/l NaCl, pH 7.2) was used as a control treatment. After 8 hr. of cultivation, the turbidity of all culture media was measured at 600 nm. Further growth analyses were done in modified LB-SEWH media (10% SEWH, 5 g/l yeast extract, pH 7.2) where SEWH replaced tryptone and NaCl.

Coliform bacteria growth analysis was done in EMB agar (10 g/l peptone, 10 g/l lactose 2 g/l K2HPO4, 0.4 g/l Eosin Y, 0.065 g/l Methylene blue and 1.5 g/l agar, pH 7.2) compared to that of modified EMB-SEWH agar using 25% SEWH (v/v) as a substitute for peptone. Water samples from a men's toilet were collected and spread on EMB or modified EMB-SEWH agars. Coliform bacterial colonies were observed after overnight incubation at 37°C.

3.5 Plasmid DNA analysis

A single colony of *E. coli* transformants harboring pUC19 or yeast shuttle vector pDYES was

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inoculated into 50 ml modified LB-SEWH or LB media containing 100 ng/ml ampicillin and incubated at 37°C, 150 rpm for 12 hours. Plasmid DNA extraction was performed according to the manufacturer instruction (VIVANTIS GF1). DNA concentration was quantified by measuring an absorbance at 260 nm.

3.6 Statistical analysis

All data in this study were statistically analyzed by Minitab ® 18.1. The one-way ANOVA were perform at critical p-value 0.05 otherwise indicated. Pairwise comparison among means was analyzed using the Tukey HSD test.

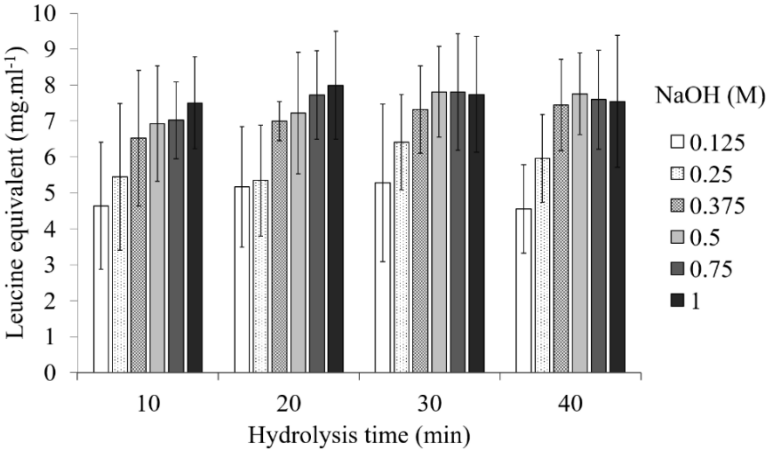

Figure 1 Degree of hydrolysis of SEW treated with 0.125-1.00 M NaOH and autoclaved for 10, 20, 30 and 40 minutes, respectively. No statistically significant differences were observed among the treatments ($p<0.05$). Bars (I) = SD.

4. Results and discussion

4.1 Alkali hydrolysis of salted egg-white generates small peptides and free amino acids

To optimize the alkali hydrolysis conditions for SEWH formulation, SEW was treated with various concentrations of NaOH and heat exposure time via autoclaving. By measuring the degree of hydrolysis or amount of available amine group represented by the leucine equivalent, the hydrolysis efficiency was compared for each treatment. The degree of hydrolysis increased significantly as the concentration of NaOH increased (Figure 1). The heating time had a slight effect on degree of hydrolysis indicating that 10-20 minutes of autoclaving was adequate for alkali hydrolysis of SEW. To ensure that an increase in the available amine group is due to alkali hydrolysis, SDS-PAGE was conducted. Clearly, all SEW samples treated with NaOH showed signs of hydrolysis (smeared bands) compared to the control untreated SEW (Figure 2, lane 6-10), suggesting that peptide bonds were broken randomly. However, only autoclaved SEW treated with NaOH displayed effective hydrolysis as shown by the major presence of peptides smaller than 20 kD (Figure 2, lane 2-5). An increase in NaOH concentration over 0.375 M (Figure 2, lane 4-5) resulted in a decrease in the amount of peptides stained by coomassie blue dye, suggesting that either they were smaller than SDS-PAGE could separate or they were degraded due to the high alkali concentration. However, since free amine group (TNBSA assay) was detected in autoclaved SEW treated with 0.375-1.00 M NaOH (Figure 1), it was hypothesized that higher alkali concentration enhanced SEW breakdown to much smaller peptides, amino acids or other amine compounds (Chen et al., 2015). To test this assumption, SEWH samples were spotted on TLC plates, developed and detected with ninhydrin and compared to standard amino acids. Based on the retardation factors (Rf), some free amino acids were preliminarily identified, e.g., valine, isoleucine, phenylalanine, glycine, methionine, tryptophan, lysine and arginine (Table 1). Intriguingly, none of the negatively charged or polar uncharged amino acids were detected, implying that these amino acids might be susceptible to alkali hydrolysis and probably underwent transformation (Masters & Friedman, 1979; Deleu, Lambrecht, & Delcour, 2019). The results suggest that alkali hydrolysis of SEW generated SEWH comprised small peptides and amino acids.

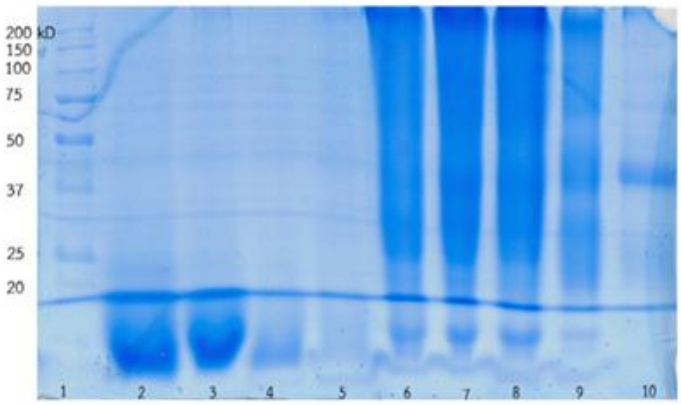

Figure 2 Effect of NaOH and heat treatment on hydrolysis of SEW. Samples from SEWH and SEW were separated in SDS-PAGE as shown by different size of polypeptides displayed on the gel. Lane 1 shows protein markers. Lane 2-5 represent SEWH samples treated with 0.125, 0.375, 0.5 and 1 M NaOH and autoclaved for 30 minute. Lanes 6-9 are SEW samples dissolved in 0.125, 0.375, 0.5 and 1 M NaOH. Lane 10 is untreated SEW control.

Table 1 TLC analysis of free amino acids in salted egg-white hydrolysate using thin-layer chromatography

Amino acid	Retardation factors (Rf)		Color
	Control	SEWH	
L-Lysine	0.03	0.05	Red-Pink
L-Glutamic acid	0.35	-	-
L-Histidine	0.19	-	-
L-Valine	0.43	0.44	Purple-Brown
L-Isoleucine	0.55	0.55	Deep purple
L-Methionine	0.49	0.47	Deep purple
L-Glutamine	0.15	-	-
L-Aspatic acid	0.21	-	-
L-Alanine	0.34	-	-
L-Leucine	0.55	0.55	Deep purple
L-Phenylalanine	0.57	0.57	Purple
L-Glycine	0.21	0.21	Purple
L-Serine	0.35	-	-
L-Proline	0.26	-	-
L-Threonine	0.32	-	-
L-Tryptophan	0.62	0.62	Purple-Grey
L-Tyrosine	0.71	-	-
L-Arginine	0.28	0.27	Purple
L-Asparagine	0.38	-	-
L-Cysteine	0.25	-	-

4.2 Facilitation of *E. coli* growth by salted egg-white hydrolysate

According to the time-course analysis, *E. coli* growth entered the mid-log phase at approximately 8 hours after inoculation (data not shown), thus *E. coli* relative growth data was collected at this point. To test whether SEWH is suitable as a tryptone replacement for bacterial growth, *E. coli* was cultured in SEWH as a sole nutrient at various concentrations and compared to tryptone broth. SEWH broth (20-25%) produced only 62-63% of the density compared to tryptone broth (Figure 3A, white boxes), suggesting a lack of key nutrients or the presence of toxic compounds in SEWH. An increase in NaOH concentration during alkali hydrolysis of SEW resulted in a shift of highest growth profile towards higher SEWH concentration, 25-30% (Figure 3A, gray boxes), implying that higher alkalinity rendered nutrients in SEWH to be degraded or transformed to recalcitrant forms unavailable to *E. coli*. To eliminate the effect of high salt concentration in SEWH on growth of *E.*

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coli (Hosotani, Noviyanti, Koseki, Inatsu, & Kawasaki, 2018), the sodium ion concentration was measured in both SEW. There were no significant difference between the sodium ion concentration in SEW and SEWH (Figure 3B). When SEWH broth comprised 20% SEWH treated with 0.5 M NaOH, it contained 10.9 g/l NaCl which was close to that typically added to LB medium (10 g/l). Therefore, the 40% lower relative cell density observed after 8 hours of incubation in SEWH broth was unlikely to be due a high salt concentration but it may explain the gradual decrease of relative cell density when SEWH was used more than 25% (Figure 3 A). To test whether enrichment of SEWH with yeast extract imitating LB medium would enhance *E. coli* growth, modified LB-SEWH media were prepared by varying concentration of SEWH where tryptone and NaCl were replaced by only SEWH. As expected, the highest relative cell density was reached 83-87% when 10-20% SEWH was supplemented as a substitution of tryptone and NaCl (Figure 4). It was likely that an increase in the relative cell density from 0-5% SEWH was due to the presence of more available nitrogen sources, whereas a decline in the relative cell density from 30-50% SEWH caused by high salt concentration as mentioned earlier. Altogether, these results indicate that SEWH was a nutrient-rich hydrolysate containing nitrogen based compounds and salts required for facilitating *E. coli* growth. However, it harbored some unavailable forms of nutrients that *E. coli* was unable to utilize readily (Friedman, 1999; Aliashkevich, Alvarez, & Cava, 2018).

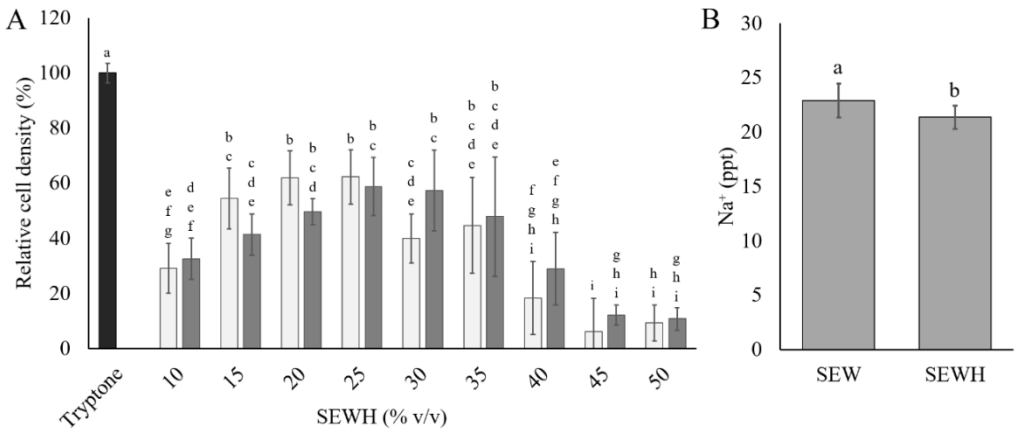

Figure 3 Effect of SEWH concentration on relative cell density of *E. coli* grown in culture media. A) Relative cell density of *E. coli* cultured for 8 hours in Tryptone broth (black), 0.5 M NaOH treated SEWH broth (white) and 1.0 M NaOH treated SEWH broth (gray). B) Sodium ion concentration in SEW and SEWH measured by atomic absorption spectroscopy. Means with the same letter are not significantly different ($p > 0.05$). Bars (I) = SD.

4.3 Replacement of tryptone and peptone by salted egg-white hydrolysate in microbiology and molecular biology work

To demonstrate that SEWH can be applied to most routines in microbiology and molecular biology, two approaches were tested, firstly identification of coliform bacteria using modified EMB-SEWH agar and secondary production of plasmid DNA using modified LB-SEWH medium. As expected, modified EMB-SEWH agar was able to distinguish coliform bacteria isolated from a toilet similar to EMB agar within 24 hours by producing dark colored colonies as a result of lactose fermentation (Figure 5, A and B). However, the laboratory strain of *E. coli* DH5α which could not utilize lactose and barely grew on modified EMB-SEWH or EMB agars (Figure 5, C and D), suggesting that modified EMB-SEWH gave comparable results to EMB and SEWH was able to replace peptone in this formulation. Furthermore, modified LB-SEWH medium demonstrated an excellent performance for the production of plasmids DNA. Even though *E. coli* transformants grew slightly slower in the modified LB-SEWH medium

(where tryptone and NaCl was replaced with SEWH) than LB medium (Figure 6A), they produced relatively similar concentration of plasmid DNA in both media (Figure 6B). Not only was the DNA quantity nearly identical, but the DNA quality was also comparable as shown by the uniform transformation efficiency of competent cells taking up these plasmids (data not shown). In summary, it was shown that SEWH was a versatile reagent that could be used in various microbiology and molecular biology experiments as a substitute for protein hydrolysate, including but not limited to tryptone and peptone.

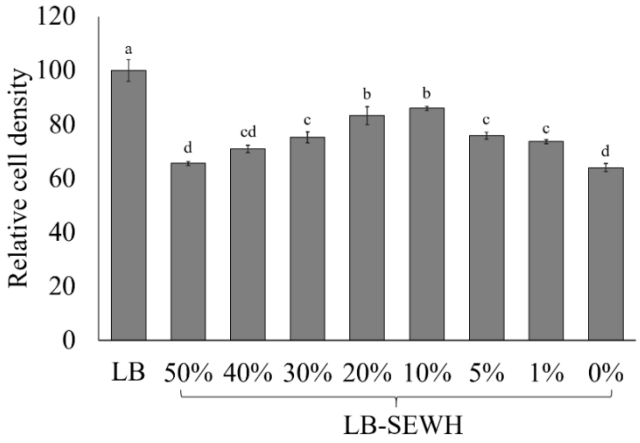

Figure 4 Relative cell density of *E. coli* grown in modified LB-SEWH media. *E coli* was cultured for 8 hours in LB medium or modified LB-SEWH media, replacing tryptone and NaCl with SEWH. Means with the same letter are not significantly different ($p > 0.05$). Bars (I) = SD.

6. Conclusion

A novel concept of waste utilization was introduced by transforming food industrial waste to use in research and biotech industries. The use of salted egg-white hydrolysate is attractive not just due to its ability to replace other protein hydrolysates, such as tryptone or peptone, but rather owing to its manufacturing simplicity. One can mix salted egg-white with NaOH and autoclave it to obtain protein hydrolysate ready to use in the laboratory. This is very useful, especially in developing countries where resources are limited. Salted egg-white hydrolysate contained small peptides, free amino acids and sodium ions vital for bacterial growth. It was also demonstrated that it could be applied to wide variety uses in ordinary microbiology and molecular biology practices. Ultimately, the entire process was simple and cost-effective thereby rendering it to be a candidate for tryptone and peptone replacement.

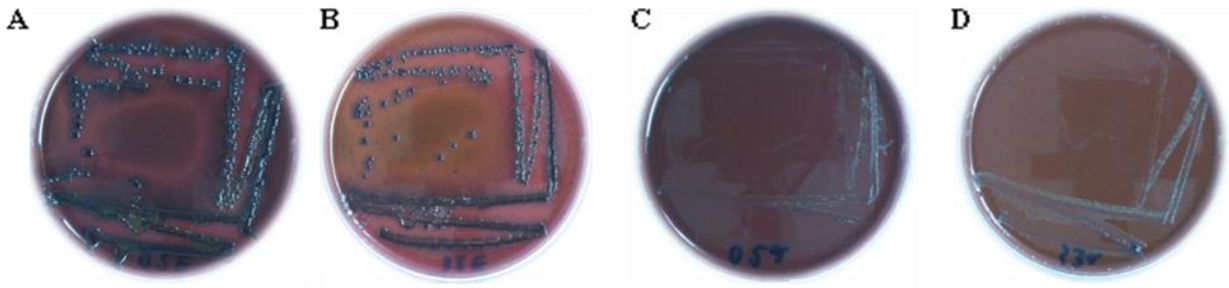

Figure 5 Screening of coliform bacteria using modified EMB-SEWH agar. Coliform bacteria isolated from a toilet were streaked on (A) EMB agar (B) modified EMB-SEWH agar (C) incubated for 24 hours. *E. coli* DH5α was used as a control strain on EMB agar and (D) modified EMB-SEWH agar.

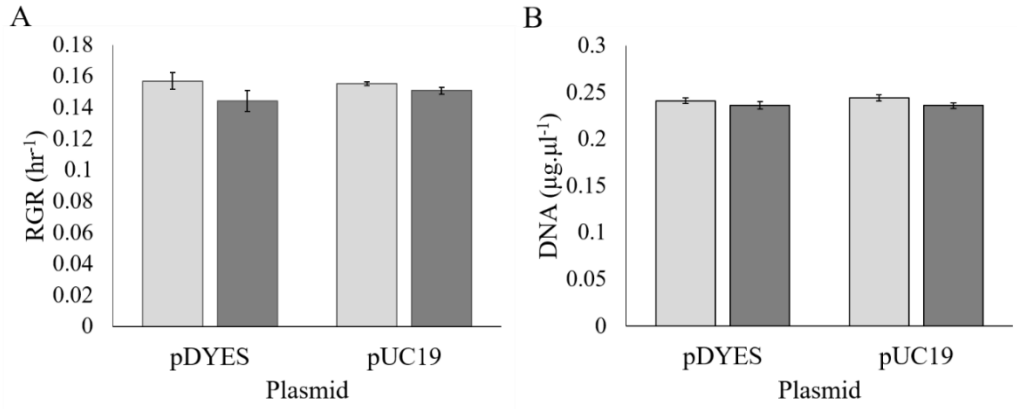

Figure 6 Growth and plasmid DNA production of *E. coli* transformants cultured in modified LB-SEWH medium. Transformants harboring pUC19 or pDYES were grown in LB medium (gray) or modified LB-SEWH medium (dark gray). Figure 6A shows relative growth rate of *E. coli* transformants grown in different media and Figure 6B illustrates concentration of plasmid DNA obtained from transformants after 14 hours of cultivation. No statistically significant differences were observed among the treatments ($p<0.05$). Bars (I) = SD.

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