

Antibacterial Characteristics of Orange Pigment Extracted from *Monascus* Pigments against *Escherichia coli*

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Abstract

ZHAO G.P., LI Y.Q., YANG J., CUI K.Y. (2015): Antibacterial characteristics of orange pigment extracted from *Monascus* pigments against *Escherichia coli*. Czech J. Food Sci., 34: 197–203.

The antibacterial characteristics of orange pigment, which is one of the *Monascus* pigments, against *Escherichia coli* were investigated. Orange pigment exhibited strong antibacterial activity against *E. coli* evidenced by an increase in the diameter of inhibition zone with orange pigment treatment. The concentration of 2.5 mg/ml was the minimum inhibitory concentration of orange pigment against *E. coli*. Scanning electron microscopy revealed that orange pigment could damage bacterial cells, eventually resulting in cell death. The increase in the electric conductivity of bacterial cell suspensions suggested that the cytoplasmic membrane was broken by treatment with orange pigment. The result of orange pigment incorporation into egg PC further demonstrated the interaction between orange pigment and the phospholipid led to the disruption of bacterial membrane.

Keywords: inhibition zone; minimum inhibitory concentration; antibacterial activity; electric conductivity; membrane disruption

Various synthetic colouring agents and preservatives have been banned due to their potential of toxicity, such as carcinogenicity and/or teratogenicity. As a result, there is an increasing demand for safe and naturally occurring edible colouring agents and preservatives (BABITHA *et al.* 2007; CHUNG & CHEN 2008; SONG *et al.* 2012; SIRIPATRAWAN *et al.* 2013; LI *et al.* 2014). *Monascus* pigments are obtained from cultures of *Monascus purpureus* grown on cooked non-glutinous rice (SU *et al.* 2003; FENG *et al.* 2014) and are the most promising natural colouring agents and preservatives for food (TAN *et al.* 2012).

Monascus pigments have been used for centuries in Asia as a natural food colorant and preservative for meat and vegetables (TAN *et al.* 2012). The Chinese Ministry of Health has incorporated *Monascus* pigments into their modern food additive standards to increase the

colour and delicacy of meat, fish, and soybean products (FABRE *et al.* 1993). Production, isolation, and purification of *Monascus* pigments have been extensively studied (LI *et al.* 2004; BABITHA *et al.* 2007; LIN *et al.* 2008; ZHANG *et al.* 2014). *Monascus* pigments include the following main compound types: ankaflavin and monascin (yellow); monascorubrin and rubropunctatin (orange); and monascorubramine and rubropuctamine (red) (MA *et al.* 2000; HEBER *et al.* 2001; CHENG *et al.* 2011; DURAKLI-VELIOĞLU *et al.* 2013).

Strains N4S, N11S, and X2P induced by neutron and X-ray irradiation, and wild type inhibited the growth of certain bacteria, especially the *Bacillus* species. Strain N11S had a higher antibacterial activity than the wild type (WONG & BAU 1977). The obvious inhibitory effects of orange pigment (rubropunctatin and monascorubrin), as well as of the novel

Supported by the National Natural Science Foundation of China. Grant No. 31371839, the Major Plan of Study and Development, Project No. 2015GSF120006, the Science and Technology Agency, Shandong Province, Science and Technology Development Plan, Shandong Province Colleges and Universities, Project No. J13LE02, and the Program for Science and Technology Innovation Team in Universities of Henan Province, Project No. 16IRTSTHN007.

metabolite ankalactone, against *Bacillus subtilis* and *Escherichia coli* were explored (NOZAKI *et al.* 1991). The orange pigment (rubropunctatin and monascorubrin) displayed antimicrobial activity against *B. subtilis* and *E. coli*, some filamentous fungi and yeasts (MARTÍNKOVÁ *et al.* 1995). Whereas the red pigments monascorubramine and rubropuctamine showed low activity. Two pigments, rubropunctatin and monascorubrin, purified from the mycelium of *Monascus purpureus*, had significant antibiotic activity against *B. subtilis* and *Candida pseudotropicalis* (MARTÍNKOVÁ *et al.* 1999). The beni-koji extract (ethanol extraction and followed by ethyl acetate extraction) prepared with *Monascus pilosus* IFO 4520 at 30 mg/ml inhibited the growth of *Aspergillus sojae* and *B. subtilis* (KONO & HIMENO 1999). These compounds were heat-stable in antimicrobial activity at 100°C for 10 minutes. The fractions isolated from red yeast rice fermented with the yellow mutant of the fungus *M. purpureus* BCRC 38038 in a thin layer chromatography (TLC) bioassay showed a potent effect against *Candida albicans* and *Saccharomyces cerevisiae* (CHENG *et al.* 2011). *Monascus* red rice powder had antibacterial activity against *Bacillus*, *Pseudomonas*, and *Streptomyces* sp. based on inhibitory zone measurements, suggesting that the pigments of *M. purpureus* had a preservative value (UNGUREANU & FERDES 2010). The yellow pigment, isolated from red yeast rice, inhibited bacteria of the genera *Bacillus*, *Pseudomonas*, and *Streptomyces* sp.

However, few reports exist on the antibacterial activity and mechanisms of orange pigment extracted from *Monascus* pigments against *E. coli* (CHUNG & CHEN 2008), which was the most common food-borne pathogen that frequently causes food-borne disease outbreaks.

The aim of this study was to investigate the antibacterial effects of orange pigment against *E. coli*. In this study, further attempts were performed to elucidate the antibacterial actions of orange pigments against *E. coli*.

MATERIAL AND METHODS

Preparation of orange pigment solution. *Monascus* pigments, obtained from Zhonghui Food Ltd. Co. (Jinan, China), were dissolved in 70% ethanol (1:10, v/v), and extracted at 50°C for 2 h, then centrifuged at 4500 g at 4°C for 15 minutes. The supernatant was collected. The extraction of orange pigment

was performed according to the previous method (WONG & KOEHLER 1981) with slight modifications. Briefly, Silica gel column chromatography was used to separate orange pigment which was eluted with ethyl acetate. The column (2 cm × 30 cm) was packed with silica gel suspended in 70% ethanol. The eluate was concentrated by rotary evaporation (RE-52AA; Yarong Biochemistry Instrument Factory, Shanghai, China) and then freeze-dried (Free Zone 6; Labconco Corporation, Kansas, USA) to get the orange pigment.

Bacteria cultures. Test bacteria, *E. coli* ATCC 8739, were obtained from the Culture Collection, Qilu University of Technology. Bacteria were inoculated on beef extract peptone (BEP) medium (0.3% beef extract, 1% peptone, 0.5% NaCl), and incubated overnight on a rotary shaker at 170 rpm at 37°C to reach a count of approximately 10⁸ CFU/ml, which were used for further study.

Measurement of inhibition zone diameters. Antibacterial activity of orange pigment against test bacteria (*E. coli*) was measured by the Oxford cup method (WANG *et al.* 2009) with some modifications. An aliquot of 100 µl of diluted inoculum (10⁵ CFU/ml) of the test bacterial culture was transferred to the surface of BEP medium plates and spread uniformly on individual plates with a glass spreader. Sterile Oxford cups (5 mm, inner diameter) were placed on the surface of plates poured with BEP medium. Aliquots of 200 µl of varying concentrations of orange pigment (0.625, 1.25, 2.5, 5.0, and 10.0 mg/ml) were transferred into Oxford cups, respectively. In addition, an aliquot of 200 µl of sterile water was used in place of orange pigment as the control. The plates were placed in the refrigerator for 2 h at 4°C to diffuse the orange pigment in the Oxford cup, and then incubated at 37°C for 2 days. The diameters of the transparent inhibition zones were measured using a transparent ruler, and then recorded in mm unit to conclude the antibacterial activity of orange pigment against *E. coli*.

Measurement of minimum inhibitory concentration (MIC). MIC was determined by the dilution method (XIAO *et al.* 2011) with a slight modification. Orange pigment and *E. coli* cells were added separately to 6 ml BEP medium, getting final concentrations of orange pigment of 0, 0.625, 1.25, 2.5, 5.0, and 10.0 mg/ml and approximately 10⁵ CFU/ml *E. coli* cells. The medium was incubated at 37°C for 24 hours. At the end of incubation time, MIC was visually identified as the lowest concentration of the orange pigment which inhibited the visible growth and confirmed by

doi: 10.17221/430/2015-CJFS

measuring the optical density (OD) at 600 nm of all treated samples. All tests were performed in triplicate.

Scanning electron microscopy (SEM) analysis of *E. coli* cells. The morphology of *E. coli* cells was assessed using the SEM analysis (SITOHY *et al.* 2012). Samples containing *E. coli* in Luria-Bertani (LB) medium (1% tryptone, 0.05% yeast extract, 1% NaCl, 2% agar) with orange pigment (10 mg/ml) were incubated at 37°C with gentle shaking for 9 h to get approximately 10^7 CFU/ml. Bacterial cells were collected by centrifugation at 6000 *g* for 15 min at 4°C. The control sample was prepared as described above in the absence of orange pigment. Bacterial cells were fixed in 2.5% glutaraldehyde for 12 hours. The fixed bacterial cells were washed with 0.1 M phosphate buffer solution (PBS) for 2 h (three times). The bacterial cells were fixed again with osmic acid for 1.5 h and washed with double-distilled water for 2 h (three times). Bacterial cells were dehydrated by two rounds of serial dehydration with alcohol solutions (50, 70, 80, 90, and 100%) at 15-min intervals, followed by a final isoamyl acetate rinse for 30 minutes. The bacterial cells were dried by CO₂ critical point drying (HCP-2), mounted, platinised with ion sputter coater (IB-5), and observed by SEM (S-570; all Hitachi, Tokyo, Japan).

Measurement of electric conductivity. Cellular leakage from bacteria was determined by measuring electrolyte leakage into the incubation medium with a conductivity meter (DDS-307; Precision and Scientific Instrument Co. Ltd., Shanghai, China) (YE *et al.* 2005) with a slight modification. After incubation in LB medium at 37°C for 12 hours, *E. coli* cells were separated by centrifugation at 6000 *g* for 10 min, washed with triple 10 mM sodium phosphate buffer (pH 7.4), and diluted with the same buffer to approximately 10^6 CFU/ml. Orange pigment was then added to the bacteria suspension to final concentrations of 10 mg/ml. The samples were mixed and incubated with shaking at 130 rpm at 37°C, and the conductivity was measured at 1, 2, 4, 6, 8, and 9 hours.

Measurement of interaction between orange pigment and the phospholipid. The interaction between orange pigment and the phospholipid was measured

following the previous method (KAJIYA *et al.* 2001) with some modifications. Phosphatidylcholine from egg yolk (egg PC) was obtained from Beilian Fine Chemicals (Tianjin, China). Egg PC was dissolved in a small amount of chloroform. The solvent was evaporated off in a round-bottomed flask with a rotary evaporator. The thin film of egg PC on the flask was dried with a vacuum pump. Then aliquots of 10 ml of aqueous glucose solution (300 mM) were poured into the flask, and the mixture was sonicated in an ultrasonicator (B5500S-MT; Branson, Binengxin ultrasound Ltd. Co., Shanghai, China) for 10 min to change the multilamellar vesicles to small unilamellar vesicles. The liposomal solution was diluted 10-fold with phosphate buffer solution (10 mM, pH 7.4). The final concentration of egg PC in the liposomal solution was adjusted to 1 mg/ml. Different amounts of orange pigment in ethanol solution were added to the liposomal solution to final concentrations of 0, 5, 10, 15, 20, 25, and 30 µg/ml orange pigment containing 10% ethanol. The amount of orange pigment in the liposomal solution was measured by spectrophotometry at 470 nm and calculated from a calibration curve obtained with the standard of rubropunctatin (98.7% purity) and monascorubrin (99.2% purity), which were purchased from Reseachem GmbH (Burgdorf, Switzerland). The amount of orange pigment incorporated into egg PC was calculated by subtracting the amount of orange pigment in the liposomal solution from the total amount of orange pigment added. The ratio (%) of orange pigment incorporated into the lipid bilayers was calculated as follows:

$$\text{Ratio incorporated (\%)} = \frac{\text{Amount incorporated}}{\text{Amount added}} \times 100$$

Statistical analysis. All experiments were carried out in triplicate, and average values with standard errors are reported. SPSS 17.0 software was used for all statistical analyses. The data were analysed by analysis of variance (ANOVA) and Duncan's post hoc analysis, graphs were produced using Microsoft Excel 2010, and regression analysis was used to de-

Table 1. Inhibition zone diameters of *E. coli* treated with varying concentrations of orange pigment

Treatments	Concentrations of orange pigment (mg/ml)					
	0	0.625	1.25	2.5	5.0	10.0
Inhibition zone diameters (mm)	5.0 ± 0.3 ^a	7.6 ± 2.1 ^a	9.5 ± 1.2 ^a	13.8 ± 1.7 ^b	17.2 ± 1.3 ^b	21.3 ± 0.6 ^b

Mean values followed by different letters represent significant difference at $P < 0.05$

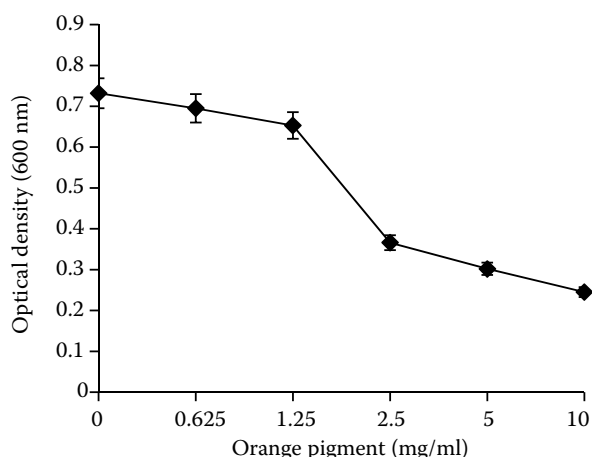


Figure 1. Effect of different orange pigment concentrations on the optical density (600 nm) of *E. coli*

termine the significant difference at 5% confidence intervals ($P < 0.05$).

RESULTS AND DISCUSSION

Antibacterial activity of orange pigment. The antibacterial activity of orange pigment against *E. coli* was evaluated by measuring the diameter of the inhibition zones. As shown in Table 1, the diameters of the inhibition zones against *E. coli* increased proportionally from 5 ± 0.3 mm to 21.3 ± 0.6 mm with an increase of the orange pigment concentration from 0 mg/ml to 10.0 mg/ml. The diameter with 10.0 mg/ml orange pigment was four times larger than in the control. As shown in Figure 1, OD values decreased with an increase of orange pigment. When the concentration of orange pigment was 2.5 mg/ml, the OD value of *E. coli* sharply decreased, which inhibited the visible growth of bacteria. Therefore MIC of or-

ange pigment against *E. coli* was 2.5 mg/ml. These results were consistent with the findings (UNGUREANU & FERDES 2010) that *Monascus* red rice powder exhibited antibacterial activity against *Bacillus*, *Pseudomonas*, and *Streptomyces* sp., suggesting that the pigments of *M. purpureus* had preservative activities. Inhibition zone diameters of *M. purpureus* from the strains N11S and X2P against *Bacillus* sp., *Streptococcus*, and *Pseudomonas* were measured and indicated that they had antibacterial activities (WONG & BAU 1977), which was consistent with the results of this study.

Effect of orange pigment on the morphology of *E. coli* cells. SEM was used to observe the morphological changes of *E. coli* cells. As seen in Figure 2, the bacterial cells of *E. coli* without orange pigment possessed regular and typical morphology, and the plump smooth surface. Moreover, these cells were uniform in size and distribution. In contrast to the control, bacterial cells of *E. coli* treated with orange pigment (Figure 2) had the thin, irregular, wrinkled, and coarse surface. Moreover, these cells were not uniform in size and distribution, but were aggregated and hollowed. These results demonstrated that the orange pigment treatment resulted in damage to *E. coli* cells. The SEM observation of *E. coli* cells treated with *Monascus* red pigments and the L-Phe derivative (KIM *et al.* 2006) showed that *Monascus* red pigments and the L-Phe derivative could induce the cells of *E. coli* to aggregate and to form large pellets, which was consistent with the present results.

Effect of orange pigment on the membrane permeability of *E. coli* cells. The electric conductivity change of *E. coli* suspension was used to evaluate the relationship between antibacterial activity and cell membrane permeability (Figure 3). The electric conductivities of *E. coli* suspensions treated

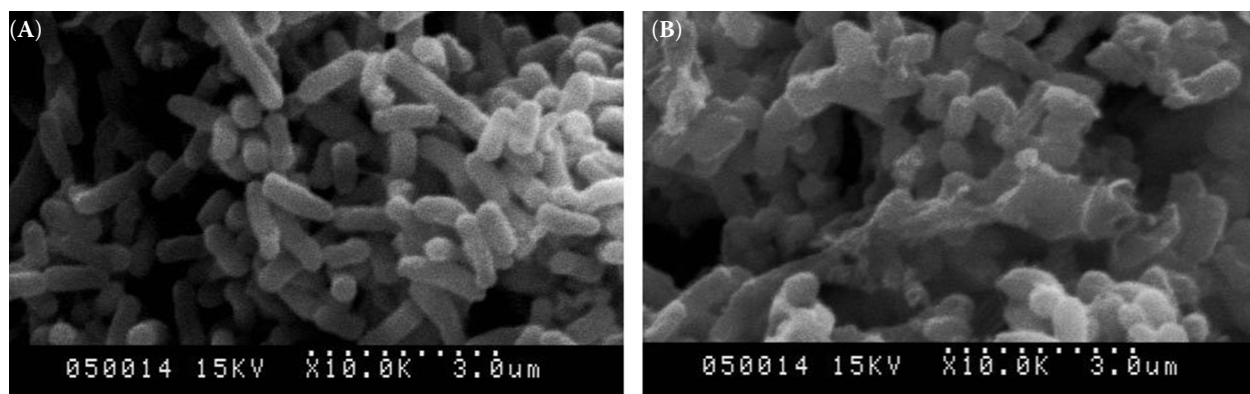


Figure 2. SEM photomicrographs of *E. coli* cells without orange pigment (A) and with 10 mg/ml of orange pigment (B)

doi: 10.17221/430/2015-CJFS

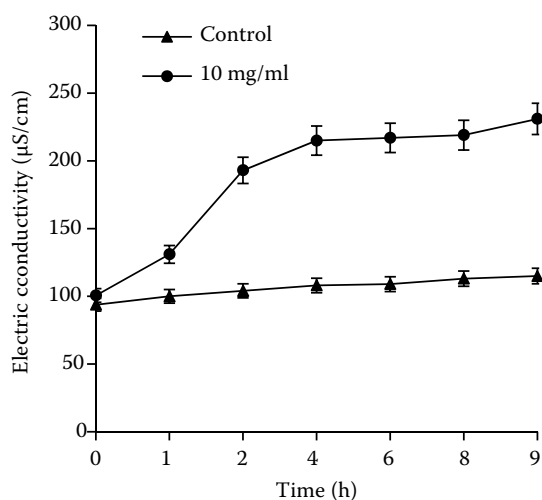


Figure 3. Effect of orange pigment on the electric conductivity of *E. coli* cell suspension

with orange pigment increased significantly ($P < 0.05$) from 100.6 to 231.0 $\mu\text{S}/\text{cm}$ with treatment time (0–9 h). The increase in the electric conductivity of the cell suspensions suggested that the cytoplasmic membranes were disrupted, which caused cellular leakage (DAYAN *et al.* 1999; GALINDO *et al.* 1999). The disruption of bacterial membrane was probably caused by the interaction between orange pigment and the phospholipid of cytoplasmic membranes. The electric conductivity of a solution of *E. coli* with 5% oleoyl-chitosan microspheres caused the permeability of *E. coli* membrane to increase to 96% compared with control (KONG *et al.* 2008), which was similar to the results in this study. The antibacterial activities of metallic oxide (ZnO, MgO, and CaO) powders against *Staphylococcus aureus* and *E. coli* were quantitatively evaluated by measuring a change

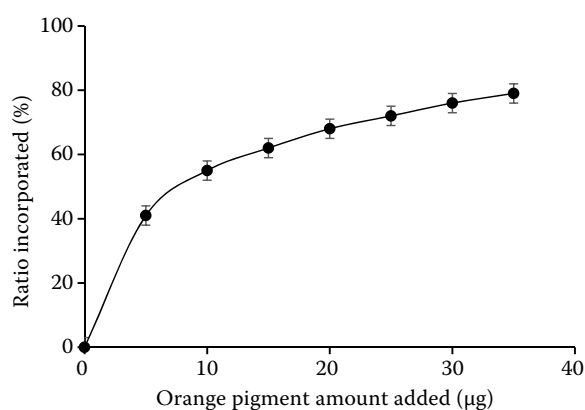


Figure 4. Effect of orange pigment amount on the ratio incorporated into liposomes

in the electric conductivity of the growth medium caused by bacterial metabolism (SAWAI 2003).

Effect of orange pigment amount on the ratio incorporated into liposomes. The effect of orange pigment amount on the ratio incorporated into liposomes is shown in Figure 4. The ratio incorporated into liposomes increased from 0% to 79.0% with an increasing amount of orange pigment added from 0 μg to 35 μg , indicating that orange pigment had a good affinity to liposomes with the dependence on the amount of orange pigment. This result indicated that the interaction of orange pigment with the phospholipid of cytoplasmic membranes resulted in the disruption of bacterial membrane. KAJIYA *et al.* (2004) found that the catechin derivatives had a very strong affinity to the membrane, and injured against the membrane by the liposome model.

In conclusion, orange pigment had antibacterial activity against *E. coli*. The antibacterial action of orange pigment was by the interaction between orange pigment and the phospholipid, thereby resulting in the disruption and permeation of bacterial membrane. These results demonstrated a possibility of using orange pigment as a natural preservative.

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doi: 10.17221/430/2015-CJFS

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Received: 2015–09–02

Accepted after corrections: 2016–05–23

Published online: 2016–06–17

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