Malaysian Journal of Microbiology, Vol 18(2) 2022, pp. 136-145
DOI: http://dx.doi.org/10.21161/mjm.211253

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

The potential use of papaya and banana peels as substrate to enhance the pigment production of Gram-positive bacterial strain isolated from *Holothuria (Lessonothuria) pardalis*

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Received 28 July 2021; Received in revised form 4 October 2021; Accepted 23 January 2022

ABSTRACT

Aims: The microbial pigment can be the best promising alternative to replace synthetic colorant. However, due to the high cost of synthetic medium for microbial pigment production, there is a need to develop a new low-cost medium of bacterial pigment production. This study aims to investigate the potential of banana and papaya peels as alternative low-cost substrates for a carotenoid-producing bacterium, B12 strain (bacteria strain isolated from *Holothuria* (*Lessonothuria*) *pardalis*).
Methodology and results: B12 strain identified as an aerobic bacterium with non-motile, diplobacilli shaped and Gram-positive bacteria. The fermentation was optimized with different parameters included the effect of temperature, time, concentrations, pHs, carbon and nitrogen sources to find the optimum relative pigment concentration produced by B12. The results showed that the B12 strain produced the highest relative pigment concentration measured at 450 nm when the strain was cultivated at 37 °C and pH 7 in the culture medium incorporated with the combination of dried papaya peels and banana peels (100% v/v with ratio 1:1) at 72 h of incubation. Lactose, peptone and yeast were observed as the best carbon and nitrogen sources to increase the pigment concentration of B12 strain. Stability of the pigment was studied at different physiochemical stress, and it showed the pigment obtained from dried papaya and banana substrates can tolerate and stable under stress condition.
Conclusion, significance and impact of study: This can be concluded that the combination of dried papaya and banana peels worked well as substrate and can be utilized as a fermentation medium to replace the synthetic medium which is more expensive and uneconomical for industry application. Besides, it also helps in managing waste and solving the pollution problem due to the increasing of biochemical oxygen demand (BOD) and chemical oxygen demand (COD).

Keywords: Fruit waste substrate, pigment, Gram-positive bacteria, optimization, stability test

INTRODUCTION

Recent concern regarding synthetic colouring due to its toxicity, carcinogenicity and harmful to health lead to the increasing demand of natural colouring in the industry (Aruldass *et al*., 2018; Sen *et al*., 2019). Natural pigment mainly obtained from plants, insects, animals, minerals and microorganisms are seen as the best option to replace the synthetic colourant in the industry because they produce biodegradable, eco-friendly and sustainable pigments (Usman *et al*., 2017; Venil *et al.*, 2020). In addition, microbial pigments are the best promising alternative to natural colourants because of their high productivity and no seasonal production compared to pigment extracted from plants or animals (Samyukhta and Mahajan, 2016).

Nowadays, natural pigments from marine bacteria have become the interest of researchers and food industries due to their biological functional attributes (Ramesh *et al*., 2020). Gram negative bacteria are the most common microorganisms found in marine environment. However, a number of studies indicated that the relative abundance, activity and diversity of marine Gram-positive bacteria may higher than previously

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ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 18(2) 2022, pp. 136-145
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thought (Leiva *et al*., 2015). A few Gram-positive and Gram-negative marine bacteria produced pigments on standard culture media and these bacteria shared the same characteristics with the members of the genera *Alteromonas*, *Corynebacterium*, *Flavobacterium* and *Pseudomonas* (Ramesh *et al.*, 2019).

Microorganisms produce various types of pigment like carotenoids, melanins, flavins, monascins, violacein and indigo (Dufossé, 2018). Carotenoids are a group of bioactive compounds that is the most widely studied pigments compared to others and responsible for bright yellow, orange and red pigments (Indra Arulselvi *et al*., 2014). Recently, many studies involving carotenoids have been conducted due to their benefits on human health (Sasidharan *et al*., 2013). Besides, carotenoids can enhance the immune system and have good antioxidant activity (Surekha *et al*., 2016). Interestingly, researchers also found that carotenoids have a great commercial potential in pharmaceutical, chemical, food and feed industries (Kamarudin *et al.*, 2020).

Fermentation medium supply the basic nutrients for the growth of microorganism. However, due to the high cost of synthetic medium and current technology of pigment production in the industrial scale, there is a need to develop new low-cost of pigment production. In conjunction with this, the use of agro-industrial waste as the substrate would provide an alternative to reduce the production cost (Babitha *et al.*, 2006; Venil *et al.*, 2013). Recently, fruit wastes like citrus fruit wastes, papaya wastes, banana wastes, mango wastes, pineapple wastes and oil palm empty fruit bunches have been used as the substrate because they are produced throughout the year and contains rich source of fermentable sugars and does not involve high-cost of chemical pre-treatment (Sarkar *et al*., 2020).

Among other fruits, bananas (*Musa cavendish*) are one of the most popular and highly consumed fruit in the world, while papaya (*Carica papaya*) has very high nutrition and vitamins (Siddique *et al.*, 2018). According to Chooklin *et al*. (2014), due to high consumption of the banana in various commercial applications, the banana peels accumulate in bulk might give a serious problem with increasing disposal and pollution issues because of high biochemical oxygen demand (BOD) and chemical oxygen demand (COD), as well as a loss of valuable biomass and nutrients (Umesh and Preethi, 2014). Besides, the improper disposal also can promote the growth of disease-causing bacteria (Shakir *et al.*, 2020). Thus, it is very important to utilize this wastage as a benefits alternative in the industry. Papaya is found abundantly in Malaysia and contains excellent sources of carbohydrates (Zahan *et al.*, 2017). According to Wall (2006), papaya contains various types of vitamins like sodium, potassium, magnesium, calcium and iron to encourage the growth of the bacterium. Thus, this study aimed to investigate the potential of banana and papaya peels as the substrate for bacteria producing carotenoid isolated from tentacles of sea cucumber to produce low-cost microbial pigment.

MATERIALS AND METHODS

Microbial strain

A bacterial strain (B12) was isolated from the tentacles of *Holothuria* (*Lessonthuria*) *pardalis* which was collected from Pulau Tinggi and maintained on tryptone glucose yeast extract (TGYE) [Ingredients: casein enzymatic hydrolysate, 5 g/L; yeast extract, 3 g/L; glucose, 1 g/L; agar, 15 g/L] agar medium by sub-culturing (Kamarudin *et al.*, 2013; Kamarudin and Rehan, 2018). The stocks were stored at 4 °C in a refrigerator. Samples were prepared for cell staining according to the general procedure (Beveridge, 2001) and morphology of bacterial strain B12 was observed under a microscope (Olympus CX 31).

Inoculum preparation

The B12 strain was grown again in TGYE broth and incubated at 37 °C overnight. A 5% (v/v) culture was used as inoculum for the next analysis.

Substrate medium preparation

Papaya (*C. papaya*) and banana (*M. cavendish*) peels obtained from the local market in Pagoh, Johor were used as substrates for six substrate media; dried and fresh papaya peels medium, dried and fresh banana peels, and the combination of dried and fresh papaya and banana peels with ratio 1:1. The fresh peels were washed using distilled water before dried at 60 °C for 48 h and then ground. The distilled water was added to the substrate with a ratio of 1:10 and soaked in a water bath with 80 °C for 30 min (de Oliveira *et al*., 2017). Then, the substrate medium was filtered using Whatman No 1 filter paper. This is because small particle size of substrate provides a larger surface area for microbial response. Hence, 0.4 and 0.6 mm are the optimal sizes for a substrate for pigment production according to Babitha *et al*. (2006). The pH of the medium was then adjusted to 7.0 ± 0.2 with 1 M NaOH and 1 M HCl. The fermentation medium was sterilized at 121 °C for 20 min (Hirayama Autoclave, HVE 50). Then, 45 mL fermentation medium was transferred to a 500 mL conical flask after they were cooled to 37 °C. After that, 5% (v/v) of B12 strain inoculum was added into medium and incubated at 37 °C for four days and 1 mL fermentation medium was sampled every 24 h. Triplicate samples were prepared for each type of medium at different time points (24, 48, 72 and 96 h), concentrations (20%, 50%, 80% and 100% (v/v)), temperature (30 °C, 37 °C and 40 °C) and pH (pH 3, 5, 7 and 9).

Pigment extraction

A total of 1 mL of each type of fermentation medium sample which had been optimized with different parameters (pH, concentration, temperature and time) were transferred into a 1.5 mL sampling tube before centrifuging at 7500 rpm for 15 min. Then the supernatant was discarded and the bacteria pellets were extracted by

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

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using the same amount of methanol of the fermentation medium. The extraction was done until colorless pellets are obtained (Ahmad *et al.*, 2012). Then, the pigment absorbance was measured with 450 nm wavelength using UV-Visible Spectrophotometry (PG T60 UV-Visible Spectrophotometer). The optimum relative pigment concentration results were expressed in absorbance unit (AU) (Suwannarach *et al.*, 2019).

Effect of different carbon and nitrogen sources on pigment estimation

Three (3)% (w/v) of carbon sources (dextrose, lactose, sucrose and glucose) and 1% (w/v) of nitrogen sources (monosodium glutamate (MSG), yeast extract, soybean meal and peptone) were supplemented into 45 mL fermentation medium before 5% (v/v) inoculum of culture was inoculated into the medium. Then, medium cultures were incubated in an incubator shaker at 37 °C at 150 rpm for 48 h. The analysis of pigment production was analysed using UV-Visible Spectrophotometry (PG T60 UV-Visible Spectrophotometer) with carotenoid absorption 450 nm wavelength.

Stability test

The pigment stability of B12 strain was analyzed according to Perumal *et al*. (2009) and Suraiya *et al*. (2018) with slight modification. Generally, test tubes were set up firstly containing 3 mL of orange pigment were incubated in a water bath at 10 °C, 30 °C, 50 °C, 70 °C, 90 °C and 100 °C for 1 h. Then, another set of test tubes were set with different pH; 2, 4, 6, 8, 10, 12 and 14, left for 1 h and absorbance were measured. Next, the pigment was tested under stress conditions including 12 h in the oven at 90 °C, 2 h under sunlight and pressure at 121 °C for 20 min. The pigment also were adjusted to 0.2%, 0.4%, 0.6%, 0.8% and 1.0% (w/v) sodium chloride (NaCl) and ammonium chloride (NH_4Cl) in water bath at 60 °C for 1 h to determine the pigment tolerance towards salt solution. The absorbance was measured using a UV-visible spectrophotometer and percent of stability, S (%) was calculated using the formulae below:

$$S\ (\%) = [1 - (A_0 - A/A_0)] \times 100$$

where A_0, absorbance before treatment and A, absorbance after different treatments. Absorbance was measured using 450 nm to observed orange pigment stability.

Statistical analysis

All the data were taken as triplicate of mean with standard deviation. The data were evaluated by two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using GraphPad Prism 9 (GraphPad Software Inc., San Diego, CA, USA). Effects of various parameters on B12 pigment production were plotted using GraphPad Prism 9 at a level of significance $p \leq 0.05$.

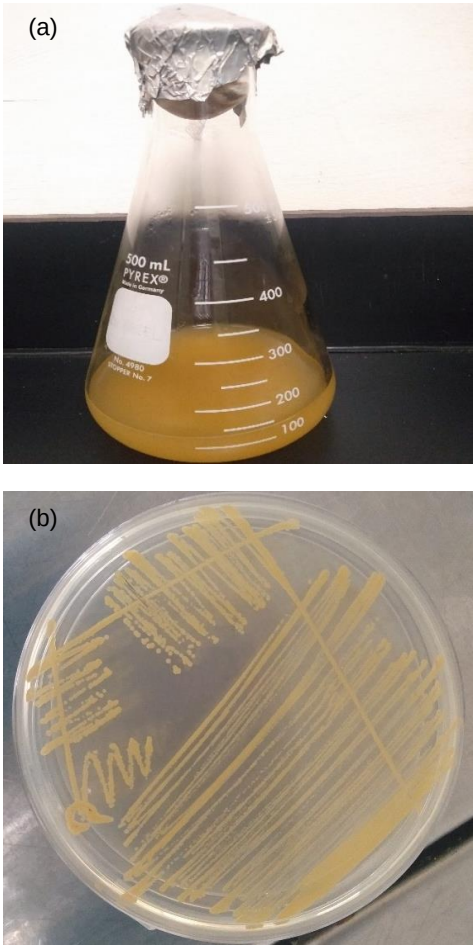

Figure 1: A bacteria strains (B12) in broth (a) and in Petri plate (b).

RESULTS AND DISCUSSION

A bacterial strain of B12 produced colony with orange pigment, moist and opaque shape in TGYE agar as in Figure 1. The fundamental cell form of the B12 strains observed was rod-shaped, diplobaccili and Gram-positive as shown in Figure 2. TGYE broth was used as a growth medium because it provides suitable nutrition and growth factors. However, the cost of this medium was expensive and did not preferable to applying it in the industry due to the uneconomical reason. In this study, the combination of banana and papaya peels as a potential media had been investigated. Banana and papaya peels are huge agro-waste that have the potential as a substrate to be used as a fermentation medium for bacteria cultivation and growth (Sharma *et al*., 2016; Zahan *et al*., 2017; Pathak *et al*., 2019). The optimization of different parameters to enhance pigment production was carried out by one variable at a time approach (Ghosh and Ghosh, 2017). Production of pigment was evaluated by absorption maxima at 450 nm using a UV-Visible Spectrophotometer because the pigment extraction

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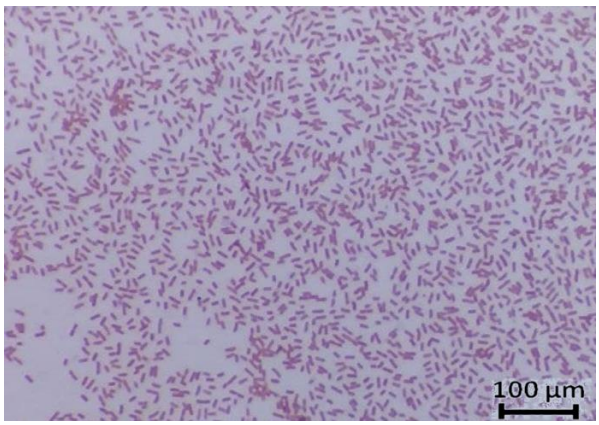

Figure 2: Gram-positive rods of B12 strain observed under microscope with 1000× magnification.

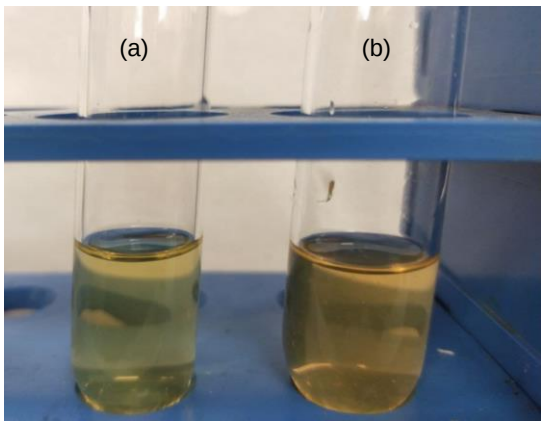

Figure 3: The B12 pigment extraction obtained from (a) TGYE broth and (b) dried papaya and banana broth.

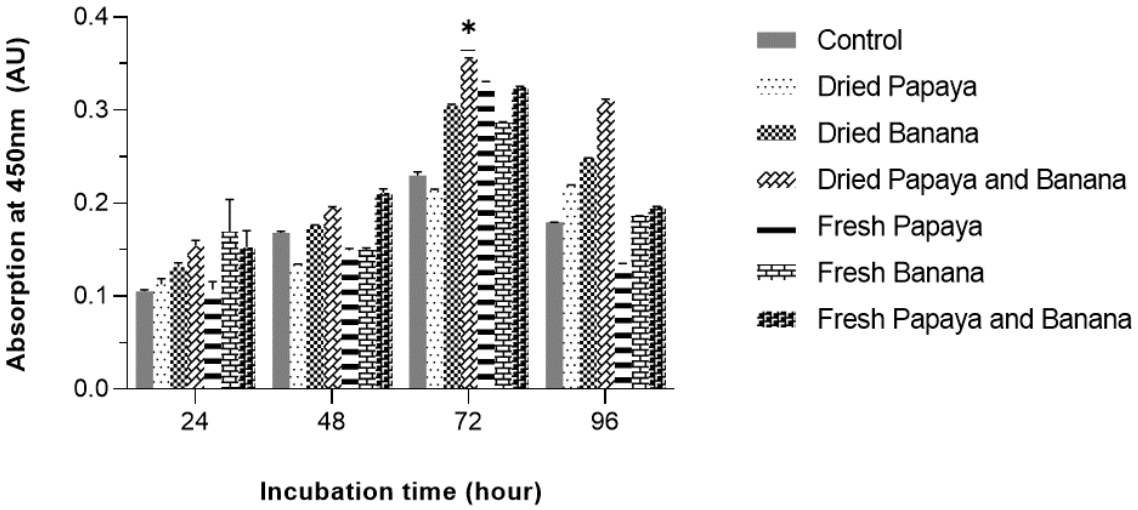

Figure 4: Effect of time on B12 pigment production. ANOVA, $p<0.0001$, p value summary based on Tukey's multiple comparison tests. * indicates highly significant with respect to the TGYE broth (control).

solution showed that the peak absorbance of the methanolic-dissolved extract was 450 nm. The different colour of B12 pigment between pigment obtained from commercial broth (TGYE) and dried papaya and banana peels media after extracted using rotary evaporator (EYELA N-1300) can be observed in Figure 3. The pigment extraction absorbance was measured at 450 nm wavelength and it showed the absorbance reading of pigment extract from combination of papaya and banana peels media was 0.588 AU_{450} while absorbance value for pigment extract from TGYE media was 0.297 AU_{450}. The results also showed the pigment obtained from dried papaya and banana substrate was darker and higher intensity colors compared to TGYE broth.

Effect of incubation time

The effect of incubation time on relative pigment concentration with papaya, banana and combination of papaya and banana peels as substrate was shown in Figure 4. The pigment absorbance reading from combination of dried papaya and banana peels was significant different than all substrate studied ($F = 61.38$, $p<0.0001$ for ANOVA). The papaya and banana peels contained high pectinase enzyme which can help as nutrient supply for the growth of microorganism (Steudler *et al*., 2019). Pigment intensity was highest in the combination of dried papaya and banana peels media at the optimum temperature of 37 °C at 72 h. The B12 strain produces relative pigment concentration the best at 72 h in all media while decreased after incubation of 72 h. This might be due to the depletion of nutrients for the growth of bacteria strain (Flores *et al.*, 1997). In addition, the shorter production cycle of pigment yield would be more cost-effective when applied to industry (Padma *et al*., 2012).

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

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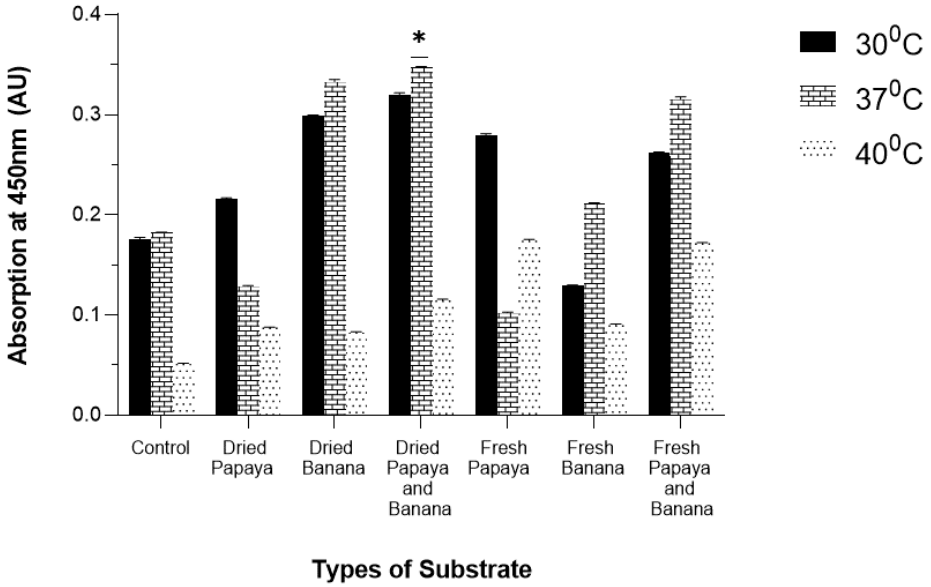

Figure 5: Effect of temperature on B12 pigment production on 72 h of incubation. ANOVA, $p = 0.0070$, p value summary based on Tukey's multiple comparison tests. * indicates highly significant with respect to the TGYE broth (control).

Effect of temperature

The effect of temperature on the relative pigment concentration was also studied. The results revealed that relative pigment concentration differs significantly ($F = 8.804$, p value = 0.0070 for ANOVA). The B12 strain showed high relative pigment concentration respectively at 30 °C and 37 °C but decreased at 40 °C in 72 h incubation. According to Arekemase *et al.* (2020), the low temperature will cause membranes to solidify while high temperature might damage microorganisms by denaturing enzymes, transport carriers and other proteins. This was also supported by Patel and Bhaskaran (2020), the key enzyme might be denatured in higher temperature. The maximum relative pigment concentration was produced in the combination of dried papaya and banana medium at 37 °C which is 0.347 ± 0.01 AU_{450} (Figure 5). It showed that these combination media is the best among other media and 37 °C is the optimum temperature to enhance the relative pigment concentration due to faster metabolic activities and extracellular enzyme production in B12 strain culture.

Effect of pH

The effect of pH is also one of the crucial factors for pigment production (Farees *et al*., 2017). Figure 6 depicts the effects of pH on the relative pigment concentration for the B12 strain. The different media were tested with a pH range from acidic to alkaline. The result showed that relative pigment concentration within pH 3 to pH 9 was significantly different ($F = 60.80$, $p<0.001$ for ANOVA). The relative pigment concentration was the lowest in acidic media. This is suggesting that the acidic media might suppress the growth of the B12 strain, consequently affecting pigment production (Zahan *et al*., 2017). It was observed that B12 strains had the maximum relative pigment concentration at pH 7 with the combination of dried papaya and banana media with pigment absorption 0.681 ± 0.011 AU_{450}. The optimum pH conditions influence the growth of microorganisms and hence influenced the pigment production activity (Méndez *et al.*, 2011). This study was supported by Afshari *et al.* (2015) and Mukesh Kumar *et al*. (2012) stated that the enzyme production of bacteria will decrease when pH is too high or too low, thus favoring pigment production. Thus, in this study the enzymatic activity seems optimum at pH 7 for pigment production.

Effect of concentration of the substrate

The effect of the concentration of substrate used was also studied as one of the factors in the relative concentration of B12 pigment. This was conducted to establish the optimum concentration at which the substrates used to produce the highest relative pigment concentration. The results showed the concentration was highly significant with respect to TGYE media (control) ($F = 20.06$, p value = 0.0002 for ANOVA). It was observed that the cultivation of B12 strain in the combination of dried papaya and banana (ratio 1:1) at 100% (v/v) concentration of media were the highest in pigment absorption which was 0.877 ± 0.012 AU_{450} compared to other media. The lowest absorption of pigment was observed at 20% (v/v) of media concentration (Figure 7). Thus, it showed an increase of concentration of substrate ultimately led to an increase of relative pigment concentration on B12 strain.

The combination of dried papaya and banana media has been chosen for the effect of supplementation of carbon and nitrogen sources. The highest absorbance

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

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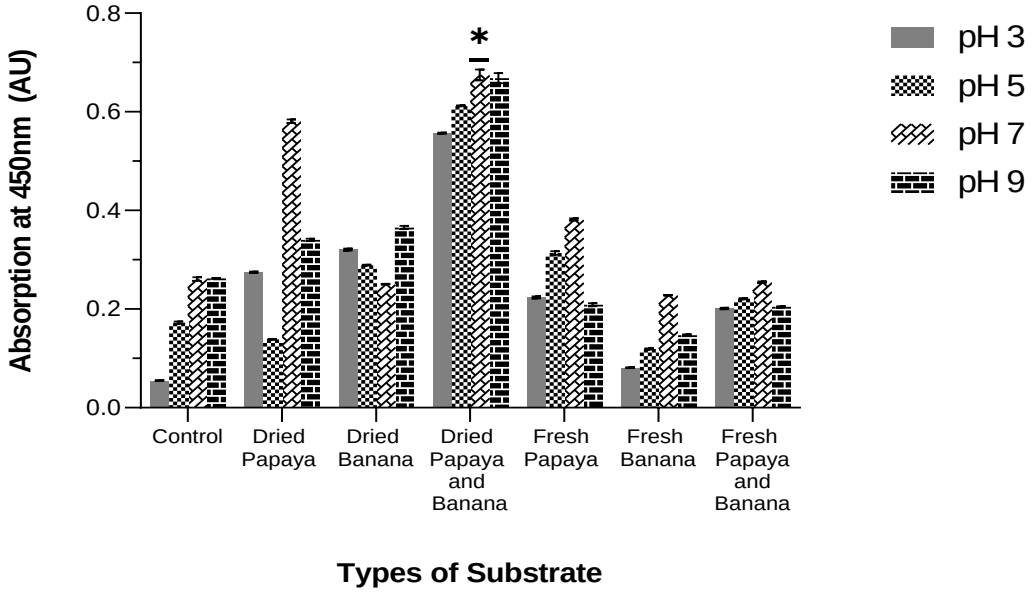

Figure 6: Effect of pH on B12 pigment production on 72 h of incubation. ANOVA, $p<0.0001$, p value based on Tukey's multiple comparison test. * indicates highly significant with respect to the TGYE broth (control).

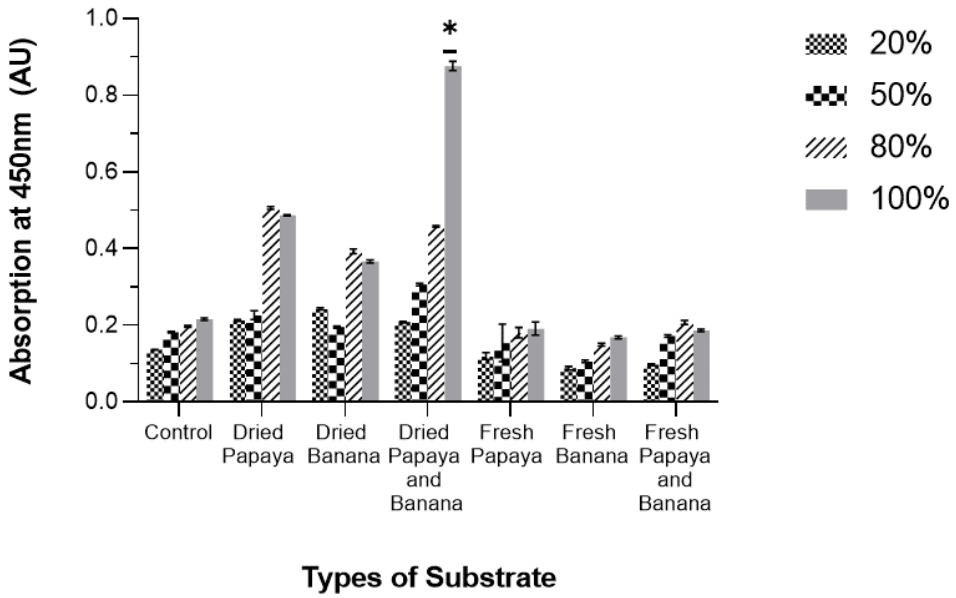

Figure 7: Effect of concentration of substrate on B12 pigment production on 72 h of incubation. ANOVA, $p = 0.0002$, p value based on Tukey's multiple comparison test. * indicates highly significant with respect to the TGYE broth (control).

peak value at 450 nm showed that the peak fell at the range of 400-500 nm which confirmed that there was the presence of carotenoids (Kaur *et al.*, 2019). Based on the analysis observed, lactose was a better source of carbon with a maxima absorption of 2.563 AU_{450} followed by glucose, sucrose and dextrose (Figure 8). According to Babitha *et al*. (2006), lactose and sucrose were found to be good supporters for pigment production. This is supported by Bhagwat and Phadalia (2020) study, the growth of bacteria was good when carbon sources like lactose and sucrose were supplied into bacteria. Four different nitrogen sources were supplemented into media at 1% (w/v). Peptone and yeast found to give the highest maxima absorption 2.494 and 2.439, respectively as shown in Figure 9. In addition, peptone and yeast had been reported as the best nitrogen sources among others (Prakash *et al.*, 2014). Spectral analysis showed that the yield of pigment with a maxima absorbance peak was

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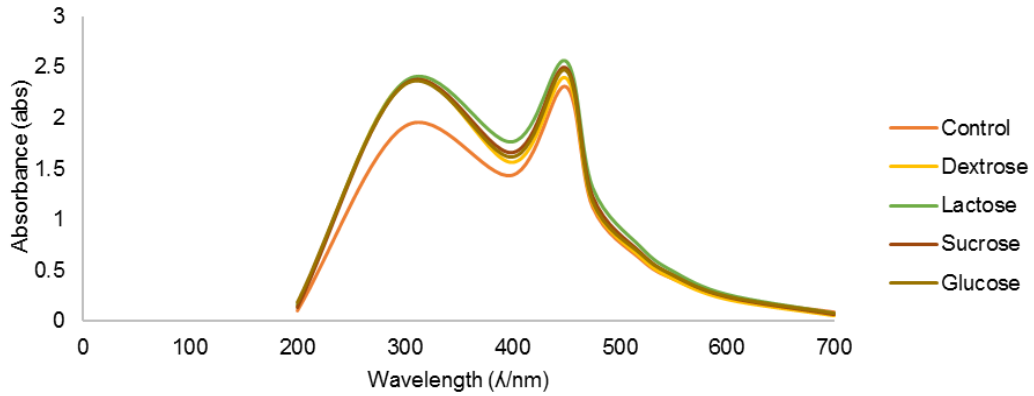

Figure 8: Effect of carbon sources on B12 pigment production.

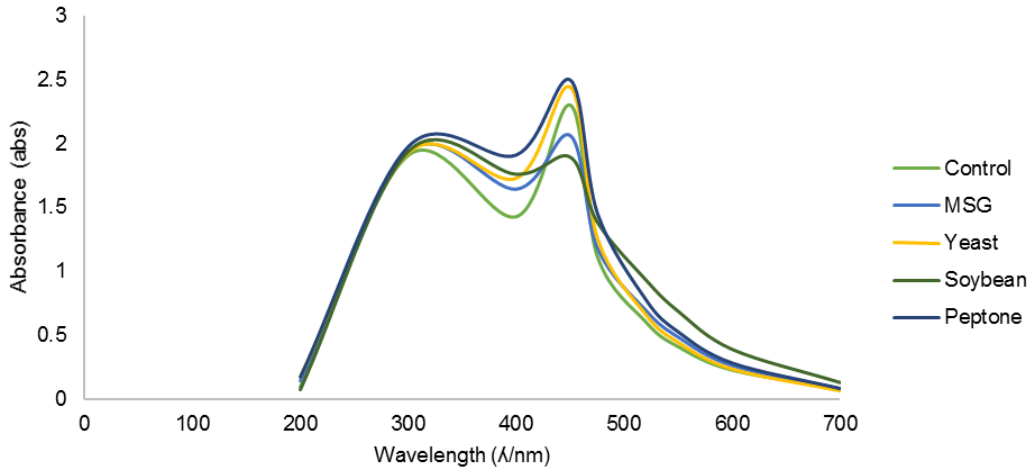

Figure 9: Effect of nitrogen sources on B12 pigment production.

observed at 450 nm and the spectral pattern were close resemblance to the characteristic of β-carotene.

Stability test

Pigment stability of B12 strains was treated chemical and physically. The results obtained from the analysis were presented in Table 1. The pigment was soluble in water and absorbed at 450 nm wavelength which indicated an orange colour. The pigment colour was remained unchanged at pH 4, 6 and 8 while slightly changed at the most acidic and alkaline conditions. The change of colour occurs due to aromatic –OH groups ionisation and tautomerism of –O(--) with –O-. Furthermore, the changes in the relative proportions of dissociated or undissipated molecules would contribute to the colour changed (Velmurugan *et al*., 2011). The retention of activity after heating an enzyme extract at a specific temperature for an extended period of time is referred to as thermo-stability (Ire and Berebon, 2016). The range of percent stability of B12 strain pigment at absorbance value at 450 nm were 87.9% to 91.7% indicated that the pigment of B12 strain were thermostable at 10 °C to 100 °C.

Besides, the pigments were stable (80% and more) when exposed to stress conditions like steaming, UV light and oven; however, the pigments were intolerable under the sunlight exposures. This was supported by Ng *et al*. (2004) who reported that pigment was not stable under the sunlight due to the changes on pigment brightness. The results also showed that the pigments were less stable under different salt treatments. The stability results of B12 strain pigments showed that the pigments remained stable across a wide pH, temperature and streaming range which similar to the results reported by Lin *et al*. (1992). Based on these findings, it is promising that the pigments derived from this Gram-positive bacterium could be used as food colourant, cosmetic or textiles colourant.

CONCLUSION

The combination of dried papaya peels and banana peels could be an effective substrate for the production of pigments by B12 strains. Banana and papaya peels are an abundant byproduct of agriculture waste and have been studied as an economical and good alternative to

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Table 1: The stability test of pigment under various treatments.

Treatment		Percent of stability (%)
Temperature (°C)	10	91.7
	30	90.5
	50	98.8
	70	93.1
	90	92.4
	100	87.9
pH	2	87.2
	4	99.2
	6	99.3
	8	95.4
	10	90.3
	12	88.1
Stress condition	Sunlight	76.3
	UV light	91.9
	Pressure 121°C at 4 min	92.3
	Oven	99.1
NaCl (w/v %)	0.2	86.3
	0.4	75.2
	0.6	75.2
	0.8	72.2
	1.0	72.0
NH_4Cl (w/v %)	0.2	83.7
	0.4	84.1
	0.6	81.7
	0.8	79.6
	1.0	80.9

replace the commercial TGYE medium. Optimization of B12 strains culture in the combination of papaya and banana media (ratio 1:1) resulted in the highest relative pigment concentration at pH 7, temperature of 37 °C in concentration of 100% (v/v) at 72 h incubation. This indicate that combination of dried papaya and banana can be an effective cultivation media of B12 strain. Therefore, a wide range of banana and papaya waste can be utilized and the cost of medium for the growth of bacteria can be reduced. Besides, lactose, peptone and yeast were the best carbon and nitrogen sources or supplements that can be used in industry in order to increase the pigment concentration of B12 strain. In the future, the other agro wastes can be considered in microbial pigment production as it can be seen as an effective cost, especially in various industrial applications.

ACKNOWLEDGEMENTS

This research was supported by Ministry of Higher Education (MOHE) through Fundamental Research Grant Scheme (FRGS/1/2019/WAB09/UTHM/03/2). We also want to thank Universiti Tun Hussein Onn Malaysia (UTHM) which provides Postgraduate Research Grant (GPPS) UTHM with code no. H416 for sponsoring the student's stipends. This research is part of a thesis which is to be submitted as a partial fulfillment to meet requirements for the Doctor of Philosophy in Science at UTHM.

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