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Original Article

Effect of additives on shelf-life of suspension of photosynthetic bacteria tested using full factorial experimental design

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Abstract

This study aimed to investigate the effects of additives, seeking to extend longevity of a viable cell count (>10^6 CFU/mL) of *Ectothiorhodospira shaposhnikovii* during storage at room temperature (30±2°C). *E. shaposhnikovii* were cultured in a 1000L-fermenter until the viable cells reached 4.85±0.50×10^6 CFU/mL. Afterward, samples were stored in 1L translucent cylindrical plastic bottles for 60 days. Effects of glycerine (0, 0.15%), $Na_2HPO_4 \cdot 2H_2O$ (0, 0.5 g/L), and Na_2CO_3 (0, 0.5 g/L) on viable cells were investigated in a full factorial experimental design. Na_2CO_3 had significant effect and maintained viable cells at 2.35±0.120×10^6 CFU/mL (a survival rate of 48.4%) and at 1.15±0.057×10^6 CFU/mL (a survival rate of 23.7%) after 45 and 60 days, respectively. In contrast, glycerine negatively affected the viable cells of *E. shaposhnikovii*, reducing the counts to 0.037-0.255×10^6 and 0.028-0.128×10^6 CFU/mL after 45 and 60 days, respectively. This indicates that Na_2CO_3 can be used as a low-cost additive to extend the storage shelf life of *E. shaposhnikovii* in commercial stage.

Keywords: *Ectothiorhodospira shaposhnikovii*, additives, viable cell, full factorial experimental design

1. Introduction

Purple photosynthetic bacteria are typical anoxygenic photosynthetic bacteria, which are classified into purple sulfur bacteria (PSB) and purple nonsulfur bacteria (PNSB). PSB are a group of Gammaproteobacteria, which have the two families *Chromatiaceae* and *Ectothiorhodospiraceae*. PSB use sulfide and hydrogen as electron donors. *E. shaposhnikovii* is a rod-spiral shaped and Gram-negative bacterium (Madigan & Jung, 2009). It is halotolerant or slightly halophilic species, often found in moderately or slightly saline lakes (Bryantseva, Tourova, Kovaleva, Kostrikina, & Gorlenko, 2010). Its optimal growth is at 30-40 ^{O}C, pH 7.5-9.5 and 1-7% salt (Ventura, Viti, Pastorelli, & Giovannetti, 2000). Photosynthetic pigments are bacteriochlorophyll a (esterified with phytol) and carotenoids of the spirilloxanthin series with spirilloxanthin as the major component. Color of cell suspensions in the absence of polysulfides and S^0 is red (Brenner, Krieg, & Staley, 2004). Currently, PSB cultivation is widespread with many advantageous applications. *E. shaposhnikovii* can remove H_2S from biogas with elemental sulfur (S_0) as the main product of sulfide oxidation (Vainshtein, Gogotova, & Heinritz, 1994). It also acts both as a bioremediation agent improving water quality and as nutrient source for shrimp larviculture (Wen, Xue, Liang, Wu, & Li, 2015). *E. shaposhnikovii* can remove COD and NH_4^+–N, and can be applied to pollutant removal in saline wastewater treatment processes (Cai, Guan, Li, Zhao, Feng, & Tang, 2019a).

E. shaposhnikovii TW02 is commercially prepared to liquid suspension form that is ready to use for many purposes, including wastewater treatment in a shrimp farm. However, the number of viable PSB in suspended state is drastically reduced after harvesting and storage at room temperature (30±2°C) in a commercial warehouse. Therefore, the shelf life of suspended viable microorganisms should be extended to maintain count higher than 10^6 CFU/mL. There have been many methods for microbial preservation for short- and long-term applications, including mineral oil storage, freeze drying, and cryopreservation (Kumar, Kashyap, Singh, & Srivastava, 2013). However, those methods reduced the activity and viable counts of microorganisms. For short-term

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storage, the cell suspension in liquid broth without further processing is preferred to maintain a high viable cell count and activity, in a ready-to-use commercial product. To extend the shelf-life of cell suspension, supplementation of low-cost additives may be a good option for short-term storage in a commercial warehouse.

In general, environmental factors affecting growth and survival of phototrophic sulfur bacteria include exposure to light, electron donors, oxygen, and carbon sources (Gemerden & Mas, 1995). The metabolism of phototrophic purple sulfur bacteria is inhibited by oxygen in one way or another. The photosynthesis of anoxygenic phototrophic bacteria is dependent on oxygen-deficient conditions (Gemerden & Mas, 1995). However, there was no light or oxygen exposure during the storage of *E. Shaposhnikovii* in suspension. It was suggested that some species continued to photosynthesize in the presence of low oxygen concentration to provide energy (Gemerden & Mas, 1995). Although carbon dioxide is commonly used as the carbon source for purple sulfur bacteria, organic compounds are also assimilated (Gemerden & Mas, 1995). Organic acids and fatty acids are the preferred substrates, but short-chain alcohols and even carbohydrates are used by certain species (Madigan & Jung, 2009).

In this study, we aimed to develop a method to extend the shelf-life of *E. shaposhnikovii* suspended in liquid medium maintaining viable cell count above 10^6 CFU/mL during the warehouse storage at room temperature (30±2ºC). The effects of glycerine as an additive nutrient at a low concentration was examined to maintain viable cells and to delay death phase. Glycerine, which is a trihydric alcohol, can absorb water and gasses such as hydrogen sulfide and sulfur dioxide, which are electron donors for purple sulfur photosynthetic bacteria (Madigan & Jung, 2009; Wernke, 2014). Glycerol is generally used as cryoprotective additive to preserve microorganisms at freezing temperatures. However, the permeation of glycerol through cell wall and plasmic membrane can damage microbial cells depending on temperature and concentration (Hubálek, 2003). Although the buffer element could remain in the liquid broth, the supplementation of $Na_2HPO_4{\cdot}2H_2O$ and Na_2CO_3 could benefit by maintaining the pH of cell suspension during the storage. Therefore, full factorial experimental design was carried out in order to study the main effects and interactions between these candidate additives on various levels of viable cells of *E. shaposhnikovii*. Moreover, only low concentrations of additives were considered for economic reasons.

2. Materials and Methods

2.1 Microorganism and cultivation conditions

E. shaposhnikovii TW02, a purple sulfur bacterium isolated from water in a shrimp cultivation pond, was obtained from CPF (Thailand) Co., LTD. The cultivation medium, obtained from our previous experiments (Sanguanmanasak, 2020), contained (per L) 2.0 g $C_5H_8NO_4Na$, 0.625 g yeast extract, 0.125 g Tryptic soy broth, 2.0 g NH_4HCO_3, 0.2 g $MgSO_4{\cdot}7H_2O$, 30.0 g NaCl, 0.5 g KH_2PO_4 and 0.0018 g $C_6H_5FeO_7{\cdot}H_2O$. The cultivation was done under light conditions (~1000 lux) in batch fermentation (sterile medium, pH 8.51±0.01) for 15 days in 1L Duran bottles. Afterwards, stepwise increases in the working volume used 50L, 500L and 1000L fermenters. After batch-mode, 7 L of non-sterile medium (pH 8.04±0.02) was added every 3 days until reaching the working volume of 28 L at day 12. For 500 and 1000 L fermenter, 60 and 100 L non-sterile medium was added every 3 days until reaching the working volume of 240 or 400 L, respectively, at day 12. In the fermenters, the cultures were exposed to halogen light (2500-4000 lux) to provide sufficient light intensity in the large fermenter.

2.2 Experimental procedure

E. shaposhnikovii TW02 were harvested from cultivation in the 1000L fermenter with the final working volume of 400 L. Afterwards, the samples were stored in 1L translucent cylindrical plastic bottles (translucent light 50%) with supplemented additive substances at an ambient temperature (30±2ºC) for 60 days. The headspace in each bottle was 2.5 cm. New bottles were used without sterilization. The transfer of the cell suspension into bottles was conducted non-aseptically. Full factorial experimental design method was applied to study the main and interaction effects among the additives (Chen, Niu, Shu, & Wan, 2015). Three additive substances were used; glycerine 99.7% v/v (0 and 0.15%), $Na_2HPO_4{\cdot}2H_2O$ (0 and 0.5 g/L or 0 and 2.8 mM) and Na_2CO_3 (0 and 0.5 g/L or 0 and 4.7 mM). The matrix of coded experiments shows high and low levels as +1 and -1, respectively (Table 1). The 2^3 full factorial design with 8 experimental runs was conducted in duplicate at room temperature (30±2ºC). The negative control is Run 1 (with no additives). The viable cell counts of *E. shaposhnikovii* TW02 in the experiments were monitored. The ORP, pH, dry cell weight (DCW), bacteriochlorophyll content (BCC) and carotenoid content (CC) were also determined. The practical shelf-life of *E. shaposhnikovii* was defined as the storage time (days) with cell survival higher than 10^6 CFU/mL.

The following equation was used to fit results from the 2^3 factorial design (Equation 1):

$$Y = X_0+X_1A+X_2B+X_3C+X_4AB+X_5AC+X_6BC+X_7ABC \quad \text{(Equation 1)}$$

where Y is the predicted response (viable cells; CFU/mL), X_0 represents the global mean, X_i are regression coefficients corresponding to the main effects and interactions, A is the amount of glycerine 99.7%v/v (%), B is the amount of $Na_2HPO_4{\cdot}2H_2O$ (g/L), and C is the amount of Na_2CO_3 (g/L).

2.3 Analytical methods

2.3.1 Viable cells

The number of viable cells was determined by plate counting method. G5 medium containing (per L) 4.0 g sodium-L-glutamate, 3.5 g DL-malic acid, 0.12 g KH_2PO_4, 0.18 g K_2HPO_4, 5 g yeast extract and 5 g peptone (Kohlmiler & Gest, 1951) was used for plate counting of PSB. The initial pH of medium was adjusted to 8.0 by using 5 N NaOH. Serial dilution of the sample was conducted using 0.85% saline. Each diluted sample was inoculated onto G5 medium and cultivated under tungsten illumination at 1000 lux and 35±5^{O}C in a desiccator for 72 h.

Table 1. The experimental ranges and levels of independent variables

Variable	Code	Low level (-1)	High level (+1)
Glycerine 99.7%v/v (%)	A	0	0.15
$Na_2HPO_4 \cdot 2H_2O$ (g/L)	B	0	0.5
Na_2CO_3 (g/L)	C	0	0.5

2.3.2 Cell biomass production

Cell biomass was determined based on dry cell weight (Prasertsan, Choorit, & Suwanno, 1993). The samples (7 mL/sample) were centrifuged at 8,000 rpm (7,878×g) and 4^{O}C for 15 min. The pellet was washed twice with distilled water and then dried at 105^{O}C until constant weight.

2.3.3 Bacteriochlorophylls and carotenoids content

The samples (7 mL/sample) were centrifuged at 8,000 rpm (7,878×g) and 4^{O}C for 15 min. The pellet was washed once with 0.85% saline. The cells collected were used for measuring the levels of carotenoids and bacterio chlorophylls. The photosynthetic pigments were extracted using acetone-methanol (7:2 v/v) solvent. Afterwards, the cell solution was sonicated at 40^{O}C for 30 min using a water bath sonicator (Elmasonic E 30 H, Elma, Germany). The photosynthetic pigments extracted were centrifuged at 3,500 rpm (548×g) for 12 min. The extracts were used for measuring photosynthetic pigments via determining the absorbances of the extracts at 480 and 770 nm, measured using a spectrophotometer (GENESYS 20, Thermo SCIENTIFIC, USA). The total carotenoids and bacteriochlorophylls contents were calculated using Equation 2 and Equation 3 (Cai *et al*., 2019a; Prasertsean, *et al.*, 1993):

$$\text{Bacteriochlorophyll content} = ((2.45 \times A770) \times 10) / Z \quad \text{(Equation 2)}$$

$$\text{Carotenoids content} = ((A480 - 0.1 \times A770) \times 3.85) / Z \quad \text{(Equation 3)}$$

where A480 and A770 are the absorbances of the extracts at 480 and 770 nm, respectively; and Z is the dry cell weight (DCW in g/L).

2.3.4 pH and ORP

pH and ORP of the samples were measured using an ORP meter (Seven2Go7, Mettler Toledo, Switzerland).

2.3.5 Statistical analysis

The Design Expert 10 (trial version) and Statistical Package for the Social Sciences (SPSS) software version 22 (IBM Corp. Released 22.0.0.0, New York, USA) were used for all analyses. Statistically significant differences between groups were determined by one-way analysis of variance (ANOVA) assessments with Duncan's *post hoc* tests, where $p<0.05$ was required for a significant difference.

3. Results and Discussion

3.1 Effects of additive substances on ORP and pH

The shelf-life of *E. shaposhnikovii* TW02 was studied for 60 days. The initial ORP and pH were on average of -53±1 mV and 8.04±0.02. During the storage of *E. shaposhnikovii* TW02, ORP increased while pH decreased in all runs (Figure 1). The increase of ORP during storage could be from the bottles not being air tight. Moreover, anoxygenic photosynthetic bacteria used fermentative reaction in Calvin-Benson cycle and anaplerotic CO_2 assimilations leading to the assimilation or release of CO_2, the hydrolysis and acidification of organic matter, and the assimilation of sulfur (Chen *et al*., 2020; Tang, Tang, & Blankenship, 2011). Although, the transfer of the cell suspension was not aseptically conducted, no contaminating microorganisms were found under microscope. It can be observed that the ORP and pH for the experiment with the glycerine supplement were constant after a storage time of 15 days. This might be caused by the glycerine addition. At the end of experiment, the highest ORP at 18±0 and 24±1 mV was found in Run No.2 (with 0.15% glycerine) and Run No. 4 (with 0.15% glycerine and 0.5% $Na_2HPO_4 \cdot 2H_2O$) with the lowest pH of 6.79±0.00 and 6.70±0.02. The addition of glycerine caused ORP increase with significant decrease of pH ($p<0.05$). Glycerine is a trihydroxyalcohol, which can attract and hold oxygen in water (National Center for Biotechnology Information, 2020). Irvan, Trisakti, B., Hasibuan, R. and Joli, M. (2018) reported

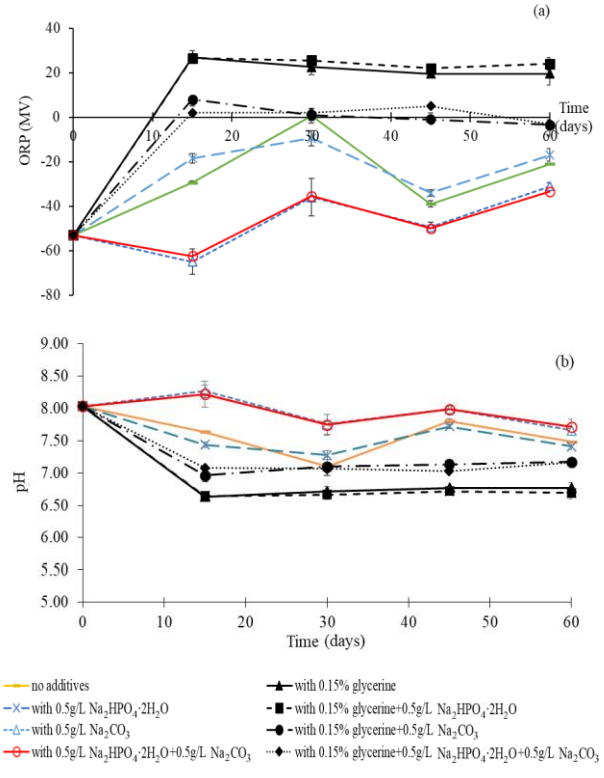

Figure 1. ORP (a) and pH (b) during the storage of *E. shaposhnikovii* TW02 in suspension form supplemented with glycerine, $Na_2HPO_4 \cdot 2H_2O$, and Na_2CO_3 as additives at room temperature.

that acetic acid was produced by the glycerine fermentation, initiated by phosphorylation of glycerine to glycerol 3-phosphate under light-anaerobic conditions. Gest (1951) reported that some purple sulfur photosynthetic bacteria produced volatile acid in a pyruvic acid medium, and *Rhodospirillum rubrum* growing in a malate-glutamate medium produced acetic acid. Therefore, glycerine, which is sugar alcohol, could be fermented during the storage of *E. shaposhnikovii* TW02 and converted to acetic acid resulting in a decrease of pH. The addition of 0.15% glycerine (Runs No. 2, 4, 6 and 8) gave lower pH than in runs without glycerine (Runs No. 1, 3, 5 and 7).

In contrast, the lowest ORP at -26±2 and -31±0 mV was found in Run No.5 (with 0.5% Na_2CO_3) and Run No.7 (with 0.5% Na_2CO_3 and 0.5% $Na_2HPO_4{\cdot}2H_2O$). Also, it was found that pH slightly changed from 8.04±0.02 to 7.57±0.03 and to 7.67±0.01 for Runs No.5 and 7, respectively. In this case, Na_2CO_3 could act as a buffer agent and neutralizing agent. In contrast, pH and ORP from the addition of 0.5 g/L $Na_2HPO_4{\cdot}2H_2O$ in Runs No. 3, 4, 7 and 8 were not different compared with the cases without $Na_2HPO_4{\cdot}2H_2O$ in Runs No. 1, 2, 5 and 6. Although $Na_2HPO_4{\cdot}2H_2O$ is a buffer agent, the amount of $Na_2HPO_4{\cdot}2H_2O$ used in this experiment may be too low to maintain pH.

3.2 Effects of additives on viable cells

The viable cells in 1L Duran bottles cultivated in batch mode for 15 days were on an average at $3.45\pm0.35\times10^5$ CFU/mL. Afterwards, the viable cells in 50 L, 500 L and 1000 L-fermenters cultivated in the fed-batch mode for 12 days were on an average at $7.27\pm1.96\times10^5$, $2.34\pm2.35\times10^5$ and $4.85\pm0.50\times10^6$ CFU/mL, respectively. The cultivation time was in the late log phase and the early stationary phase, which gave the highest viable cells. Consequently, the initial viable cells in all runs were on an average at $4.85\pm0.50\times10^6$ CFU/mL. After 45 and 60 days, the viable cells of *E. shaposhnikovii* TW02 were determined and the counts are presented in Table 2. After the storage for 45 days, it was found that the viable cell count of *E. shaposhnikovii* TW02 in a suspension without any additives (Run 1) was at $1.62\pm0.856\times10^6$ CFU/mL with a survival rate of 33.3%. However, Na_2CO_3 as the sole additive (Run 5) maintained the viable cells of *E. shaposhnikovii* TW02 at $2.35\pm0.120\times10^6$CFU/mL with a survival rate of 48.4%. It can be noted that there was no significant difference among Runs No.1 (without any additives), No.3 (with 0.5% $Na_2HPO_4{\cdot}2H_2O$ addition) and No.7 (with 0.5% $Na_2HPO_4{\cdot}2H_2O$ and 0.5% Na_2CO_3 addition) ($p>0.05$ in Duncan's test). This indicates that there was enough buffer capacity in the liquid medium. The storage of the suspension at ambient temperature for 45 days could give viable cells > 10^6 CFU/mL without any additives. In contrast, the addition of glycerine (Run 2, 4, 6, 8) reduced the viable cells of *E. shaposhnikovii* TW02 to 0.037-0.255 $\times10^6$ CFU/mL and the viable cell counts were lower than without glycerine as the additive (Runs 1, 3, 5, 7) significantly. This might be due to the lower pH and the limitation of nutrients (Figure 1). *E. shaposhnikovii* preferably grows under anoxic conditions in the light at pH 8.0-9.0 (Brenner *et al*., 2004). The ORP can influence microbial metabolism, and a favorable low ORP is necessary during anaerobic fermentation (Cai *et al*., 2019b). To prolong stationary phase, other nutrient supplements might be required instead of glycerine. CN105543094B (2020) claimed that collagen protein was used as slowly energy-releasing substrate for the preservation of photosynthetic bacterium. After storage for 60 days, viable cells decreased in all runs with a survival rate of less than 10%, except for the experiment with $Na_2HPO_4{\cdot}2H_2O$ and Na_2CO_3 as additives (Run 7). The viable cells remained higher than 10^6 CFU/mL, which might be caused by more buffer capacity than in the other Runs. In addition, carbonate ions can form alkaline complexes with the metal ions, such that are much more soluble than hydroxides (Sorokin & Kuenen, 2005). Carbonate and bicarbonate are involved in carbon cycle for the growth of Ectothiorhodospiraceae (Sorokin & Kuenen, 2005). Tang, Tang, and Blankenship (2011) reported that anaerobic anoxygenic phototrophics can use the Calvin–Benson cycle for CO_2 fixation. At the exhaustion of nutrient in liquid broth, Na_2CO_3 could be an inorganic carbon source for *E. shaposhnikovii*. Bryantseva, I. A., Tourova, T. P., Kovaleva, O. L., Kostrikina N. A., & Gorlenko, V. M. (2010) reported that the best growth for *Ectothiorhodospira magna* sp. nov. was on sulfide in the presence of acetate and bicarbonate.

3.3 Full factorial experimental design

To study the main effects and interactions between the factors, statistical analysis was conducted assessing the viable cell counts at the storage times of 45 and 60 days. The ANOVA for the main and interaction effects are shown in Table 3. The sums of squares used to estimate factor effects,

Table 2. Viable cells and pigment contents of *E. shaposhnikovii* TW02 during storage at room temperature for 45 days in a suspension supplemented with glycerine, $Na_2HPO_4{\cdot}2H_2O$, and Na_2CO_3 as additives.

Run	A	B	C	Viable count ($\times10^6$CFU/mL)	
				at 45 days	at 60 days
1	-1	-1	-1	1.62 ± 0.856^{a}	$0.194\pm0.028^{b,c}$
2	+1	-1	-1	0.255 ± 0.032^{b}	$0.073\pm0.088^{b,c}$
3	-1	+1	-1	1.65 ± 0.141^{a}	$0.246\pm0.233^{b,c}$
4	+1	+1	-1	0.064 ± 0.006^{b}	$0.128\pm0.020^{b,c}$
5	-1	-1	+1	2.35 ± 0.120^{a}	0.260 ± 0.028^{b}
6	+1	-1	+1	0.104 ± 0.000^{b}	$0.100\pm0.000^{b,c}$
7	-1	+1	+1	1.86 ± 0.127^{a}	1.15 ± 0.057^{a}
8	+1	+1	+1	0.037 ± 0.005^{b}	0.028 ± 0.018^{c}

Note: Different superscripts in the same column indicate statistically significant difference ($p<0.05$, Duncan test)

Table 3. Analysis of variance (ANOVA) for viable cell counts of *E. shaposhnikovii* TW02 after storage in 1L translucent cylindrical plastic bottle for 45 or 60 days

Storage time	Source term	Sum of Squares	df	Mean Square	F Value	*p*-value Prob>F
45 days	Model	1.30×10^{13}	7	1.86×10^{12}	18.99	0.0002*
	A	1.23×10^{13}	1	1.23×10^{13}	125.4	<0.0001*
	B	1.26×10^{11}	1	1.26×10^{11}	1.28	0.2902ns
	C	1.45×10^{11}	1	1.45×10^{11}	1.48	0.2582ns
	AB	9.12×10^{9}	1	9.12×10^{9}	0.093	0.7681ns
	AC	3.13×10^{11}	1	3.13×10^{11}	3.19	0.1119ns
	BC	3.92×10^{10}	1	3.92×10^{10}	0.40	0.5447ns
	ABC	1.04×10^{11}	1	1.04×10^{11}	1.06	0.3337ns
	Pure Error	7.84×10^{11}	8	9.80×10^{10}		
	Cor Total	1.38×10^{13}	15			
	R^2= 0.9432, Adj. R^2=0.8936, Pred. R^2=0.7729					
60 days	Model	1.86×10^{12}	7	2.65×10^{11}	31.46	<0.0001*
	A	5.78×10^{11}	1	5.78×10^{11}	68.65	<0.0001*
	B	2.14×10^{11}	1	2.14×10^{11}	25.39	0.0010*
	C	2.02×10^{11}	1	2.02×10^{11}	23.93	0.0012*
	AB	2.30×10^{11}	1	2.30×10^{11}	27.29	0.0008*
	AC	2.73×10^{11}	1	2.73×10^{11}	32.34	0.0005*
	BC	1.26×10^{11}	1	1.26×10^{11}	14.96	0.0048*
	ABC	2.33×10^{11}	1	2.33×10^{11}	27.69	0.0008*
	Pure Error	6.74×10^{11}	8	8.43×10^{9}		
	Cor Total	1.92×10^{12}	15			
	R^2= 0.9650, Adj. R^2=0.9343, Pred. R^2=0.8598					

Note: Significance of estimated coefficient; ns = not significant; * = significant at $p<0.05$. R^2, coefficient of determination; R^2_{adj}, the coefficient adjusted for degrees of freedom; *F*-value, ratio between mean squares of regression and residuals.

Fisher's *F* ratios (defined as the ratio of mean square effect and the mean square error) and *P* values (defined as the level of significance leading to the rejection of the null hypothesis) are presented. The *F*-values of 18.99 and 31.46 for the storage times 45 and 60 days, respectively, with low probability values ($p < 0.05$) indicate that the models are significant for viable cell counts during the storage. Furthermore, the fitted models had coefficients of determination (R^2) of 0.9432 and 0.9650, in good agreement with the statistical model. At storage time of 45 days, only glycerine (*A*) was a significant factor negatively affecting the viable cells of *E. shaposhnikovii* TW02 (Figure 2a). However, $Na_2HPO_4{\cdot}2H_2O$ (B), Na_2CO_3 (C) and an interaction of BC showed a positive effect on the viable cells after storage for 60 days. In contrast, glycerine and the interactions AC, BC and ABC showed negative effects on the viable cells (Figure 2b).

The following are predictive model equations of viable cell counts after storage of *E. shaposhnikovii* TW02 in 1L translucent cylindrical plastic bottle for 45 days (Equation 4) and 60 days (Equation 5):

$$Y = 9.911\times10^5 - 8.764\times10^5A - 8.8625\times10^4B + 9.525\times10^4C + 2.3875\times10^4AB - 1.398\times10^5AC - 4.95\times10^4BC + 8.05\times10^4ABC \quad \text{(Equation 4)}$$

$$Y = 2.721\times10^5 - 1.901\times10^5A + 1.156\times10^5B + 1.122\times10^5C - 1.199\times10^5AB - 1.305\times10^5AC + 8.875\times10^4BC - 1.207\times10^5ABC \quad \text{(Equation 5)}$$

where Y is the predicted response (viable cells in CFU/mL), *A* is the amount of glycerine 99.7%v/v (%), *B* is the amount of $Na_2HPO_4{\cdot}2H_2O$ (g/L), and *C* is the amount of Na_2CO_3 (g/L).

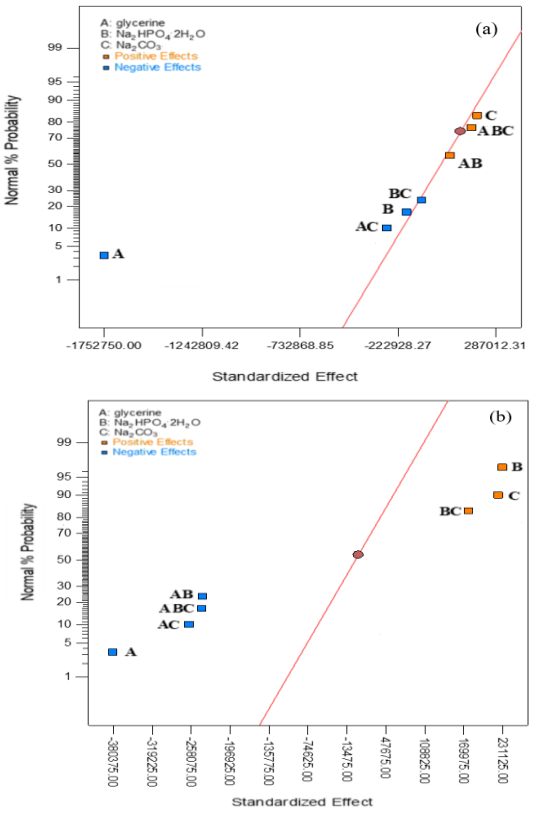

Figure 2. Standardized effects on the viable counts during storage of *E. shaposhnikovii* TW02 in suspension supplemented with glycerine, $Na_2HPO_4{\cdot}2H_2O$, or Na_2CO_3 as additives at room temperature for 45 days (a) and for 60 days (b).

In Equation 4 and Equation 5, Na_2CO_3 (C) has positive coefficients for the storage times of 45 and 60 days. This means that Na_2CO_3 as an additive increased longevity of the viable cells of *E. shaposhnikovii*: the addition Na_2CO_3 at 0.5 g/L in the suspension of *E. shaposhnikovii* gave larger viable cell counts. Moreover, the addition of Na_2CO_3 at 0.5 g/L and $Na_2HPO_4{\cdot}2H_2O$ at 0.5 g/L in the suspension of *E. shaposhnikovii* prolonged the shelf life (with over 10^6 CFU/mL of viable cells) to 60 days of storage. Na_2CO_3 could act as a neutralizing agent and contribute to the carbon cycle of purple sulfur bacteria (Sorokin & Kuenen, 2005).

Equation 4 indicates that the main effect of (A) was significant. This large negative main effect was quite intense, and could not be ignored from the model. Equation 5 indicates that the main effects (A, B and C), some two-variable interactions (AB, AC and BC), and the three-variable interaction (ABC) were significant. The large positive B and C main effects and (BC) interactions were quite intense, thus could not be ignored from the model. It can be noted that Equation 4 and Equation 5 can predict the viable cells (CFU/mL) for the storage times of 45 and 60 days. These equations allow predicting effects of various scenarios with some given (low) concentrations of the additives.

3.4 Effects of additive substances on pigments

Photosynthetic pigments are bacteriochlorophyll a (esterified with phytol) and carotenoids of the spirilloxanthin series, with spirilloxanthin as the major component accumulated in PSB (Brenner *et al*., 2004). After cultivation of *E. shaposhnikovii*, bacteriochlorophyll (BCC) and carotenoid (CC) contents were on average 65.0±3.0 and 8.23±0.18 mg/g_{DCW}. During the storage of *E. shaposhnikovii*, the BCC and CC levels gradually increased in all runs (Figure 3). This was caused by dead microbial cells. Dry cell weight decreased but the pigments were at constant levels. At the end of the experiment, the highest BCC and CC contents of respectively 95.1±1.3 and 12.7±0.1 mg/g_{DCW} were found for Run No. 7 with the addition of $Na_2HPO_4{\cdot}2H_2O$ and Na_2CO_3. However, there was no statistically significant difference between the suspensions of *E. shaposhnikovii* with and without additives. It was noted that the supplementation with additives did not affect the pigment contents of *E. shaposhnikovii* during storage at room temperature.

4. Conclusions

Based on experiments following a full factorial design, it could concluded that 0.5% Na_2CO_3 as an additive enhanced the longevity of viable *E. shaposhnikovii* cells during storage in liquid suspension at room temperature (30±2°C). On the other hand, the addition of glycerine and the interactions of glycerine with other additives significantly decreased viable cell counts. The addition of Na_2CO_3 at 0.5 g/L and $Na_2HPO_4{\cdot}2H_2O$ at 0.5 g/L in the suspension of *E. shaposhnikovii* extended the shelf life of viable cells (requiring over 10^6 CFU/mL) to allow storage for 60 days. This indicates that for storage, *E. shaposhnikovii* in commercial stage could be supplemented with effective low-cost substances. However, more experiments are required to determine such concentrations of additives that would maximize the viable cell counts. Also, the suspension of *E. shaposhnikovii* after storage at room temperature should be examined for its efficacy in applications, including the removal of H_2S, BOD, or COD from shrimp aquaculture ponds, or for serving as a nutrient source for a shrimp larviculture.

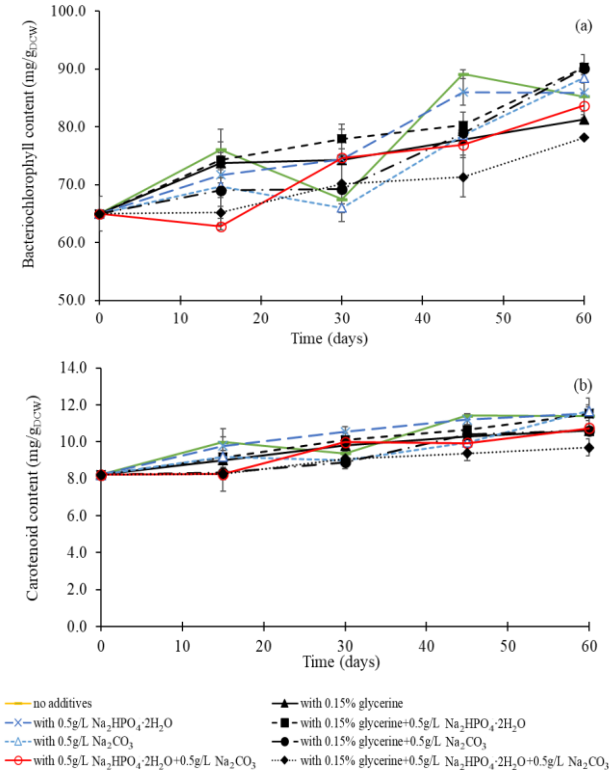

Figure 3. Bacteriochlorophyll content (a) and carotenoids content (b) during the storage of *E. shaposhnikovii* TW02 in suspension supplemented with glycerine, $Na_2HPO_4{\cdot}2H_2O$, or Na_2CO_3 as additives at room temperature.

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