

The potential of papaya and banana peels as a substrate for *Sphingomonas* sp. pigment production: optimisation and antimicrobial activity

¹Solehin, S.N., ^{1,*}Kamarudin, K.R., ¹Badrulhisham, N.S. and ²Rehan, A.M.

¹Centre of Research for Sustainable Uses of Natural Resources (SUNR), Faculty of Applied Sciences and Technology (FAST), Universiti Tun Hussein Onn Malaysia (UTHM), Pagoh Campus, Pagoh Education Hub, KM 1, Jalan Panchor, 84600 Muar, Johor Darul Takzim, Malaysia.

²Department of Chemical Engineering Technology, Faculty of Engineering Technology (FTK), Universiti Tun Hussein Onn Malaysia (UTHM), Pagoh Campus, Pagoh Education Hub, KM 1, Jalan Panchor, 84600 Muar, Johor Darul Takzim, Malaysia

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Abstract

The need for colourants derived from natural sources and the change in consumer perception of synthetic colourants have opened the new potential for the natural colourant industry. As a result, due to its stability, high yield, and no seasonal variation, pigment derived from microorganisms is the best replacement for sources from plants and animals. Due to the high cost of synthetic media for microbial pigment production, it is necessary to design a new low-cost medium for microbial pigment production. This research aimed to examine the viability of banana and papaya peels as an alternative low-cost substrate for the yellow pigment-producing bacterium, AX1 strain (bacterium strain isolated from the anus of *Holothuria (Lessonothuria) pardalis*. AX1 was identified as *Sphingomonas* sp., an aerobic bacterium having Gram-negative, non-motile, rod-shaped bacteria. The optimal relative pigment concentration was achieved when the culture was cultivated in a medium containing the combination of dried papaya and banana with a ratio of 1:1 (100% v/v) at 37°C and pH 7 after 72 hrs incubation. The wavelength absorption was recorded at 468 nm. Various parameters including the effect of temperature, time, concentration, and pHs were used in this study. The pigment extraction showed a positive effect of antimicrobial activity against *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 25923, and *Staphylococcus epidermidis* ATCC 51625. The investigation of this study has shown that the combination of dried papaya and banana peels performed well as a substrate and may be used as a fermentation medium to replace the synthetic medium which is more expensive and less cost-effective for industrial application

1. Introduction

Colour is vital to consumers as it can improve the acceptability and appearance of foods or beverages, making them more attractive to consumers (Neves *et al.*, 2021). The demand for natural colourants instead of synthetic colourants that have been used widely in the last decade has increased and become a research trend because of the awareness towards health and immunity especially after the COVID-19 pandemic lurks into life. According to the global market for natural food colourants, natural food colouring reached \$ 3.71 billion in 2017 and is projected to rise at a Compound Annual Growth Rate (CAGR) of 5.7% from 2017 to 2023 (Lombardelli *et al.*, 2021). Natural colourants are derived from sources like plants, animals, minerals, and

microorganisms. Microorganisms are seen as the best alternative sources among the natural sources because of no seasonal variation, high stability, and high yield production. Besides, using a strategic fermentation medium like substrates instead of synthetic medium can develop the low-cost process of pigment production.

Substrate from waste like agro-residue is considered a good fermentation medium alternative. In recent years, fruit wastes like banana waste, papaya waste, mango waste, oil palm empty fruit bunches, and pineapple waste have been utilised as substrate due to their rich supply of fermentable sugars and low chemical pre-treatment costs (Sarkar *et al.*, 2020). The Department of Statistics of Malaysia (DOSM) showed that agricultural growth had decreased by 0.2% in 2021 as compared to -

*Corresponding author.

Email: kamarulr@uthm.edu.my

2.4% in 2020. However, papaya and banana had recorded the largest per capita consumption (PCC) crops with banana having been listed as the third highest of PCC which is 9.3 kg/year (Mohamad, 2022). In Malaysia, about 1.2 million tonnes of agro-waste are produced and disposed of in landfills annually. Bananas (*Musa cavendish*) and papaya (*Carica papaya*) peels were used in this study because they are the world's most popular and widely consumed fruits besides containing high nutrition and vitamins (Siddique *et al.*, 2018). Furthermore, papaya and banana are found abundantly in Malaysia. According to Thiviya *et al.* (2021), the use of cheap and abundant ago-waste for microorganisms can help reduce the organic waste disposal in the environment which can contribute to the increase of biochemical oxygen demand and chemical oxygen demand in the soil. Thus, the aim of this study is to investigate the potential of a combination of dried papaya and banana peels performance as a substrate for *Sphingomonas* sp.

2. Materials and methods

2.1 Materials

A yellow pigmented bacterial strain, AX1 was isolated from the anus of *Holothuria (Lessonothuria) pardalis* collected from Pulau Tinggi (Figure 1). The culture was maintained on Tryptone Glucose Yeast Extract (TGYE) agar medium [ingredients: casein enzymatic hydrolysate, 5 g/L yeast extract, 3 g/L glucose, 1 g/L agar 15 g/L] by sub-culturing once per month (Kamarudin *et al.*, 2013; Kamarudin and Rehan, 2018). The stocks were kept in the refrigerator at 4°C. The morphology of bacterial strain AX1 was studied under a microscope (Olympus CX 31) after the cell staining process had been done according to the Gram-staining general procedure (Beveridge, 2001).



Figure 1. Sampling location of *Holothuria (Lessonothuria) pardalis* at Pulau Tinggi (2.3047° N, 104.1176° E). Source: Department of Survey and Mapping Malaysia (2015).

2.2 Identification of pigmented bacterium

Genomic DNA for AX1 strain was extracted using FavorPrep™ Tissue Genomic DNA Extraction Mini Kit (Favorgen Biotech Corp. Taiwan) following the manufacturer's instructions. The 16S rRNA gene was amplified by PCR with the bacterial primers with V3-V4 target regions; S-D-Bact-0341-b-s-17 (5'-CCTACGGGNGGCWGCAG-3') and S-D-Bact-0785-a-A-21 (5'-GACTACHVGGGTATCTAATCC-3) (Klindworth *et al.*, 2013). PCR was performed in 25 µL of the reaction of a mixture containing 12.5 µL of PCR master mix, 1.5 µL of each primer, and 9.5 µL of genomic DNA. The optimal conditions for PCR were set as follows: an initial denaturation step at 3 mins at 95°C, 25 cycles of a denaturation step at 95°C for 30 s, an annealing temperature at 56°C for 30 s, and extension at 72°C for 30 s and a final extension step at 72°C for 5 mins. PCR products and DNA ladder were resolved in 1.5% agarose gel electrophoresis at 80 V for 40 mins. Sequencing of the 16s rRNA gene of the bacterial strains was performed at the Apical Sdn Bhd, Seri Kembangan, Malaysia. The sequences received in raw format were edited using Chromas software Version 2.6.6 by Technelysium Pty. Ltd. to remove terminal noise response during the sequencing. Sequence homology of the bacterial isolates was determined using the web-based Basic Local Alignment Search Tool (BLAST) program of the NCBI, <http://blast.ncbi.nlm.nih.gov> (Altschul *et al.*, 1990). The bacterial strain was determined to belong to the same genus if the 16s rRNA gene sequence was 97% identical to the reference strains of the same species. The identified homologous strains were aligned using MUSCLE in MEGA 11 software for multiple sequence alignment. The final phylogenetic tree was generated from this multiple alignment result using the bootstrap method by the neighbour-joining algorithm and maximum likelihood algorithm in MEGA11 software. For the construction of the phylogenetic trees, *Candida albicans* was considered an outgroup (NG070791).

2.3 Inoculum preparation

The *Sphingomonas* sp. strain was cultured in TGYE broth and incubated overnight at 37°C. A starter inoculum of 5% (v/v) culture was used for the next analysis.

2.4 Substrate medium preparation

Peels from papaya (*Carica papaya*) and banana (*Musa cavendish*) purchased from the local market in Pagoh, Johor were used as substrates for six different types of media: dried papaya peels, dried banana peels, combination of dried papaya and banana peels with ratio

1:1, fresh papaya peels, fresh banana peels and combination of fresh papaya and banana peels with ratio 1:1. Half of the fresh peels were dried at 60°C for 48 hrs before grinding. All the substrates were immersed in distilled water at a ratio of 1:10 for 30 mins and heated to 80°C in a water bath (de Oliveira Rodrigues *et al.*, 2017). Then the Whatman No.1 filter paper was used to filter the substrate medium. This is because the larger surface area due to the small particle size might provide good microbial cultivation. Therefore, 0.4 mm and 0.6 mm were the ideal diameters for a substrate to increase pigment production (Babitha *et al.*, 2006). Then, 1 M NaOH and 1 M HCl were used to adjust the pH medium to 7.0±0.2 and sterilised at 121°C for 20 mins (Hirayama Autoclave, HVE 50). The fermentation medium was then transferred to a 500 mL conical flask after being cooled to 37°C. The medium was then supplemented with 5% (v/v) of the *Sphingomonas* sp. strain inoculum and incubated at 37°C for four days with 1 mL of the fermentation media being sampled every 24 hr. Each type of media had triplicate samples prepared at various temperatures which were 30°C, 37°C and 40°C, pH 3, 5, 7 and 9, concentrations; 20%, 50%, 80% and 100% (v/v) and incubation hour; 24, 48, 72 and 96 hrs respectively.

2.5 Pigment extraction

A 1.5 mL sampling tube was filled with a total of 1 mL of each type of fermentation optimum medium sample (pH, concentration, temperature, and time) and the tube was then centrifuged at 7500 rpm for 15 mins. After removing the supernatant, the bacteria pellets were extracted using the same amount of methanol as the fermentation medium. The extraction was continued until a colourless pellet was obtained (Ahmad *et al.*, 2012). The pigment absorbance was then measured by UV-visible spectrophotometry at 468 nm wavelength (PG T60 UV-visible spectrophotometer). Absorbance unit (AU) results for the optimal relative pigment concentration were recorded (Suwannarach *et al.*, 2019).

2.6 Determination of antimicrobial activity

The antimicrobial susceptibility assay was carried out using the Kirby-Bauer disc diffusion method with standard Mueller Hinton agar (MHA). Pigment extract of AX1 was conducted against human pathogenic bacterial strains; Gram-negative bacterium (*Escherichia coli* ATCC 8739) and Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923 and *Staphylococcus epidermidis* ATCC 51625) (Lashin *et al.*, 2021). A total of 100 µL of each bacterial suspension with a turbidity of 0.5 MacFarland standard was spread into MHA agar. A sterile blank disc with 6 mm diameter was loaded with 100 µg/mL of pigment extract and put onto the MHA agar (Silva *et al.*, 2018). Discs with

antibiotics were used as positive controls while discs with methanol were marked as negative controls. The plates were incubated at 37°C for 24 hrs. The inhibition zones showing the antimicrobial activity of the pigment extract against pathogenic bacteria were measured. The test was performed in triplicate.

3. Results and discussion

A yellow-pigmented bacterial strain of AX1 produced a colony with yellow bright pigment, a moist and opaque shape on TGYE agar as shown in Figure 2 (a). As shown in Figure 2(b), AX1 strains have been discovered as non-motile aerobic bacterium, rod-shaped and Gram-negative bacterium

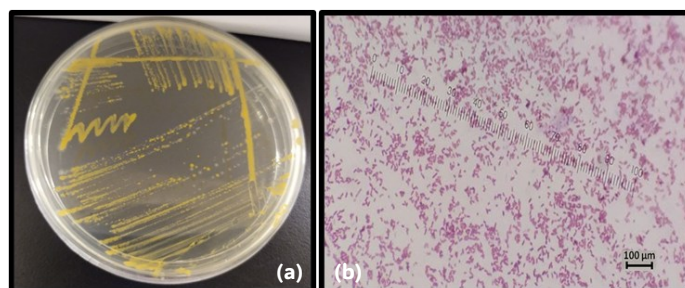


Figure 2. (a) A bacteria strain of AX1 on TGYE plate and (b) Gram-negative of AX1 strain observed under microscope with 1000× magnification.

3.1 16S rRNA sequencing

A partial 16S rRNA gene sequence (427 bp) was aligned with the other species of the genus *Sphingomonas*. The strains of *Sphingomonas olei* NR157757, *Sphingomonas aurantiaca* NR042128 and *Sphingomonas aerolata* NR042130 were most closely related to the bacterial strain AX1 with 100% similarity gene sequence. Phylogenetic trees were reconstructed by the maximum-likelihood method and the neighbour-joining method by using the MEGA 11 software and applying Tamura-3-parameter model, including proportion of gamma distribution with complete deletion of gaps or missing data treatment and codon position including 1st+ 2nd+3rd + Noncoding with bootstrap values based on 1000 replications (Tamura, 1992; Saitou and Nei, 1987; Xue *et al.*, 2018). The tree with the highest log likelihood (-3644.38) is shown in Figure 3. The phylogenetic tree of maximum likelihood tree also indicated that strain AX1 clustered with the species of the genus *Sphingomonas* which is also supported by the neighbor-joining tree (Figure 4). This can be concluded that the bacterial strain was confirmed in the *Sphingomonas* genus and can be identified as *Sphingomonas* sp.

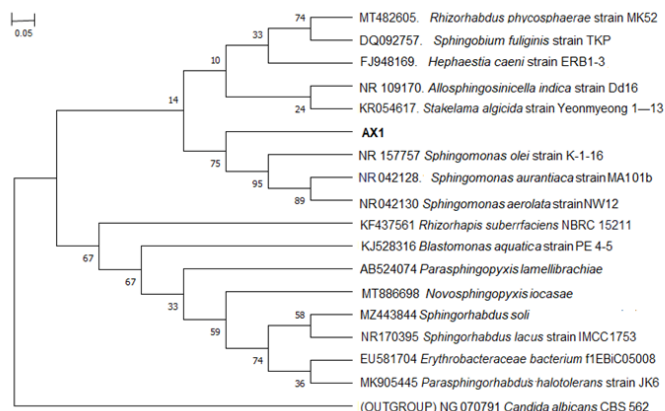


Figure 3. Maximum likelihood phylogenetic tree based on 16S rRNA gene sequences showing the phylogenetic relationships of strain AX1 and related taxa. Bootstrap values based on 1000 replications are shown at branch nodes. *Candida albicans* NG070791 formed the outgroup.

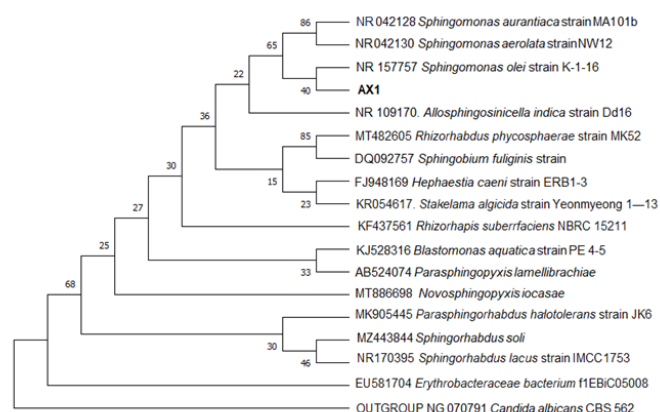


Figure 4. Neighbour-Joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships of strain AX1 and related taxa. Bootstrap values based on 1000 replications are shown at branch nodes. *Candida albicans* NG070791 formed the outgroup.

3.2 Optimisation of *Sphingomonas* sp. pigment concentration

TGYE media is very important to microbial strains to supply sufficient nutrients and growth of the microorganisms. However, this synthetic media is highly expensive and unfavourable to use in the industry due to uneconomical reasons (Solehin et al., 2022). Thus, the potential of using the combination of papaya and banana peels as one of the alternative fermentation media has been studied. According to Zahan et al. (2017) and Sharma et al. (2019), banana and papaya peels are one of the largest agricultural wastes that have the potential to be used as fermentation media to supply nutrients for bacteria growth. The pigment production was optimized with various factors by using one variable at a time approach method (Ghosh and Ghosh, 2017). The methanolic-dissolved extract was measured at 468 nm wavelength due to the peak absorbance reading of yellow pigment measured at 468 nm using UV-visible

spectrophotometer. The different colours of pigment extraction using commercial synthetic media (TGYE) and a combination of papaya and banana peel media have been observed in Figure 5. The pigment extraction absorbance reading from TGYE media and the combination of papaya and banana peel media was $0.13 \pm 0.004 \text{ AU}_{468}$ and $1.56 \pm 0.07 \text{ AU}_{468}$ respectively. The pigment produced with the combination of papaya and banana peels showed higher intensity compared to TGYE media.

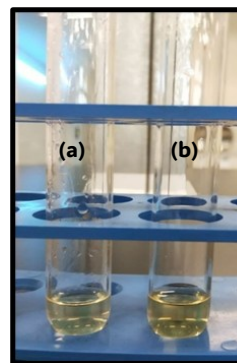


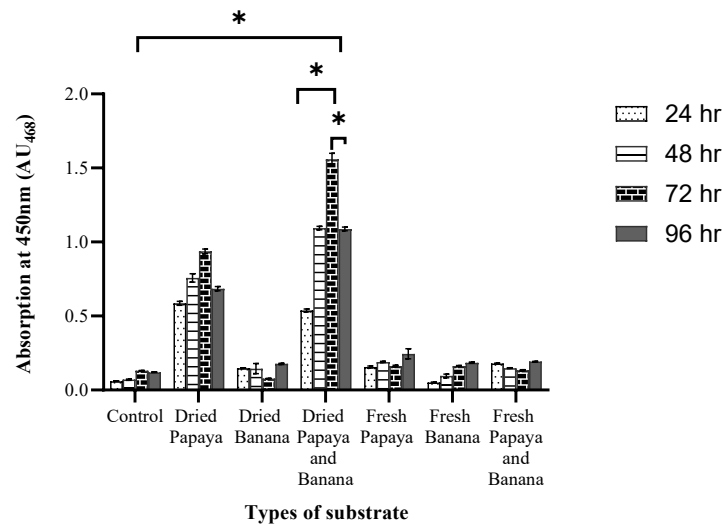
Figure 5. Pigment extraction when using fermentation media of (a) TGYE and (b) a combination of papaya and banana peels.

3.2.1 The effect of incubation hour

The relative pigment concentration reading with the effect of incubation hour for all various media is shown in Figure 6. The results showed the combination of dried papaya and banana peels was highly and significantly different than all substrates studied ($F = 118.31$, $p < 0.000$) when two-way ANOVA was conducted. According to Steudler et al. (2019), the high pectinase enzyme in papaya and banana peels can help to supply nutrients in microorganism growth. The combination of dried papaya and banana peel media showed the highest pigment intensity when the optimum incubation hour at 72 hrs. Besides, all the media showed the best relative pigment concentration at 72 hrs and then depleted after 72 hrs incubation. This could be a result of the decreasing nutrients for the growth of *Sphingomonas* sp. (Solehin et al., 2022). Besides, this can be supported by Venkatachalam et al. (2020) stated that the decrease in pigment production due to long incubation hours might be the result of the decomposition of pigments because of degradation of the chromophore pigment group or the structure of pigment had been changes. Additionally, the shorter the pigment production the more cost-effective when applied to the food industry (Padma et al., 2012).

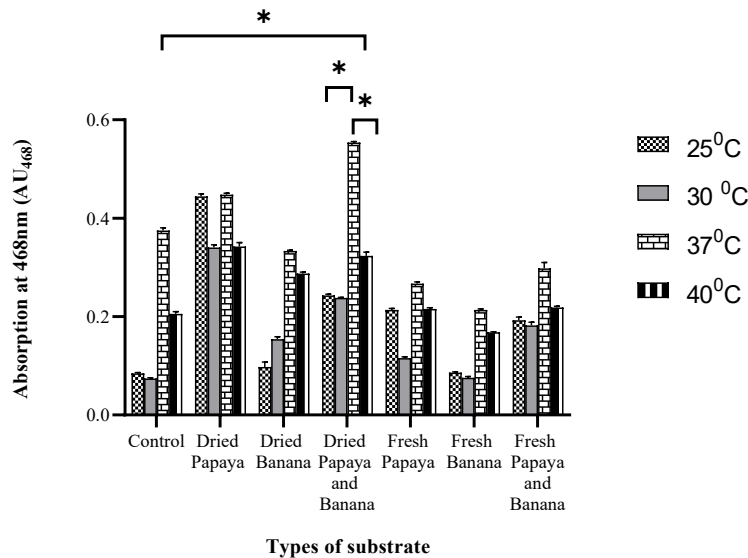
3.2.2 The effect of temperature

The result of the temperature effect is shown in Figure 7. It showed that the relative pigment concentration differs significantly ($F = 429.42$, $p < 0.0001$). The relative pigment concentration of



* $p < 0.001$ when compared between combination of dried papaya and banana substrate to TGYE broth, when compared between optimum incubation hour (72 hr) to the longest incubation hour (96 hr) and shortest incubation hour (24 hr)

Figure 6. Effect of incubation hour on *Sphingomonas* sp. pigment production. $p < 0.0001$, p -value summary based on Tukey's multiple comparison tests. * indicates highly significant to the TGYE broth (control).



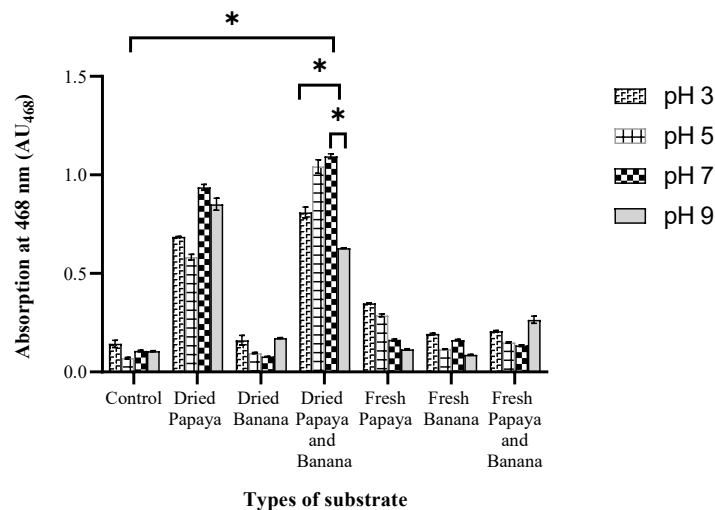
* $p < 0.001$ when compared between combination of dried papaya and banana substrate to TGYE broth, when compared between optimum temperature (37°C) to the highest (40°C) and lowest (25°C) temperature

Figure 7. Effect of temperature on *Sphingomonas* sp. pigment production. $p < 0.0001$, p -value summary based on Tukey's multiple comparison tests.

Sphingomonas sp. was the highest reading when incubated at 37°C and lowest for all substrates at 30°C and 40°C. Low temperature will cause the membranes to thicken while high temperature might damage microorganisms by denaturing enzymes, transporters, and other proteins (Arekemase *et al.*, 2020). In addition, Patel and Bhaskaran (2020) also conclude that high temperatures might denature the main enzyme of microorganisms. As a result, the optimum temperature for pigment produced was with the combination of papaya and banana peel media at 37°C which is 0.554 ± 0.01 AU₄₆₈.

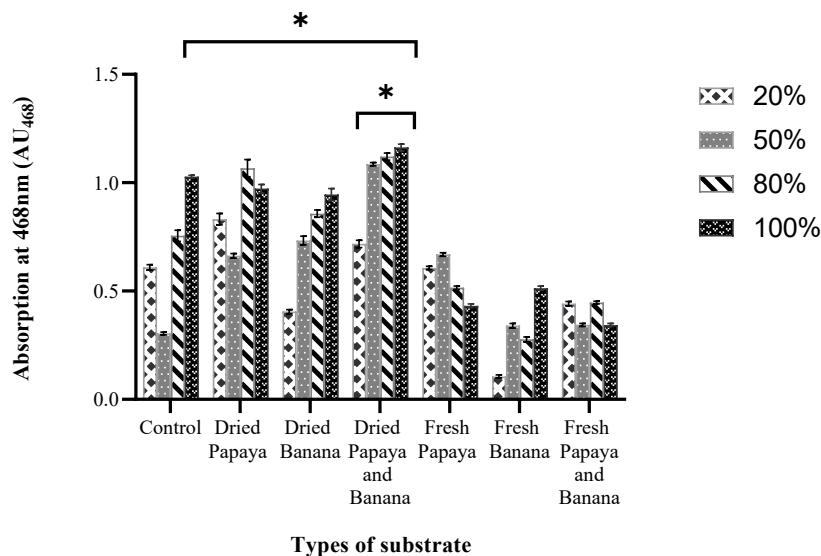
3.2.3 The effect of pH

The fermentation time and pH had a substantial interaction that affected the pigment synthesis. Bezirhan Arikan *et al.* (2020) stated that optimising pH was one of the crucial factors in increasing pigment production. Figure 8 shows the relative pigment concentration for *Sphingomonas* sp. pigment extract. The pH range of media from acidic to alkaline was chosen. The relative pigment concentration between pH 3 to pH 9 showed a highly significant difference ($F = 80.85$, $p < 0.001$). The combination of dried papaya and banana peel media showed the highest relative pigment concentration for pigment extraction at pH 7 with absorption 1.095 ± 0.01 AU₄₆₈. The relative pigment concentration showed low



* $p < 0.001$ when compared between combination of dried papaya and banana substrate to TGYE broth, when compared between optimum pH (pH 7) to the highest (pH 9) and lowest (pH 3) pH.

Figure 8. Effect of pH on *Sphingomonas* sp. pigment production. $p < 0.0001$, p -value summary based on Tukey's multiple comparison tests.



* $p < 0.001$ when compared between combination of dried papaya and banana substrate to TGYE broth, when compared between optimum concentration (100%) to the lowest concentration (20%).

Figure 9. Effect of pH on *Sphingomonas* sp. pigment production. $p < 0.0001$, p -value summary based on Tukey's multiple comparison tests.

pigment absorption in acidic and alkaline media. According to Méndez *et al.* (2011) and Afshari *et al.* (2015) stated that the growth of microorganisms was influenced by the optimum pH conditions and the pigment production activity. The enzyme synthesis from bacteria might decrease when pH levels are excessively high or low thus affecting the pigment production (Kalaichelvan, 2012). Zahan *et al.* (2017) also suggested that the acidic condition might inhibit the growth of bacteria which would have an impact on pigment production.

3.2.3 The effect of concentration

Another parameter that affected the pigment

production was the effect of the substrate concentration. The results showed the concentration of all substrates was significantly different from each other ($F = 356.48$, $p < 0.0001$). The combination of dried papaya and banana peel media with a ratio of 1:1 was found and this combination produced the maximum pigment absorption (1.163 ± 0.01 AU₄₆₈) when compared to other media as shown in Figure 9. The lowest pigment absorption was shown when the media concentration was 20% (v/v). As a result, it was demonstrated that the relative pigment concentration on the *Sphingomonas* sp pigment extraction increased as substrate concentration increased (Solehin *et al.*, 2022).

3.3 Antimicrobial activity

The observed results on the *Sphingomonas* sp. pigment extract for the disk diffusion assay are shown in Table 1. The *Sphingomonas* sp. pigment extract was the most effective against Gram-positive bacterium, *S. epidermidis* ATCC 51625 with an inhibition zone of 22±1.0 mm. According to the results, *Sphingomonas* sp. pigment extract showed significant antimicrobial activity against selected Gram-positive and Gram-negative bacteria. According to Sato *et al.* (2003), some *Sphingomonas* produce anticyanobacterial chemicals argimicins. In addition, this also might be the ability of *Sphingomonas* strains to digest organic compounds or contaminants and their biosynthetic production of extracellular polymers (Romaneko *et al.*, 2007). *Sphingomonas* pigment extract was effective against Gram-positive also due to bacteriocins produced which are important as food preservatives and antimicrobial agents (Romanenko *et al.*, 2009).

Table 1. Inhibition zone of *Sphingomonas* sp. pigment extract against pathogenic bacteria.

Pathogenic bacteria	Inhibition zone
<i>Escherichia coli</i> ATCC 8739	21±1.2
<i>Staphylococcus aureus</i> ATCC 25923	20±0.7
<i>Staphylococcus epidermidis</i> ATCC 51625	22±1.0

4. Conclusion

The combination of dried papaya and banana peels may work well together as a substrate for *Sphingomonas* sp. strains to produce colours. Papaya and banana peels are a common byproduct of agricultural waste and have been studied as cost-effective to replace the commercial TGYE media. After 72 hrs incubation in a combination of dried papaya and banana peels with a ratio 1:1 media, the maximum relative pigment concentration was observed at pH 7, 37°C, and 100% (v/v). This indicates that the combination of dried papaya and banana peels can be effective for the cultivation media of *Sphingomonas* sp. As a result, papaya and banana waste can be used effectively hence reducing the cost of the medium for bacterial growth. Besides that, *Sphingomonas* sp. pigment extract showed positive antimicrobial activities against pathogenic bacteria. Therefore, agricultural waste can be considered as a substrate in the future for microbial pigment synthesis as it can be considered the most efficient and effective cost to implement in the industry.

Conflict of interest

The authors declare no conflict of interest.

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