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Pigments from halophilic bacteria isolated from salty fermented foods for further development as bio/natural-food additives

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Abstract-Pigments producing strains *Halobacillus yeomjeoni* (81-1) *Salinicoccus* sp. (82-1) *Bacillus infantis* (63-11) *Bacillus amyloliquefaciens* (60-5) and *Staphylococcus carnosus* (48-10) were isolated from salty fermented foods. As per FTIR analysis, IR spectrum of pigment from each isolate were similar to IR spectrum of xanthophyll as previously reported. This study concluded that the pigments generated from these different halophilic strains with different color shade were all derivatives of carotenoid. Dominant bacterial pigments of these strains analyzed by High-Performance Liquid Chromatography (HPLC) compared to standard reagent included lycopene, lutein and β-carotene. Antimicrobial assays of crude pigment extract of 81-1 isolate exhibited highest inhibitory effect on *Staphylococcus aureus*, while 82-1 and 60-5 isolates best inhibited *Escherichia coli* and *Bacillus cereus*, respectively. DPPH assay of 60-5 isolate demonstrated significantly higher antioxidant activity as compared to others crude pigments. Furthermore, virulence analysis of strains depicted no key biogenic amine genes and no hemolytic activity in any of the isolates.

Keywords: Halophilic bacteria, bacterial pigment, carotenoid profiles, safety analysis

1. Introduction

Recent awareness in human wellbeing had led to consumer's demand for high quality food preservatives. Natural food preservatives such as coloring agent are considered safe for human consumption as compared to synthetic coloring agent, as they cause no deleterious effect. They can be categorized into plant-derived products (herb and spices) and microbial-derived pigments, with microbial pigments being promising and desirable alternative. Microorganisms offer certain distinctive advantages due to their short life cycle, their low sensitivity to seasonal and climatic changes, their easy scaling as well as ability to produce pigments of various colors and shades depending on species for numerous applications in food to cosmetics (Pankaj *et al.*, 2016). Microbial pigments have been applied in both food and feed products so far. Particularly, carotenoids produced from microorganisms have been already commercialized and wildly used. For example, astaxanthin produced by *Xanthophyllomyces dendrorhous* has been used as feed supplement for salmons, crabs, and shrimps. Similarly, zeaxanthin produced by *Flavobacterium* sp. has been used as an additive in poultry feed to increase yellow color of animal's skin and egg yolk (Pankaj *et al.*, 2016). Microorganisms like *Blackesleatrispora*, *Mucorcircinelloides,* and *Phycomyces blackesleeanus* have also been used as a food additive in vegetable oils, orange drink, margarine, various emulsions, and microencapsulated bead lets (Nigam & Luke, 2016).

Apart from coloring agent, microbial pigments due to their key bioactivity such as anti-microbes and anti-oxidation are a promising attractive agents for new biotechnological applications, ranging from functional food production to the generation of new drugs and biomedical therapies. Thus, to extend the application and/or find alternative beneficial natural pigment for food industries, identification of new alternative microbes, utilization of low-cost substrates, and optimization of process parameters are the areas under focus towards economical pigment production. (Pankaj *et al.*, 2016; Nigam, 2016; Ventosa *et al.*, 1998).

Microbial pigments are of great interest due to their stability and year around availability and easy cultivation methods (Dhere & Dharmadhikari, 2017). Criteria for microbial strains selection for pigment production include, ability to adapt in extreme environment, protection from solar radiation, photosynthesis and stress like drought condition and especially the condition with high salt. Studies support that microbial-derived pigments can be isolated from different environmental sources like soil, sea water or salty fermented foods such as Thai fermented fish (pla-ra) (Det-udom *et al.*, 2022) and soy sauce (Pankaj *et al.*, 2016; Ventosa *et al.*, 1998).

Though, several microbial pigments have been studied and categorized based on their composition molecular weight and structure, they are yet to be approved for application in commercial food systems. Therefore, it is essential to accurately identify and evaluate strain selection process prior to intensive strain development. Microbial metabolites and microbial culture used in food may produce both beneficial and toxic metabolites (Pariza & Johnson, 2001). Thus, the pathogenicity and toxicity of microbes is evaluated before considering as food grade. Prior to recognition as probiotics,

preservatives, starter or protective culture in food, the minimum safety assessment of the strains for any toxin/allergen production and hemolytic potential is required as per regulations. In ensuring the safety of the pigment producing strains, it is also essential that the microbes do not carry any pathogenic, or allergen/toxin genes (Gueimonde *et al.*, 2013).

This study was aimed to characterize pigments produced by halophilic bacteria isolates having different color shades and to assess their bioactivity and safety for further development as bio/natural-food additives.

2. Materials and methods

2.1 Bacterial strains and culturing conditions

Halophilic pigment producing bacteria were isolated from salty fermented fish (pla-ra) and soy sauces samples. The strains stability were tested and further identified by 16S rDNA analysis as previously described by Sricharoen and research group in year 2019. Five representative strains from different color shades were selected for further investigation including, *Halobacillus yeomjeoni* (81-1) *Salinicoccus* sp. (82-1) *Bacillus infantis* (63-11) *Bacillus amyloliquefaciens* (60-5) and *Staphylococcus carnosus* (48-10). The isolates were cultivated in nutrient broth (NB) (Himedia, India) containing 3% sodium chloride (NaCl) under orbital shaking 150 rpm, at room temperature for 4 days. The cell pellet was collected by centrifugation at 1000 xg, for 20 min and frozen at least 24 h prior to pigment extraction.

2.2 Pigment extraction

Pigment extraction method was modified from Khaneja *et al.* (2010). The frozen cell was suspended in 1M sodium hydroxide (NaOH) and sonicated at room temperature for 5 min and centrifuged to remove NaOH. Methanol and Chloroform were added (ratio 1:2) and followed by addition of water. The suspension was vortexed and let to create a phase separation. After centrifugation, the organic layer (lower) was collected, and the aqueous (upper) layer re-extracted twice with chloroform. The organic solvent was evaporated in vacuum evaporator. Pigment extract was stored at -20°C for further analysis.

2.3 Pigment analysis

The crude pigment extracts were identified based on the absorption spectra by scanning the absorbance in the range of 300-600 nm using UV-Vis spectrophotometer (Biopectrometer basic, Eppendof, Geremany) (Ramachandran *et al.*, 2014). The extracts were subjected to FTIR spectroscopy (FTIR-200, PekinEemer, USA). Pigment sample was prepared by mixing the extract with small amount of KBr. The prepared sample was then pressed in a sample holder and analyzed by computerized Fourier Transform Infrared Spectroscopy system (Spectrum One) which generates the transmitting spectra in the range of 4000-400 cm^{-1}. (Usman *et al.*, 2018). The extracts were further subjected to HPLC analysis by dissolved in methanol/acetonitrile (1:1, v/v) and diluted with methanol/acetonitrile (50:50) to a final volume of 4 ml. The final solution was filtered through 0.45 µm membrane filters and 20 µl were injected for HPLC analysis. Analysis was

performed using a Shimadzu LC-20AC (Shimadzu, Kyoto, Japan) pumps, SPD-M20A with diode array detector and chromatographic separations were performed on a LUNA C-18 column (4.6 · 250 mm i.d., 5 μm). The mobile phase consisted of methanol (solvent A)/ acetonitrile + triethylamine (TEA) 9 μM (solvent B) 90:10 (v/v) at a flow rate of 0.9 ml/min. The column temperature was set at 30 °C and the absorbance was read at 475 nm (Siriamornpun *et al.*, 2012).

2.4 Bioactivity assay

The antioxidant activity of the extracts was determined using DPPH assay followed the method described by Braca *et al.* (2001). Aqueous extract (0.1 ml) was added to 3 ml of 0.001 M DPPH in methanol. Absorbance at 517 nm was determined after 30 min, and the percent inhibition of activity was calculated as $[(A_o - A_e)/ A_o] x100$ (A_o = absorbance without extract; A_e = absorbance with extract).

Antimicrobial activity against potential pathogens, *Staphylococcus aureus* ATCC 25923, *Bacillus cereus*, *Escherichia coli* ATCC 25922 and *Salmonella Typhimurium* ATCC 1331 was conducted by agar well diffusion method. Nutrient agar were streaked (lawn culture) with pathogen strains following 6 mm well preparation, to which 150μl of crude extract were added and inoculated for 24h at 37°C and zone OD inhibition (ZOI, mm) was determined.

2.5 Safety screening

The isolates as was evaluated for any potential production of biogenic amine and hemolysins. Biogenic amine encoding genes, associated with histamine, tyramine, and putrescine productions, were tested by multiplex PCR with primers as mentioned by Coton *et al.* (2004) and Fernández *et al.* (2007). The screening for hemolytic activity was tested by spotting of the overnight culture onto 5% blood agar plate and incubating at 37 °C for 24 h. Hemolysis zone, (α, β, and λ) for each isolate were inspected following the methods of Mukry *et al.* (2010).

2.6 Statistical analysis

Statistical analysis of variance (ANOVA) and multiple comparisons by Tukey's test were performed using IBM-SPSS statistics package version 22 (SPSS Inc., Chicago, IL, USA). A probability at $p < 0.05$ was considered statistically significant.

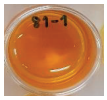
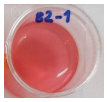
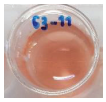
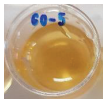
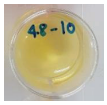
3. Results and discussion

3.1 Pigment producing strains, pigment color shades and composition

The functional group as identified by FTIR analysis were summarized in table 1. The IR spectra of the extracted yellow pigment from *Staphylococcus* group, yellow-orange pigment from *Halobacillus* group, orange-red pigment from *Bacillus* group and pink pigment from *Salinicoccus* sp. showed the presence of carbonyl functional group by a sharp peak at 1,745.75, 1,745.13, 1,744.00 and 1,745.78 cm^{-1}, respectively. This carbonyl group might belong either to aldehyde, ester or carboxylic families. An observation of a weak and broad signal as around 2,957.57, 2,955.64, 2,956.32 and 2,954.79 cm^{-1} of each group provide further evidence that a carboxylic family was present in the compound. This information demonstrated that these pigmented bacterial

isolates produced carotenoids which might mainly be xanthophyll group. This information demonstrated that these pigmented bacterial isolates produced carotenoids which were mainly xanthophyll group. The standards in a xanthophyll group will be therefore selected as standard in further quantitative analysis (Ramachandran *et al.*, 2014; Namitha & Negi, 2010). Majority of carotenoids are derived from a 40-carbon polyene chain which could be considered as the backbone of the molecule and divided into 2 groups (carotene and xanthophylls). Carotenes, such as beta carotene and alpha carotene, are purely hydrocarbon, whereas xanthophylls, such as lutein and zeaxanthin, are oxygenated. Thus, carotene, lutein and lycopene were used as representative carotenoid standards to qualify crude pigment extracts by HPLC analysis. The results in Table 2 and Figure 1 exhibited lycopene as the main composition of crude pigment extract in all strains, followed by lutein**.** Crude extract of *Halobacillus yeomjeoni* with intense orange color shade contained the highest amount of lycopene up to approximately 5,000 µg/g followed by *Bacillus amyloliquefaciens* with pale orange shade containing approximately 2,000 µg/g while the other strains with different color shades contained lycopene lower than 1,000 µg/g. Lutein detected in all strains varied around 10-200 µg/g. The strains with yellow shade, *Staphylococcus carnosus* contained significantly higher amount of the lutein (around 200 µg/g) relative to the other strains. Carotene was detected in crude pigment extract of *Bacillus infantis*. From the composition observed in pigment of these isolates, the bacteria could be a source of natural pigment color in the carotenoid group. Carotenoids have attracted great attention for their functional properties, health benefits and prevention of several major chronic diseases (Cooper, 2004). Carotenoids contribute to the yellow color found in many fruits and vegetables. The color appearances depend on conjugated double bonds and the various functional groups contained in the carotenoid molecule (Rodriguez-Amaya & Kimura, 2004). Some studies also reported that greater the number of conjugated double bonds, higher the absorption maxima (λ_{max}). As a result, the color ranges from yellow, red to orange (Bartley & Scolnik, 1995; Rodriguez-Amaya, 2001). Lycopene, main carotenoid composition observed in these bacterial isolates is one of the popular pigments highly accepted by food industry as a food additive and also for its health benefits (Kong *et al.*, 2010). As a red colorant and antioxidant agent, the demand for lycopene is still increasing. According to Kong *et al.* (2010), total world consumption of lycopene was tripled to 15,000 tonnes in 2004 compared to 5000 tonnes in 1995. Thus, alternative sources for the production of natural lycopene are warranted. Lutein (β, ε-Carotene- 3, 3' diol) is a naturally occurring oxygenated derivative of hydrocarbon carotenoids (Tsao *et al.*, 2004). It has been reported in epidemiological studies that lutein reduces the risk of some chronic diseases such as cancer, heart disease and age-related eye diseases. A lot of information regarding the importance of lutein on human health has been gathered (Hajare *et al.*, 2013). Carotenes are polyenes with the ends consisting of either one or two unsaturated cycles. They are considered tetraterpenes, having the general formula $C_{40}H_{56}$. Carotenes are present in plants and are related to several other compounds of biological importance such as retinal and vitamin A (retinol). The most common carotenes are α-carotene and β-carotene (Landrum, 2010).

Table 1. Functional group as identified by FTIR analysis and carotenoid profiles in crude pigment extracts of isolates

Strains of isolate	Pigment color	FTIR analysis		HPLC analysis		
		Identifying functional group	Tentative identification	Contents (µg/g)		
				Lycopene	Lutein β-	Carotene
Halobacillus yeomjeoni		2955.64 and 2871.25 (C-CH_3/O-H stretch); 2920.88 and 2848.21 (-CH_2/O-H stretch); 1745.13 (C=O stretch); 1462.75 and 1445.83 (-CH_2- and -CH_3 bend); 1423.48 (-CH_2 bend); 1377.74 (-CH_3 bend)	Xanthophylls	4991.41±40.40	17.54±0.75	Not Detected
Salinicoccus sp.		2954.79 (C-CH_3/O-H stretch); 2920.59 and 2848.57 (CH_2/O-H stretch); 1745.78 (C=O stretch); 1637.31 (C=C stretch); 1445.81 (-CH_2- and -CH_3 bend)	Xanthophylls	852.13±28.12	41.12±1.03	Not Detected
Bacillus infantis		2955.65 and 2872.26 (C-CH_3/O-H stretch); 2920.21 and 2848.21 (-CH_2/O-H stretch); 1743.67 and 1728.80 (C=O stretch); 1652.52 and 1635.64 (C=C stretch); 1462.79 and 1445.40 (-CH_2- and -CH_3 bend); 1422.50 (-CH_2 bend)	Xanthophylls	938.57±5.39	6.50±1.34	13.19±0.56
Bacillus amyloliquefaciens		2956.32 and 2873.69 (C-CH_3/O-H stretch); 2921.26 and 2848.57 (-CH_2/O-H stretch); 1744.00 (C=O stretch); 1636.56 (C=C stretch); 1462.66 and 1446.01 (-CH_2- and -CH_3 bend); 1422.57 (-CH_2 bend); 1378.84 (-CH_3- bend)	Xanthophylls	2225.36±63.84	124.83±0.50	Not Detected
Staphylococcus carnosus		2956.23 and 2873.49 (C-CH_3/O-H stretch); 2921.15 and 2848.51 (-CH_2/O-H stretch); 1745.20 (C=O stretch); 1641.03 (C=C stretch); 1462.91 and 1445.92 (-CH_2- and -CH_3 bend) 1423.20 (-CH_2 bend); 1378.11 (-CH_3 bend)	Xanthophylls	496±6.10	199.43±4.78	Not Detected

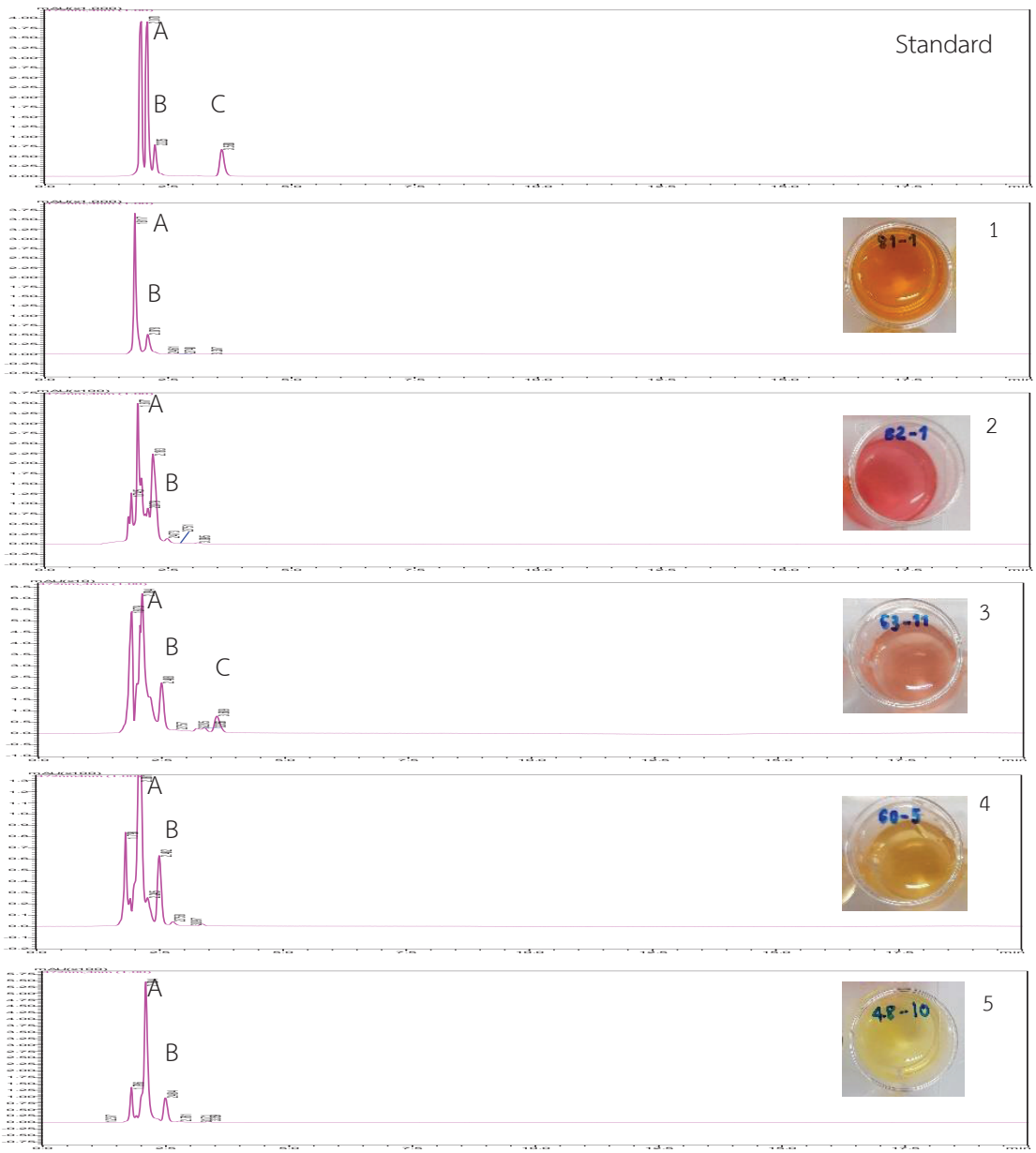


Figure 1. HPLC chromatograms of crude pigment extracts from 5 pigment producing strains

3.2 Pigment extracts and biological properties

Due to increasing demand of natural food and alternative for synthetic food colorants, carotenoids from these bacterial isolates might be interesting for use as both natural antioxidants and antimicrobial agents in food products. Therefore, in this study, crude pigment extracts from the isolates were studied for their antibacterial and antioxidant activity. Inhibitory effect of pigment extracts (5 mg/ml) on representative food pathogens, gram positive *Staphylococcus aureus*, spore forming *Bacillus cereus* and Gram-negative *Escherichia coli,* were investigated.

Tetracycline was used as standard antibiotic. Results in table 2, demonstrated significant inhibitory effects of *Halobacillus yeomjeoni* (81-1) and *Bacillus infantis* (63-11) on three tested strains. *Salinicoccus* sp. (82-1) could inhibit S. *aureus* and *E. coli* and *Bacillus amyloliquefaciens* (60-5) inhibited *B. cereus* and *E. coli* while Staphylococcus *carnosus* (48-10) could not inhibit any tested strains. The results observed corresponded to the report of D. C. Mohana *et al.* (2013) who demonstrated that pigment extract (contained carotenoids) of *M. roseus* inhibited *S. aureus* and *Rhodotorula glutinis* extract inhibited *S. aureus, B. cereus* and *E. coli* with similar inhibition zones to this study.

Antioxidation activity of pigment extracts by DPPH radical scavenging analysis, reported as% inhibition, displayed highest% inhibition in *Bacillus amyloliquefaciens* (60-5) at approx. around 15. This demonstrated that all extracts had antioxidation activity much lower than standard ascorbic (0.30 mg/ml) that had%inhibition up to around 96. From previous report that evaluated pigment extracts of *M. roseus* (1-10 mg/ml) and *M. luteus* (1-10 mg/ml),% DPPH radical scavenging observed as 32.8-88.5 and 29.1-82.1 were also much lower than standard. These low activities might be due to the concentration of crude extracts used in the test were too low allowing% inhibition detected was lower than the true value (D. C. Mohana *et al.*, 2013). Since DPPH* is relatively not active like free radical generated in in vivo allowing its reaction caused slower. In addition, the impurity in the crude extract could interfere the reaction and reduce the color of DPPH* reaction. Thus, for accurate antioxidation activity assessment, purification of the crude extract would be crucial. The results suggested that carotenoids producing isolates could be alternative source of natural pigments with antioxidant and antimicrobial, colorants, and nutraceutic

Table 2. Antimicrobial and antioxidant activities of crude pigment extracts of isolates.

Strains	Antimicrobial Activity Inhibition Zone (mm)			DPPH analysis (% of Inhibition)
	S. aureus	***B. cereus***	***E. coli***	
Halobacillus yeomjeoni (81-1)	8.10±0.07	6.77±0.04	6.30±0.07	12.72±0.25
Salinicoccus sp. (82-1)	6.85±0.28	NI	7.75±0.64	11.99±0.27
Bacillus infantis (63-11)	6.05±0.00	6.38±0.18	6.95±0.00	7.73±0.33
Staphylococcus carnosus (48-10)	NI	NI	NI	6.01±1.35
Bacillus amyloliquefaciens (60-5)	NI	7.33±0.67	7.30±0.49	14.93±1.82
Tetracycline (30 µg)	23.65±0.70	23.98±0.95	24.35±0.71	Ascorbic Acid (0.30 mg/mL) = 95.50±2.74

Remark NI means No Inhibition Zone

3.3 Safety property screening

All bacterial isolates in this study have been reported as halophilic bacteria generally found and/or isolated from salt contained foods. In addition, some species such as *Staphylococcus carnosus* have been known as important bacteria used in food (Götz, 1990). *Bacillus amyloliquefaciens*, a GRAS (generally recognized as safe) probiotic microbe (Yohannes *et al.*, 2020), has the ability to synthesize various bioactive components (Cai *et al.*, 2017), and it has been widely applied in soybean fermentation to produce novel lycopene-rich soybean food (Yohannes *et al.*, 2020); *Bacillus infantis* as probiotic strains in aquaculture production. *Salinicoccus* sp. has been reported as bacteria associated to traditional fermented foods such as salty shrimp paste and kimji (Pakdeeto *et al.*, 2007) and has much importance in biotechnology applications (Ventosa, 2015). *Halobacillus yeomjeoni* with its ability to grow under halophilic conditions, the presence of a clearly defined carotenoid biosynthetic pathway, and the probable potential toward enzyme production makes *Halobacillus* an organism of industrial importance (Joshi *et al.*, 2013). Based on their source of isolates in this study and information from many previous reports, these bacteria should be primarily categorized in GRAS status and potential to be developed for application in food, biotechnological, agricultural and even nutraceutical industries. However, in development of the pigment production isolates for further industrial use, the safety of strains should be firstly evaluated. Pariza and Foster (1983) stated the importance of food safety, including food enzymes, and in particular the importance to study on safety of candidate strains to be developed as food grade. The minimum safety assessment of the food microorganism should be determined for toxin production and hemolytic potential as recommended by Food and Agriculture Organization (FAO) US and European Food Safety Authority (EFSA, 2005). Thus, the safety of the five isolates were screened for allergens (biogenic amines), including histamine, tyramine, putrescine, hemolysin production to evaluate the pathogenicity. Biogenic amines are functionally important low molecular weight nitrogenous bases and metabolic compounds in living organism and occasionally present in some food products causing considerable toxicological risks as potential human carcinogens when consumed in excess concentrations (Eom *et al.*, 2015). The presence of histamine, tyramine and putrescine in five strains was tested following multiplex PCR method, using primers mentioned by (Chhetri *et al.*, 2019). Although, Han *et al.* (2007) reported that *Bacillus* spp. including *B. amyloliquefaciens* were potential to have decarboxylase activities and could produce high biogenic amines from free amino acids available in fermentation system. However, the strains in this study and another 4 strains, none of the biogenic amines (histamine, tyramine or putrecine) genes were found as shown in Figure 2. Therefore, these bacteria have no potential to generate BA in fermentation system making it safe to be used in food systems in term of allergens production.

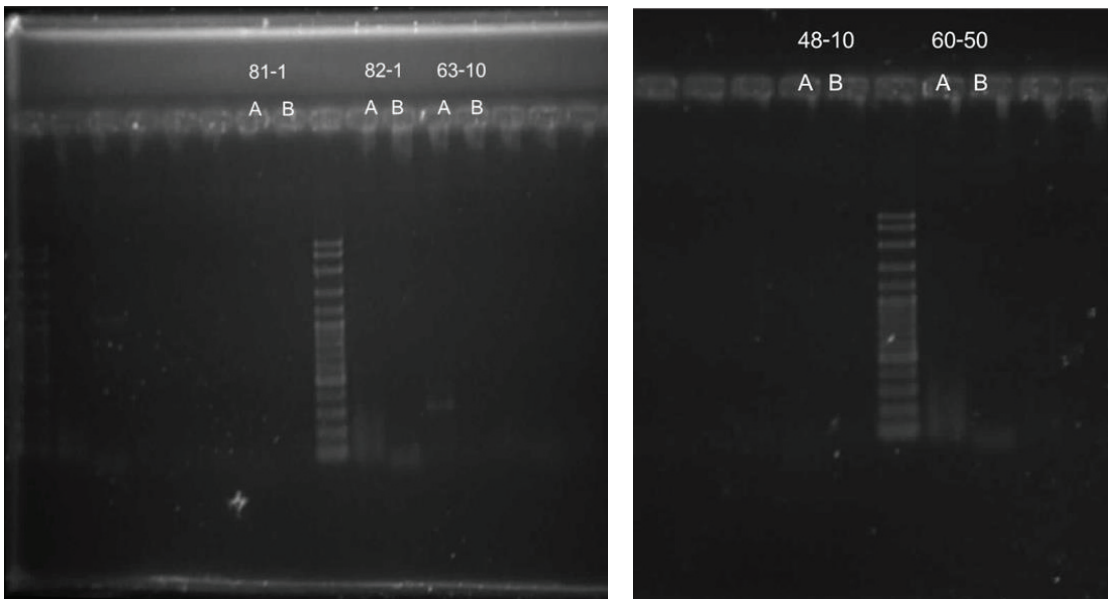


Figure 2. 1.5% agarose gel for biogenic amines gene assay
(A) Primer TD2/5 + TD-F/R + PUT2-F/R + HDC 3 / 4
(B) Primer set PUT1-F/R + TDC 1 / 2 + JV16HC/17HC

Hemolysin are the compounds that contributes to the pathogenicity of organism. The most extensively studied hemolysins produced by *Bacillus* strains are cereolysin (Hemolysin I) and hemolysin BL (HBL) beside other group of hemolysin enzymes produced (Mukry *et al.*, 2010). Strains of *Bacillus* spp, particularly, *B. cereus* are important as they cause food-spoilage and food-poisoning by producing hemolysin (subtilysin) enzymes apart from the production of biogenic amines (Bernheimer & Avigad, 1970). Thereby, screening of the hemolysin production of *Bacillus* spp. and candidate strains is important to avoid threat to food industry and public health. In this study, none of the isolates produce any hemolysin, as shown in figure 3. The isolates did not depict any zone of hemolysis, also called as λ-hemolysis (Savardi *et al.*, 2018), considering it to be safe in term of non-hemolytic strains.

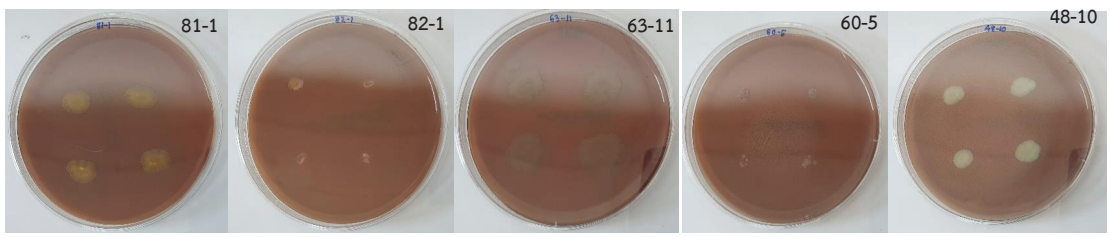


Figure 3. Blood agar to study hemolysis production

4. Conclusion

In this study, pigments generated from different halophilic strains of *Halobacillus yeomjeoni* (81-1) *Salinicoccus* sp. (82-1) *Bacillus infantis* (63-11) *Bacillus amyloliquefaciens* (60-5) and *Staphylococcus carnosus* (48-10), with different color shade were all derivatives of carotenoid. Dominant bacterial pigments of these strains included lycopene, β-carotene and lutein. Antimicrobial assays of crude pigment extract of 81-1 exhibited highest inhibitory effect on *Staphylococcus aureus*, while 82-1 and 60-5 best inhibited *Escherichia coli* and *Bacillus cereus*, respectively. As per DPPH assay, 60-5 demonstrated significant higher antioxidant activity relative to others crude pigments. Evaluation of potential virulence of strains, none of the strains contained key biogenic amine genes and had no hemolytic activity. The results from this study demonstrated that these carotenoids producing isolates could be a potential alternative source of natural pigments with antioxidant and antimicrobial, colorants and nutraceutical properties, and thus require further intensive investigation.

5. Acknowledgement

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