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ANTIBACTERIAL EFFECT OF ORANGE *MONASCUS* PIGMENT AGAINST *STAPHYLOCOCCUS AUREUS*

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The objective of this work was to research the antibacterial effects of orange pigment, which was separated from *Monascus* pigments, against *Staphylococcus aureus*. The increase of the diameter of inhibition zone treated with orange pigment indicated that orange pigment had remarkable antibacterial activities against *S. aureus*. Orange pigment (10 mg ml^{-1}) had a strong destructive effect on the membrane and structure of *S. aureus* by the analysis of scanning electron microscopy as well as transmission electron microscopy. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) further demonstrated that the cell membrane was seriously damaged by orange pigment, which resulted in the leakage of protein from *S. aureus* cells. A significant decrease in the synthesis of DNA was also seen in *S. aureus* cells exposed to 10 mg ml^{-1} orange pigment. All in all, orange pigment showed excellent antibacterial effects against *S. aureus*.

Keywords: antibacterial effects, orange pigment, *S. aureus*, cell membrane, DNA synthesis

The microbial contamination problem in food safety is still a fundamental concern of both consumers and the food industry (Tang et al., 2009; Li et al., 2014a). Food preservatives can inhibit the growth of pathogenic and spoilage microorganisms such as *S. aureus*, thereby prolonging shelf life and maintaining freshness of foods (Li et al., 2014a; Yang et al., 2016). Due to the potential toxicity of chemical antimicrobial agents, there is a pressing need to discover natural antimicrobial preservatives (Navarro García et al., 2003; Kim et al., 2010; Li et al., 2014b).

Red yeast rice, as a traditional Chinese food, is the fermented product of rice on which *Monascus purpureus* has been grown (Li et al., 1998; Xie et al., 2012). *Monascus purpureus* can produce many secondary metabolites, such as pigments, monacolin analogs, γ-aminobutyric acid (GABA), dimerumic acid, and citrinin (Wild et al., 2002; Su et al., 2005). *Monascus purpureus* is well known for its ability to produce pigments ranging from bright yellow to deep red. The types of *Monascus* pigments are yellow pigments, including ankaflavin and monascin; orange pigments, including monascorubrin and rubropunctanin; and red pigments, including monascorubramine and rubropuctamine (Wong & Koehler, 1981; Blanc et al., 1994). The pigments have been used in food as a natural preservative or to preserve taste and colour in fish and meat (Yang & Mousa, 2012).

The antimicrobial activity of *Monascus purpureus* was first reported by Wong and Bau (1977). Wild type of *Monascus purpureus* showed antimicrobial activities against *Bacillus, Streptococcus*, and *Pseudomonas*, which are spoilage bacteria in foods (Wong & Bau, 1977). Two yellow pigments, isolated from *Monascus purpureus*, had antibacterial activity against

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Bacillus subtilis (Wong & Koehler, 1981). The orange pigments had antimicrobial activities against *Bacillus subtilis*, *Escherichia coli*, even some filamentous fungi and yeasts (Sato et al., 1997). It had also been reported that *Monascus* pigments inhibited the growth of *Escherichia coli* through reducing availability of oxygen in the cells (Kim et al., 2006b). To the best of our knowledge, little information is available about the antibacterial activities of orange pigment against *S. aureus*, which could produce enterotoxin and be resistant to several antibiotics.

The purpose of this study was to study antibacterial effects of orange pigment against *S. aureus* by detecting inhibition zones and by analysis of morphology, ultrastructure, proteins, and DNA of *S. aureus* cells.

1. Materials and methods

1.1. Preparation of orange pigment solution

Monascus pigments, purchased from Zhonghui Food Ltd. Co. (Jinan, China), were dissolved in 70% ethanol to a final concentration of 10% (v/v) and extracted at 50 ºC for 2 h. The extracting solution was centrifuged at 4500 × *g* at 4 ºC for 15 min, then the supernatant was collected in order to extract the orange pigment according to our previously described method (Zhao et al., 2016). Silica gel column chromatography was used to separate orange pigment from the supernatant. The column (2 cm × 30 cm) was packed with silica gel suspended in 70% ethanol. Orange pigment was eluted with ethyl acetate. The eluate was concentrated by rotary evaporators (RE-52AA, Yarong Biochemistry Instrument Factory, Shanghai, China) and then freeze-dried (Free Zone 6, Labconco Corporation, Kansas, USA) to get the orange pigment.

1.2. Bacteria strain and cultivation

The test strains of *S. aureus* ATCC 6538 were obtained from the Culture Collection in the Qilu University of Technology (Shandong Academy of Sciences). Freeze-dried bacteria were activated according to the ATCC guidelines. The strain was maintained on the slants of beef extract peptone (BEP) medium (0.3% beef extract, 1% peptone, 0.5% NaCl, and 2% agar boiled to dissolve in 100 ml of distilled water) and inoculated into 100 ml of sterile BEP broth. The BEP broth was cultivated with shaking (130 r.p.m.) at 37 ºC for 9 h to yield a final cell concentration of 10^7–10^8 CFU ml^{-1} used for further study.

1.3. Measurement of inhibition zone diameters

Orange pigment was dissolved in sterile distilled water to final concentrations (w/v) of 0.625, 1.25, 2.5, 5, and 10 mg ml^{-1}, respectively. Antibacterial activities of orange pigment against *S. aureus* were measured by the Oxford cup method (Wang et al., 2009) with some modifications. *S. aureus* cells (approximately 10^8 CFU ml^{-1}) were harvested and diluted with phosphate buffer solution (PBS, 10 mM, pH 7.4) to a final concentration of 10^5 CFU ml^{-1}. An aliquot of 0.1 ml (10^5 CFU ml^{-1}) of the diluted inoculum from the bacterial culture was transferred to the surface of BEP medium plates. Each strain was spread evenly onto individual plates through a glass spreader. Sterile Oxford cups (5 mm) were placed on the surface of plates poured with BEP medium in a regular triangle distribution. Aliquots of 200 µl of varying orange pigment concentrations (0.625, 1.25, 2.5, 5.0, and 10 mg ml^{-1}) were

transferred into each Oxford cup, respectively, and 200 µl of sterile water was added in place of orange pigment as control. These plates were placed in the refrigerator at 4 ºC for 2 h and incubated at 37 ºC for 2 days. Then the diameters of the transparent inhibition zones were measured.

1.4. Scanning electron microscopy (SEM) analysis of S. aureus *cells*

SEM analysis was performed to observe the morphological changes of *S. aureus* cells (Booyens et al., 2014). Aliquots (5 ml) of the *S. aureus* culture treated with orange pigment at 0 and 10 mg ml^{-1} were incubated at 37 ºC with gentle agitation for 4 h. After centrifugation at 6000 × *g* for 15 min, the precipitated *S. aureus* cells were washed twice in PBS (10 mM, pH 7.4) and fixed in 2.5% glutaraldehyde for 12 h. The fixed cells were washed three times with PBS (10 mM, pH 7.4) and dehydrated in alcohol (30, 50, 70, 80, 90, and 100%, one time at 20 min for each dilution and three times at 30 min at the last step). The dehydrated cells were dried by CO_2 critical point drying, mounted onto SEM specimen stubs and coated with gold, then observed by SEM (S-570, Hitachi, Tokyo, Japan).

1.5. Transmission electron microscopy (TEM) determination of S. aureus *cell ultrastructure*

The cell ultrastructure of *S. aureus* was studied using TEM according to the previously described method (Zhao et al., 2017). A series of quantity of orange pigment were added to the bacterial suspensions (5 ml) to get final concentrations of 0 to 10 mg ml^{-1}. The bacteria treated with orange pigment were incubated at 37 ºC for 4 h. The treated cells were harvested by centrifugation at 6000 × *g* for 15 min and washed twice in PBS (10 mM, pH 7.4). Then the cells were fixed with 2.5% glutaraldehyde at 4 ºC for at least 2 h and oxidised by adding 200 µl of 1% osmium tetroxide at 4 °C for 3 h. After oxidation, the cells were dehydrated sequentially using different acetone solutions (30%, 50%, 70%, 90%, and 100%). Finally, the dehydrated pellets were transferred into embedding plate, then permeated twice with 1,2-epoxypropane for 10 min and with epoxy resin (DER: DMAE: ERL: NSA = 1.80: 0.06: 2.00: 5.00) at 4 °C for 45 min before drying (70 ºC, 18 h) to form specimen blocks. TEM (JEOL-JEM-1200 EX, Japan) was used to observe the section of samples.

1.6. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of the proteins of S. aureus *cells*

SDS-PAGE of the bacterial proteins was carried out after incubation with the antimicrobial agent (Sitohy et al., 2012). Aliquots (2 ml) of different concentrations of orange pigment were added to 18 ml bacterial culture solutions (approximately 10^8 CFU ml^{-1}) to final concentrations of 0 to 10 mg ml^{-1}. The bacterial suspensions of the control group were immediately centrifuged at 6000 × *g* for 15 min. The treated bacterial suspensions were incubated at 37 °C for 4 h and then centrifuged at 6000 × *g* for 15 min. The obtained pellets were washed twice with PBS (10 mM, pH 7.4) and resuspended in 1 ml PBS (10 mM, pH 7.4). Aliquots (50 µl) of bacterial suspensions were mixed with 25 µl of the sample buffer (pH 6.8; 1 M Tris-HCl, 50% glycerol, 10% SDS, 10% β-mercaptoethanol, and 0.1% bromophenol blue), heated at 100 °C for 3 min, cooled to 25 °C, then treated with ultrasonic for 3 min, and loaded onto a 3% stacking and 12% resolving gel. The proteins were run at 10 mA for 30 min on the stacking gel and at 20 mA for 2 h on the resolving gel. The gel was dyed by Coomassie Brilliant Blue R250 and decolorized by decolouring agent. After 4 days, protein bands were visualized on the gels.

1.7. DNA assay

Aliquots (5 ml) of the *S. aureus* culture treated with orange pigment at 0 to 10 mg ml^{-1} were incubated at 37 ºC with gentle agitation for 4 h, then harvested by centrifugation at 6000 × *g* for 15 min and washed twice in PBS (10 mM, pH 7.4). DNA was extracted with the bacterial DNA extraction kit (Bioteke corporation, Beijing, China). Lastly, samples were applied to a 1.0% agarose gel electrophoresis and run at 200 V for 15 min, then observed under UV illumination through gel imaging system.

1.8. Statistical analysis

Every experiment was performed in triplicate, and average values with standard errors are reported. SPSS17.0 software was used for all statistical analyses. The data were analyzed by analysis of variance (ANOVA) and Duncan's post-hoc analysis, and regression analysis was used to determine the significant difference at 5% confidence intervals ($P<0.05$).

2. Results and discussion

2.1. Antibacterial activities of orange pigment against S. aureus

The antibacterial activities of orange pigment against *S. aureus* were evaluated by the diameter of the inhibition zones (Table 1). As observed in Table 1, the diameters of the inhibition zones against *S. aureus* increased from 5.0±0.3 mm to 34±1.3 mm with increasing concentrations of orange pigment from 0 to 10.0 mg ml^{-1}. These results demonstrated that orange pigment had antibacterial activities against *S. aureus*. Moreover, its antibacterial activities increased with the increase of orange pigment concentration.

Table 1. Inhibition zone diameters of *S. aureus* treated with varying concentrations of orange pigment

Treatments	Concentrations of orange pigment (mg ml^{-1})					
	0	0.625	1.25	2.50	5.00	10.00
Inhibition zone diameters (mm)	5.0±0.3^{a}	24±1.8^{a}	26±1.5^{a}	27±2.0^{a}	33±0.9^{b}	34±1.3^{b}

Different superscript lowercase letters (^{a,b}) indicate significant differences at $P<0.05$.

Ungureanu and Ferdes (2010) reported antibacterial activities of *Monascus* red rice powder against *Bacillus*, *Pseudomonas*, and *Streptomyces* spp., proposing that the pigments of *Monascus purpureus* might be a preservative applied in food. A similar report indicated that the *Monascus* red pigments exhibited high antibacterial activities against *S. aureus*, *Escherichia coli*, and *Bacillus subtilis* by using the minimum inhibitory concentration (Kim et al., 2006a).

2.2. Effect of orange pigment on the morphological changes of S. aureus *cells*

The morphological changes of *S. aureus* cells were evaluated by SEM analysis. Figure 1 displays SEM photomicrographs of *S. aureus* cells treated with and without orange pigment. As observed in Figure 1A, the untreated *S. aureus* cells had typical regular and plump

appearance with uniform size and distribution. However, *S. aureus* cells treated with orange pigment had irregularly wrinkled outer surface and were not uniform in size and distribution. Furthermore, there were some adhesion and aggregation of damaged cells or cellular debris (Fig. 1B). These changes in the morphology of *S. aureus* indicated that orange pigment treatment resulted in the destruction of the bacteria.

A B

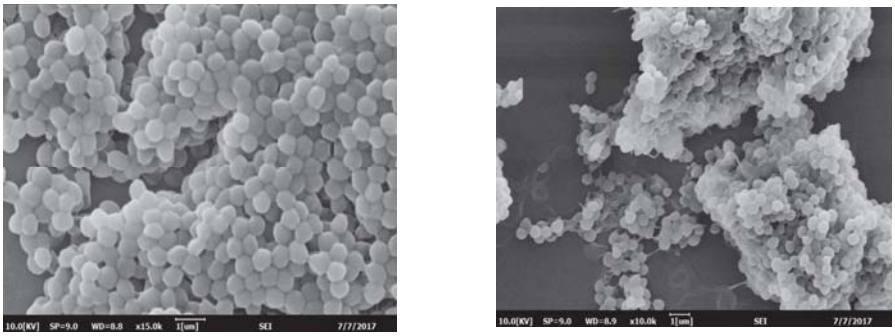

Fig. 1. SEM photomicrographs of *S. aureus* cells treated with orange pigment. (A) *S. aureus* treated without orange pigment, (B) *S. aureus* treated with 10 mg ml^{-1} orange pigment

Our SEM photomicrographs of *S. aureus* treated with orange pigment were similar to *Escherichia coli* cells, which had irregular and wrinkled surfaces when treated with orange pigment, compared to regular and smooth surfaces of untreated cells (Zhao et al., 2016). The *Escherichia coli* cells in the presence of *Monascus* red pigments were obviously damaged with large pellets and aggregation, just as the *S. aureus* exposed to orange pigment with cellular debris and adhesion (Kim et al., 2006b).

2.3. Effect of orange pigment on the ultrastructure of S. aureus *cells*

TEM was used to observe the ultrastructural changes of the orange pigment treated cells. TEM imaging of the control experiments exhibited intact membrane and uniform morphology (Fig. 2A). Compared with the control, various morphological and intracellular changes of orange pigment-treated cells were seen in the cell membrane and cytoplasm (Fig. 2B). *S. aureus* cells were irregular and wrinkled, with outflow of some part of cytoplasm from the cells. These phenomena further indicated the damage of orange pigment to the ultrastructure of *S. aureus* cells. This present study was in agreement with the finding that cells showed a normal rod or spherical shape in the control group, whereas cells had a thin and wrinkled layer of pigment on the cell surface in *Monascus* red pigment treated *S. aureus* via TEM observation (Kim et al., 2006b).

2.4. Effect of orange pigment on the leakage of the bacterial proteins

SDS-PAGE of the bacterial proteins from the *S. aureus* cells treated with orange pigment is shown in Figure 3. The types and amounts of protein in *S. aureus* treated with orange pigment were less than the control. There were two bands with molecular weight of approximate 25 kDa in the control. However, the two bands almost disappeared in the treated samples. The

reason of the decrease or disappearance in bacterial proteins of the treated samples might be that orange pigment seriously damaged the *S. aureus*, thus resulted in the leakage of protein from the bacterial cells, which was consistent with the result of the ultrastructure of *S. aureus* cells (Fig. 2).

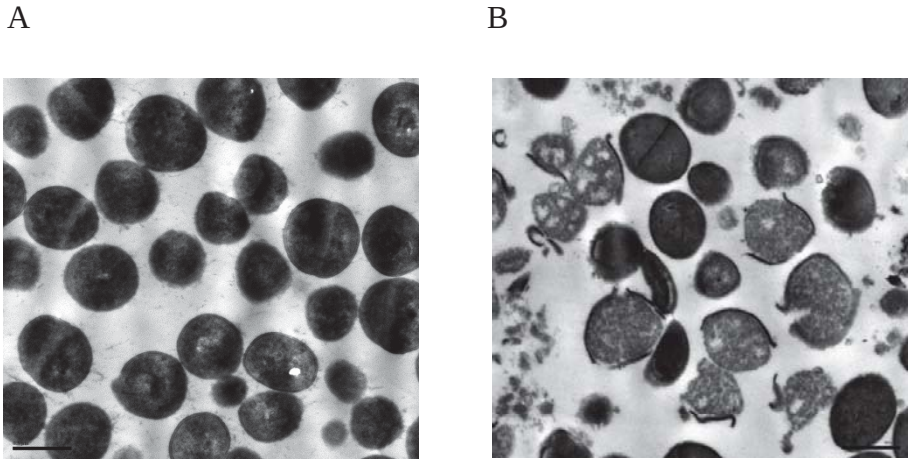

Fig. 2. TEM imaging of *S. aureus* cells treated with orange pigment. (A) *S. aureus* treated without orange pigment, (B) *S. aureus* treated with 10 mg ml^{-1} orange pigment

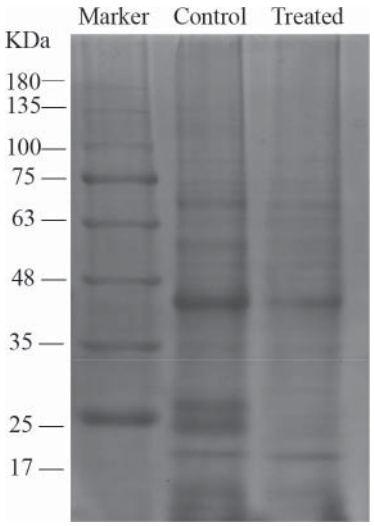

Fig. 3. Effect of orange pigment on proteins of *S. aureus* cells

CLOETE and co-workers (2009) suggested that anolyte caused bacterial death by complete destruction of or partially degrading proteins. It has also been reported that protein patterns of *Listeria monocytogenes* and *Salmonella enteritidis* treated with 100 μg ml^{-1} of glycinin basic subunit showed early the fading of most bacterial protein bands, indicating glycinin basic subunit caused the leakage of the bacterial protein (SITOHY et al., 2012).

2.5. Effect of orange pigment on the bacterial DNA

The effect of orange pigment on the bacterial DNA is showed in Figure 4. The amounts of DNA in *S. aureus* treated with orange pigment were less than that in the control group. The reason of the decrease in bacterial DNA might be that orange pigment could destroy the *S. aureus* cells, further influence its growth and synthesis of DNA. The present result was consistent with the finding of WANG and co-workers (2010), who indicated soybean isoflavone affected nucleic acid synthesis and inhibiting bacterial growth, proved by the agarose gel electrophoresis analysis of DNA.

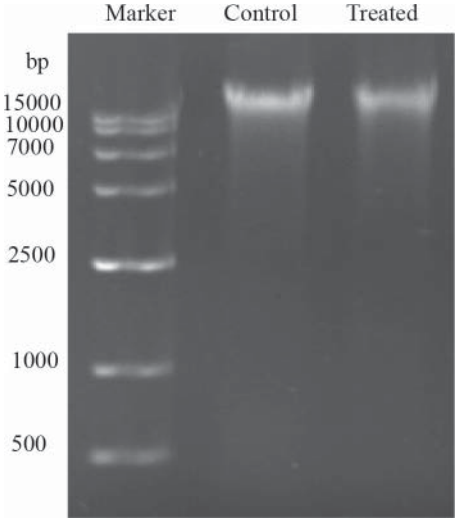

Fig. 4. Effect of orange pigment on DNA of *S. aureus* cells

3. Conclusions

In a word, these results showed that orange pigment exhibited antibacterial effects on *S. aureus*. The antibacterial actions of orange pigment might be that it damaged cell membrane of bacterial cells, and made some cellular components such as proteins and DNA let out, consequently resulting in the death of bacterial cells. Thus orange pigment might be a potential natural food preservative in food industry.

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