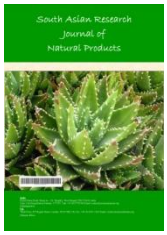

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Stability and Antioxidant Activity of Natural Red Pigment by *Pseudomonas stutzeri* ZH-1

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Natural pigments are widely used in textiles, leather, food and other materials, due to they are non-toxic and easy to extract. In the present study, stability and antioxidant activity of a natural red pigment was investigated from *Pseudomonas stutzeri* ZH-1 which was first isolated and identified from the sludge of Fenhe River, Linfen, Shanxi Province. The results of stability tests showed the red pigment had a relative good stability at pH 5-12 and 30-90°C. The contents of pigments from *P. stutzeri* ZH-1 were all not significant differences ($p>0.05$) in a certain concentration of H_2O_2, $Na_2S_2O_8$ and $NaSO_3$ at 30 °C for 1h, respectively. In the metal ion solutions being tested for 2h, the pigments retained a residual rate above 80% except for Mg^{2+} and K^+; Additional results of antioxidant activity assays displayed that the nature pigment had the strong scavenging activities against three free radicals including DPPH, ABTS and hydroxyl, and the highest scavenging rates at 0.5 g/L concentrations of pigments were up to 20.58%, 19.23% and 37.69%, respectively. Obviously, the above results suggest the red pigment produced by *P. stutzeri* ZH-1 which is a promising microbial resources provides a new strategy for the promotion and application of natural pigments.

Keywords: P. stutzeri ZH-1; pigment; stability; antioxidant activity.

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1. INTRODUCTION

Natural pigments has attracted a lot of attention and are widely used in textiles, leather, food and other materials, especially in food. The international demand for pigments of food is achieved to US$27.5 billion in 2018 [1]. The colorant is remarkable in the food industry by providing enhancement, masking or the act of copying the natural color of food [2]. Pigments can be classified as natural, nature identical and synthetic on account of source [3]. In the past, synthetic pigments were used optional in food. However, Synthetic pigments have disadvantages such as teratogenicity and carcinogenicity [4]. At present, natural pigments have many advantages over synthetic pigments. They are environmentally friendly, on the other hand, they are safer, so that nature pigment loved by producers and businesses [5]. Natural pigments are sourced from animals, plants and microbes.

Compared to those from plants and animals, production of natural colorants by microbial fermentation has several advantages, such as in large quantities of raw materials, not affected by seasons, cost effective production and higher concentration with easier to purify products. It is beneficial to produce natural pigments from microorganisms. Such as red pigments by *Monascus* was studied in 1996 [6], natural melanin from submerged cultures of the mushroom *Auricularia auricular* was studied [7], production of natural edible melanin by *A. auricula* and its physicochemical properties was experimented [8], a new blue pigment produced from *Streptomyces* was found [9]. Obviously, the bacteria producing pigment are mostly fungus in the current researches. But the demand for natural pigments is very large, which means the microbe-pigments is also play an extreme important on making natural pigment resources richer. Moreover, bacteria have a shorter growth cycle and faster reproduction than fungus, and the development of bacteria producing pigment is considerable necessary, Such as *Serratia* [10]. However, the following problems still exist in the development of natural pigments by microorganisms: shortage of resources, insufficient types of pigments, low stability and yield, few real applications and so on. This means that it is a crucial part to develop the natural pigments from bacteria. In our previous study, a bacterial strain named *Pseudomonas stutzeri* ZH-1, which exhibits the ability of efficient heterotrophic nitrification and aerobic denitrification, was isolated from the sludge of Fenhe River (in Shanxi Province, China) and identified by 16S-rDNA sequencing as a strain of *P. stutzeri.* The strain ZH-1 was a Gram-negative, non-motil and short rod-shaped [11], and *P. stutzeri* ZH-1 had a broad spectrum of degradation of organophosphorus pesticides [12].

There is another interesting thing in our previous researches that the *P. stutzeri* ZH-1 can produce red pigment in the Nutrient agar medium. In this study, we investigated deeply the characteristic of stability and antioxidant activity of the red pigment to reveal the potential application of the strain ZH-1. It would lay a theoretical foundation for the development and utilization of microbial resources in the field of natural pigments. Meanwhile, it would also open up a new approach for the application of *P. stutzeri* ZH-1.

2. MATERIALS AND METHODS

2.1 Strain and Media

P. stutzeri ZH-1 was separated from the sludge of the Fenhe River in Shanxi province of China and was given the NCBI accession DQ513513. The stock culture was maintained on Nutrient agar, and seed culture medium used Nutrient broth. Nutrient Agar and Nutrient Broth were bought from Aoboxing (Beijing , China).

2.2 Extraction of the Red Pigment

A single colony was transferred from the Nutrient agar to a 250 ml erlenmeyer flask containing 100 ml of Nutrient broth and agitated at 120 rpm at 37 °C for 50 h. After centrifugation at 6000 rpm for 30 min, the supernatant was adjusted to pH 12.0 using NaOH. Then, take the supernatant to adjust to pH 2.0 using HCl, collect the deposits, and dissolve in 40% ethanol (According to the solubility test results, the pigment have the best solubility in 40% ethanol) [7]. At last, the ethanol solution was rotary evaporated and dried. Solid pigments were obtained for the subsequent uses.

2.3 Stability Analysis

Solid red pigments were prepared a 2 g/L-solution with 40% ethanol for the tests of stability. The liquid pigments (2 g/L) were stored with different pH (2-12) at 25 ℃ for1 h and then the absorbance of the solutions was measured at 500 nm. For the same reasons, the liquid

pigments were deposited at various temperatures (30、40、50、60、70、80、90、100℃) for 1 h, and the residual ratio of the pigments were estimated. Different concentrations of metal ions (K^+、Na^+、Ca^{2+}、Cu^{2+}、Zn^{2+}、Al^{3+}、Mg^{2+} and Mn^{2+}), oxidizer (H_2O_2、$K_2S_2O_8$) and reducer (Na_2SO_3) were added to the pigment solutions (2 g/L) and incubated at 30 ℃ for 0.5 h, 1 h, 1.5 h and 2 h, respectively. And then the residual ratio of the pigments was determined [9]. The concentrations of metal ions, oxidizer and reducer were set at 6 levels of 5 g/L, 10 g/L, 15 g/L, 20 g/L, 25 g/L and 30 g/L. Three experiment was repeated three times. Residual rate can be calculated by the following equation:

Residual rate (%) = $A_1/A_0 \times 100\%$

A_0: OD value of pigment at 25 ℃
A_1: OD value of pigment at different temperature

2.4 Antioxidant Analysis

Samples of solid red pigments were prepared different solutions with 40% ethanol for the tests of inoxidizability. The concentrations of pigment sample were set at 6 levels of 0, 0.1,0.2, 0.3, 0.4 and 0.5 g/L. V_C solutions (40% ethanol) of the corresponding concentrations was used as the positive control.

2.4.1 DPPH analysis

The solutions of DPPH radical (DPPH·) were prepared at 0.2 mmol/L with the 40% ethanol solution and stored in the dark. Add DPPH· solutions to the pigment solutions with the volume ratio 1:1 and incubate at 25 °C for 1 h. Then, the absorbance at 517nm was measured spectrophotometrically. The scavenging activities were calculated by the following formula [13]:

Scavenging rate (%) = $[1 – (A_1/ A_0)] \times 100\%$

A_1: absorbance value of sample
A_0: absorbance value of control

2.4.2 ABTS analysis

The ABTS radicals (ABTS·) solutions were generated by reacting ABTS (7mmoL/L) with potassium persulfate (2.45 mmol/L) at 4 ℃ for 16 h in the dark and was diluted with 40% ethanol to OD_{734} of 0.70 ± 0.05. A 0.1 ml-sample was added to 3.9 ml of ABTS· solution at 25 °C for 6 min and was measured OD_{734} [7]. The scavenging activities were calculated by the following equation:

Scavenging rate (%) = $[1- (A_1-A_2) /A_0] \times 100\%$

A_0: absorbance of the blank group (40% ethanol + ABTS·)
A_1: absorbance of the sample reaction (sample + ABTS·)
A_2: absorbance of the sample

2.4.3 Hydroxyl radical analysis

To determine hydroxyl radical (OH·) scavenging activity, 1 ml-sample was mixed with 3.0 ml H_2O_2 (9 mmol/L), 3.0 ml $FeSO_4$ (3 mmol/L), 3.0 ml salicylic acid (6 mmol/L) and was incubated at 37 °C for 15 min [9]. After be centrifuged (2000 r/min) at room temperature for 10 min the absorbance was measured at 510 nm. The detailed formula is as follows:

Scavenging rate (%) = $(1-A_1/A_0)\times 100\%$

A_0: absorbance of the blank group
A_1: absorbance of the sample

2.5 Statistical Analysis

All data values are expressed as the mean values of at least three independent experiments. All statistical analyses were performed using SPSS 17.0. Duncan Data from the experiments were subjected to ANOVA.

3. RESULTS

3.1 Stability Analysis

3.1.1 Effect of pH and temperature on the stability of pigment

Natural pigments characteristically fade or change color, depending on pH. To study the influence of pH on the stability of pigment from *P. stutzeri* ZH-1, the NaOH and HCl were employed to adjust pH of the pigment solution. The results were shown in Fig. 1.

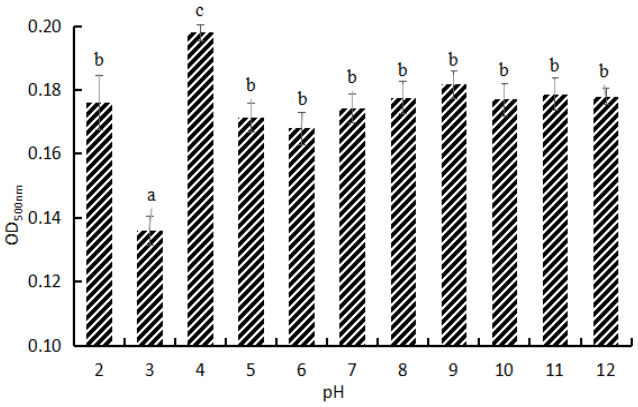

Fig. 1. OD_{500} of pigments from *P. stutzeri*ZH-1 at different pH. Different lowercase letters represent a significant difference between different pH ($p < 0.05$)

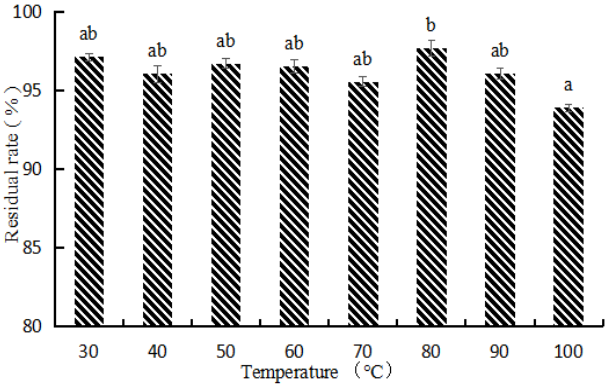

Fig. 2. Residual rate of pigments from *P.stutzeri* ZH-1 at different temperature. Different lowercase letters represent a significant difference between different temperature ($p < 0.05$)

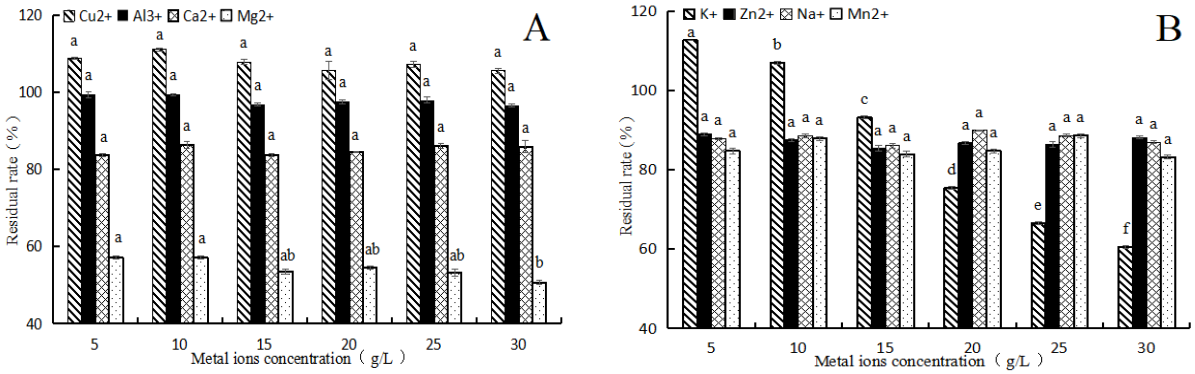

Fig. 3. Residual rate of pigments from *P. stutzeri*ZH-1 at various metal. A for Ca^{2+}, Mn^{2+},Na^{+} and Al^{3+}; B forCu^{2+}, K^{+}, Mg^{2+} and Zn^{2+}. Different lowercase letters represent a significant difference between respective different concentrations ($p < 0.05$)

In general, the change of OD value is used to reflect the stability of pigment. The pigment contents were different in the range of pH 2-12, and they were significant difference ($p<0.05$) when pH<5. But the pigment contents were no significantly difference ($p>0.05$) when pH was higher than 5. That would indicate that the pigment was stable at a pH greater than 5.

Thermal stability is used to ascertain the potential functions of a natural colorant and the results of residual rate were shown in Fig.2.

From Fig. 2, we can see the residual rate of pigments were over 90% under the test conditions and the minimum value was 93% at 100 ℃. But significance analysis showed there was no difference ($p>0.05$) in the residual rate when the temperature was lower than 90℃. It revealed that the pigment was relative stable below 90 ℃.

3.1.2 Effect of metal ions on the stability of pigment

Eight metal ions were employed to study the stability of natural pigments in this investigation and the results were shown in Fig.3 and Fig.4.

Fig. 3 showed that the residue rate of pigments is different when they were incubated with different metal ions at 30 ℃ for 1 h. After being treated most metal ions with different concentrations, the residual rate of pigments were more than 80 %. There were 6 metal ions (Ca^{2+}, Mn^{2+},Na^{+}, Al^{3+},Cu^{2+} and Zn^{2+}) which correspond data of residual rate were not significant ($p>0.05$) between all their respective test concentration. The residual rate of K^{+} was up to 112.69 % at a concentration of 5 g/L, and with the increase of its concentration, the residue rate of pigments decreased significantly ($p<0.05$) (Fig. 3A). In addition, the residue rate of Mg^{2+} remains at a very low level and varies significantly within a range of test concentrations ($p<0.05$) (Fig. 3B). It was suggested that the natural pigment from *P. stutzeri* ZH-1 had good stability in metal ion solutions except K^{+} and Mg^{2+}.

Fig. 4 showed a variation curve of the OD_{500} over time. It can be seen there was no significant change ($p>0.05$) in the OD_{500} value of pigments under metal ion solutions (Mg^{2+}, Mn^{2+},Na^{+}, Al^{3+}, and Cu^{2+}) within 2 h in Fig.4A, and the OD_{500} of Mg^{2+} had been very low (Fig. 4A). However, for Ca^{2+}, Zn^{2+} and K^{+}, there were significant differences ($p<0.05$) between their OD_{500} values of respective times in Fig.4B. Combined with the results of upper experiments, the stability of natural pigment form *P. stutzeri* ZH-1 in metal ion respective solutions of Mn^{2+},Na^{+}, Al^{3+} and Cu^{2+} was desirable.

3.1.3 Effect of oxidants and deoxidizer on the stability of pigment

The oxidizer of H_2O_2 and $Na_2S_2O_8$ and the reducer of $NaSO_3$ were selected in the experiments and the results were in Fig. 5. It can be seen that the residue rates were not significant difference ($p> 0.05$) along with the increase of the concentration of H_2O_2, $Na_2S_2O_8$ and $NaSO_3$. Moreover, $Na_2S_2O_8$ had the smallest effect on the pigment residue rates among three agents, and the pigment residual rates were all above 90%.The conclusion was that the pigment from *P. stutzeri* ZH-1 was relatively stable in the solutions of H_2O_2, $Na_2S_2O_8$ and $NaSO_3$.

3.2 Antioxidant Analysis

Most natural pigments show some antioxidant activities. Three free radicals (DPPH·, ABTS· and OH·) were tested in this survey and the result were shown in Fig. 6.

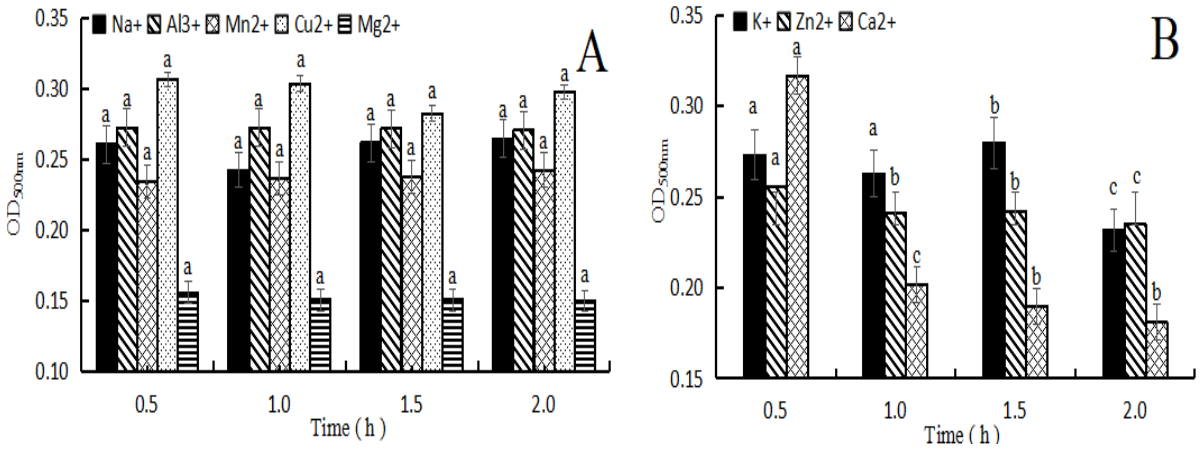

Fig. 4. OD_{500} of pigments from *P. stutzeri*ZH-1 at different time in different metal ions solution. A for Cu^{2+}, Mn^{2+}, Na^{+}, Mg^{2+} and Al^{3+}; B for K^{+}, Ca^{2+} and Zn^{2+}. Different lowercase letters represent a significant difference between respective different times ($p < 0.05$)

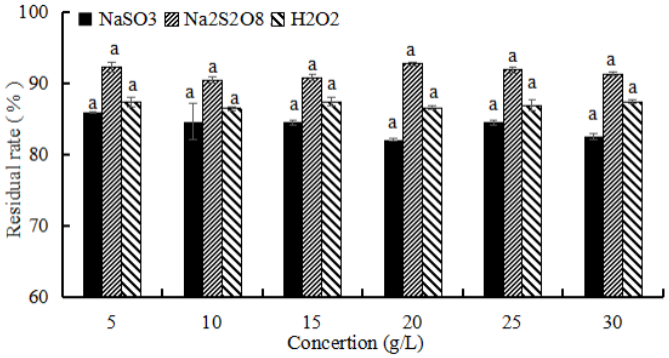

Fig. 5. Effects of various oxidants and deoxidizer on the stability of pigment *P. stutzeri*ZH-1. Different lowercase letters represent a significant difference between respective different concentrations ($p < 0.05$)

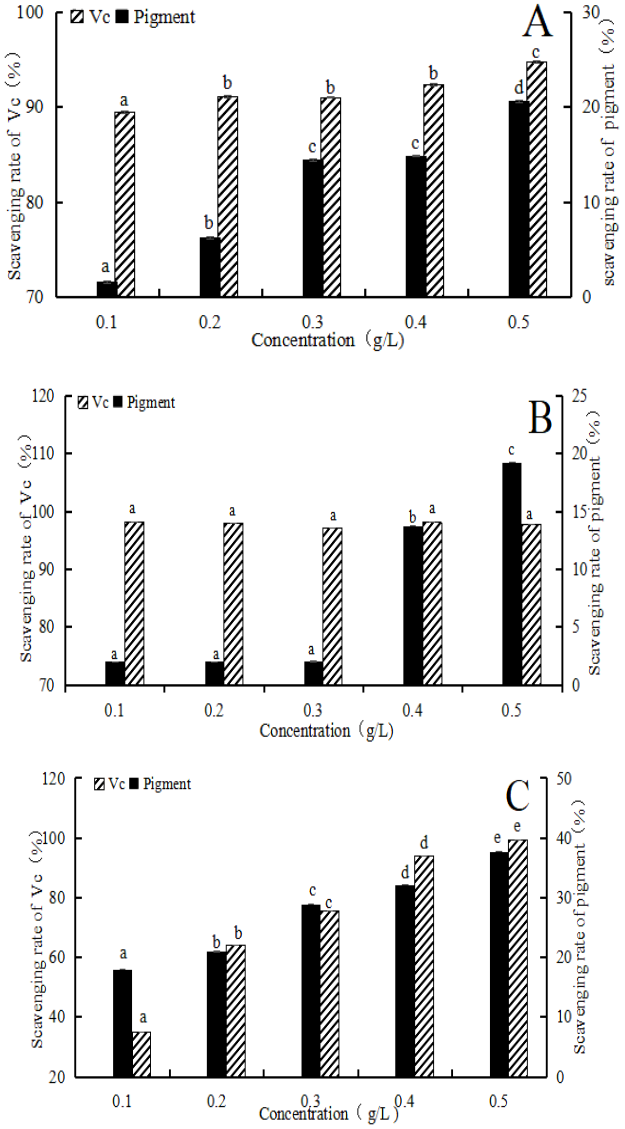

Fig. 6. The radical scavenging rate of natural pigment *P. stutzeri*ZH-1. A for DPPH·, B for ABTS· and C for OH·. vitamin C (Vc) as control

There is positive direct correlation between the pigment concentrations and the scavenging rates like Fig. 6. Pigments possessed some scavenging activity on the DPPH, ABTS·and OH· and acted as an antioxidant. The scavenging effect was increased with increasing concentration. However, at the concentration of 0.5 g/L, the scavenging rate of pigment on DPPH·, ABTS· and OH· increased to the highest value, up to 20.58 % (Fig. 6A), 19.23 % (Fig. 6B) and 37.69 % (Fig. 6C), respectively. The results also provided a direct comparison of the antioxidant activity between the pigment and vitamin C. Compare to the vitamin C, the ability of scavenging of 1 g pigment on DPPH·, ABTS· and OH· were equal to that of 0.217、0.196 and 0.379 g vitamin C (Vc), respectively. In short, this study suggested that the pigment of *P. stutzeri* ZH-1 could potentially be used as a natural antioxidant and as substitute for synthetic dyes.

4. DISCUSSION

In the international market, the demand of natural colorant increases continuously, especially in the food, pharmaceutical, textile [14-15] and cosmetic industries. Natural pigment stability was studied to widely used. In previous studies, the blue pigment form *Streptomyces coelicolor* color was changed with pH value, from red at pH < 7, through amaranth at pH 7–8, to blue at pH 8 [4]. In our results showed that the red pigment from *P. stutzeri* ZH-1 exhibited a good stability both in pH 5-12, and some components are easily precipitated by acids in pigment solution when pH<5. Moreover, a red pigment from seeds of *O. fragrans* was was stable to heat in the temperature range of 25–100 ℃ [16] and pigment from peel of *C. burmannii* was stable in the range of 25–100 °C [17]. Similarly, the color from *P. stutzeri* ZH-1 was stable within a widely range of temperatures, which is one of the typical characteristics of various pigment from plants, microorganism and animals. Additionally, Mg^{2+} and K^{+} should be avoided when the pigment was extracted and used because of its no resistant to Mg^{2+} and K^{+}. Furthermore, no evident influence on the pigment stability was observed in oxidizer and reducer. There are a large number of reports on the application of natural pigments in the textile industry, by way of contrast, the pigment from *P. stutzeri* ZH-1 have good stability and it will be widely used in textiles, leather, food and other materials, especially in the textile industry.

The assay of scavenging capacity could reflect the electron or hydrogen donating ability of antioxidant, and it is broadly used to evaluate the antioxidant activity of natural product by ABTS·discoloration [18]. Hydroxyl radical is known to be very harmful to cellular components as a precursor of more reactive oxygen species [19]. The pigment from *P. stutzeri* ZH-1 has a strong antioxidant potential according to the evaluation of its scavenging rates on ABTS free radical, hydroxyl radical and DPPH free radical *in vitro*. Pigment from *P. stutzeri* ZH-1 have some antioxidant properties, but not nearly as much as that of Vc. This may have some possibilities which is due to the purity of the pigment.

The result show that the pigment of *P. stutzeri* ZH-1 is similar to eumelanin of the DOPA pathway, In order to develop more extensive application, further research will be made on the Identify the structure of the red natural pigment from *P. stutzeri* ZH-1.

5. CONCLUSION

The experiment results showed that the red pigment that we have separated from *P. stutzeri* ZH-1 have good stability in widely temperature range of 30-90°C , acid-base (pH = 5 – 12) solutions, metal ion solutions except for Mg^{2+} and K^{+} and H_2O_2, $Na_2S_2O_8$ and $NaSO_3$ solutions. Besides, the red pigment exhibit the strong scavenging activities against three free radicals including DPPH, ABTS and hydroxyl through antioxidant activity assays. It is be suggested that the red pigment from *P. stutzeri* ZH-1 is potentially be used as a natural antioxidant and as substitute for syntheticdyes.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

COMPLIANCE WITH ETHICAL STANDARDS

This article does not contain any studies involving animals or human participants performed by any of the authors.

CONSENT

It is not applicable.

ACKNOWLEGEMENTS

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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