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Antibacterial Activity of *Monascus*-fermented Sorghum Extracts against *Staphylococcus aureus* and *Escherichia coli*

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Abstract

This research was aimed to investigate the antibacterial activity of *Monascus*-fermented sorghum extract (MFSE). Three different solvents were used for extraction: ethyl acetate, ethanol, and water. Antibacterial activity was assessed by determining minimum inhibitory concentration (MIC). Broth microdilution method was used to determine the MIC of the extract against *Staphylococcus aureus* and *Escherichia coli*. Additionally, moisture content, color, and biomass of *Monascus*-fermented sorghum (MFS) were analyzed, along with the pigment and total phenolic content of MFSE. Antibacterial activity was observed in MFSE extracted with all three solvents, displaying MIC values of 0.018, 0.216, and 0.794 mg/L against *S. aureus* and 0.996, 1.205, and 1.138 mg/L against *E. coli* for ethyl acetate, ethanol, and water extract, respectively. The MFSE extracted with ethyl acetate exhibited lowest MIC, indicating highest antibacterial activity.

Keywords: *Monascus*, sorghum, extract, antibacterial, MIC.

1. Introduction

In recent years, there has been a growing preference for the development and utilization of natural compounds over synthetic ones (Gökmen et al., 2021). *Monascus purpureus* is one of the few types of edible fungi. Through solid-state fermentation, *M. purpureus* produces pigments and compounds known for their abundant bioactivity, such as antioxidants, anti-inflammation, anticancer, antidiabetic, antiobesity, and antimicrobial (Srianta et al., 2017; Choe et al., 2020; Hong et al., 2005; Hsu and Pan, 2012; Kim et al., 2007; Lee et al., 2011; Ding et al., 2022; Kim et al., 2010; Kim et al., 2006; Feng et al., 2019; Gökmen et al., 2021). Commonly, rice is used to produce red-yeast rice (angkak), which has been used as natural food colorant, preservative, food supplement and traditional medicine in Asia. However, any other cereal grains containing sufficient fermentable carbohydrates for *M. purpureus* can also be considered as suitable substrates.

Sorghum (*Sorghum bicolor* (L.) Moench) is the fifth most important cereal globally, following maize, rice, wheat, and barley. It requires less water and shows higher resilience to significant shifts in climate compared to other cereals (Balakrishna et al., 2019). In spite of that, sorghum is still underutilized. Sorghum has a composition similar to multiple cereal grains. For every 100 grams, sorghum, rice, and maize contain carbohydrates at 73g, 79g, and 72g respectively, protein at 11g, 7g, and 9g, fat at 3.3g, 0.7g, and 4.5g, fiber at 2.3g, 1.0g, and 2.7g, calcium at 28mg, 6mg, and 9mg, phosphorus at 287mg, 147mg, and 380mg, and iron at 4.4mg, 0.8mg, and 4.6mg (Sirappa, 2003). Srianta and Harijono (2015) had successfully fermented sorghum using *M. purpureus*. The fermentation process yielded yellow, orange and red pigments at concentration of 61.00, 29.10, 35.60 AU/g respectively, for ethanol extract and 16.30, 12.40, 13.30 AU/g for water extract.

Monascus pigments have been reported to possess antibacterial properties (Kim et al., 2006; Feng et al., 2019; Gökmen et al., 2021). *M. purpureus* produces 6 pigments, classified into three groups: orange pigments [monascorubrin ($C_{23}H_{30}O_5$) and rubropunctanin ($C_{21}H_{22}O_5$)], yellow pigments [ankaflavin ($C_{23}H_{30}O_5$) and monascin ($C_{21}H_{26}O_5$)], and red pigments [monascorubramin ($C_{23}H_{27}NO_4$) and rubropunctamine ($C_{21}H_{23}NO_4$)] (Feng et al., 2012). Antibacterial activity was observed on *Monascus* red pigment (Gökmen et al., 2021), orange pigment (Feng et al., 2019), and amino acid derivatives of *Monascus* pigment (Kim et al., 2006). Antibacterial activity was investigated against Gram-positive bacteria *Staphylococcus aureus* and Gram-negative bacteria *Escherichia coli*. *S. aureus* and *E. coli* were pathogens causing various human infection and foodborne diseases (Tong et al., 2015; Braz et al., 2020; Bintsis, 2017)

This research was aimed to investigate the antibacterial activity of MFSE against *Staphylococcus aureus* and *Escherichia coli*. It is the first to examine antibacterial activity of *Monascus*-fermented product that utilizes sorghum as substrate.

2. Materials and methods

2.1 Microorganism, substrate, and chemical

M. purpureus M9 culture was used for solid-state fermentation of sorghum. It was isolated from commercial *Monascus*-fermented rice in Surabaya, Indonesia and identified as *M. purpureus* M9 (NCBI Accesion Number: HM188425.1). *Staphylococcus aureus* ATCC 25920 and *Escherichia coli* ATCC 25927 were used for antibacterial assay. The bacteria were suspended in DMSO 20%. The sorghum used was commercial dehulled sorghum. It was vaccum packed until used. All chemical and media used in the analysis was analytical grade.

2.2 Starter culture preparation

M. purpureus M9 was monthly cultured on potato dextrose agar (PDA) slant. Starter culture was prepared with inoculating 8 loops of the culture scrabbed from the PDA slant, then incubated at 30^{o}C for 7 days. Before use, the culture was suspended in 10 mL of sterile distilled water for 1 hour and homogenized with a vortex.

2.3 Sorghum solid-state fermentation

The fermentation process was carried out according to Srianta et al. (2016) with modification. Dehulled sorghum was washed and steamed at 90^{o}C for 60 minutes. Fifty g of steamed sorghum was weighed and put inside an Erlenmeyer flask, then sterilized at 121^{o}C for 20 minutes. Solid state fermentation was carried out by inoculating 5 mL of *M. purpureus* M9 starter culture containing 5 x

10^5 spores/mL into each flask containing sterilized substrate. It was then incubated at 30°C for 14 days with daily shaking of the flask to ensure thorough fermentation. The fermented material was then dried at 45°C for 24 hours, grounded into powder, and sieved (size 45 mesh). The MFS powder was analyzed for moisture content, color and biomass.

2.4 Moisture content, color and biomass analysis of MFS

Moisture content of MFS was analyzed using oven drying method (AOAC, 2005). The sample underwent drying for a specific duration at a constant temperature. The loss in weight due to moisture evaporation was then used to calculate the moisture content of the sample. The color of MFS was measured using color reader (Konica Minolta, 2015). The MFS was packed inside a transparent plastic and measured for L*, a*, b*, C, and °h value. These measurements were repeated in triplicate.

Biomass analysis was conducted according to Srianta et al. (2016). The fungal biomass was estimated by determining the amount of N-acetyl glucosamine released through the acid hydrolysis of chitin, a component within the cell wall of the mycelia (Babitha et al., 2006). Chitin hydrolysis was carried out by using 10 M HCl and autoclaving at 130°C for 2 h. The hydrolysate was neutralized to pH 7.0, mixed with acetyl acetone reagent and followed by Ehrlich reagent. Optical density was measured at 530 nm against the reagent blank. N-acetyl glucosamine (Sigma Aldrich Co. LLC) was used as a standard.

2.5 MFS Extraction

The MFS was extracted with three different solvents: ethyl acetate, ethanol, and water. One gram of the powder was weighed and extracted with ethyl acetate/ethanol/water with ratios of 1:50 (w/v) (one gram in 50 mL solvent) with Soxhlet apparatus at 80°C for 2 hours. The extract obtained was evaporated at 200 mbar, 50 rpm for 30 minutes using a rotary evaporator, producing a concentrated extract. The MFSE was analyzed for antibacterial activity assay, pigment and total phenolic contents.

2.6 Antibacterial activity assay

Broth microdilution test was carried out according to Klančnik et al. (2010). Fifty µL of *S. aureus* and *E. coli* suspension in Müeller Hinton Broth (MHB) medium was added to the wells of a sterile 96-well microtitre plate containing 50 µL of two-fold serially diluted MFSE or amoxicillin. The final volume in each well was 100 µL. Control wells were prepared with culture medium, bacterial suspension only, MFSE only and DMSO 20% in amounts corresponding to the highest quantity present. The contents of each well were mixed on a microplate shaker at 900 rpm for 1 minute prior to 24-hour incubation. The MIC was the lowest concentration where no viability was observed after 24 hours on the basis of metabolic activity (Mourey and Canillac, 2002). Viability could be indicated by the presence of respiratory and ATP activity. To indicate respiratory activity, the presence of colorr was determined after adding 10 µL/well of TTC (2,3,5-triphenyltetrazoliumchloride) dissolved in water (TTC 20 mg/mL). The microplate was then incubated under appropriate cultivation conditions for 30 minutes in the dark (Ellof, 1998). To determine the ATP activity, the bioluminescence signal was measured by a microplate reader after adding 100 µL/well of BacTiter-Glo™ reagent and after 5 min incubation in the dark (Klančnik et al., 2009). Positive controls were wells with a bacterial suspension in an appropriate growth medium and a bacterial suspension in an appropriate growth medium with DMSO 20% in amounts corresponding to the highest quantity present in the broth microdilution assay. Negative controls were wells with growth medium and MFSE or amoxicilin. All measurements of MIC values were repeated in triplicate.

2.7 Pigment content analysis

Pigment content of MFSE analysis was carried out according to Srianta et al. (2016). The MFSE was dissolved in respective solvent and the absorbance was measured with UV-vis spectrophotometer at λ 400 nm, 470 nm, and 500 nm for yellow, orange, and red pigment, respectively. The pigment content was express as absorbance (nm) at the wavelength per gram of dry matter (AU/g).

2.8 Total phenolic content

The total phenolic content of MFSE was analyzed according to Srianta et al. (2014). Folin-Ciocalteu was used as a reagent and Gallic acid was used as a standard. A total of 0.1 mL of extract was put into a 10 mL volumetric flask and 0.5 mL of Folin-Ciocalteu reagent was added. The samples were left at room temperature for 5 minutes then added with 1.5 mL 20% (w/v) Na_2CO_3. The mixture was added with distilled water until the volume reached 10 mL. After 30 minutes at room temperature, absorbance was measured at a wavelength of 765 nm versus blank using a spectrophotometer (Shimadzu UV 1800, Japan). Total phenol content was expressed in mg GAE/g.

3. Results and discussion/Results

3.1 Moisture content, color and biomass content of MFS

Table 1. Moisture content, color and biomass content of MFS

Moisture content (%)	Color					Biomass (mg/g)
	L* value	a* value	b*value	C	°h	
8.72	46.5	23.6	13.8	27.4	3.3	825.786

The MFS powder had a moisture content of 8.72% (Table 1.). Moisture content of food powder below 10% has a high stability for storage (Zambrano et al., 2019). The lightness (L*), redness (a*), yellowness (b*), chroma (C), and hue (°h) values of the MFS powder presented in Table 1. The positive a* and b* values of all the fermented-products reflected that the powder color is combination of red and yellow. Hue indicate a red color and chroma value indicate a dull-brownish red color. L* value below 50 indicate low lightness, resulting in a dark red color.

Fungal biomass, branched fibrous mycelium of *Monascus*, was form during the fermentation. Generally, biomass comprise of protein, lipids, polysaccharides, chitin (or chitosan), and various inorganic salts in different proportions (Isaza-Pérez et al., 2020). The biomass yield of MFS was recorded at 825.786 mg/g or 0.825 g/mL. It was higher than the biomass obtained from *M. purpureus* fermentation on potato waste, which ranged from 0.046 to 0.262 g/mL (inoculum concentration 7×10^5 spores/mL) (Abdel-Raheam et al., 2022). High biomass implies that sorghum was a suitable substrate for *M. purpureus* fermentation. Additionally, the high biomass could have resulted from the assistance of the steaming process (90°C for 60 minutes), which helped soften the sorghum and facilitated easier utilization for the growth of *M. purpureus* (Zhao et al., 2018).

3.2 Antibacterial activity

Table 2. MIC of amoxicillin and MFSE against *S. aureus* and *E. coli*

Bacteria	Amoxicillin (µg/ml)	Ethyl acetate extract (mg/ml)	Ethanol extract (mg/ml)	Water extract (mg/ml)
Staphylococcus aureus ATCC 25920	3.125	3.125	12.5	100
Escherichia coli ATCC 25927	12.5	6.25	50	200

MIC value is defined as the lowest concentration of the assayed antimicrobial agent that inhibits the visible growth of the microorganism tested (Balouiri et al., 2016). Low MIC value indicates a high antibacterial activity. The extraction using ethyl acetate produced MFSE with the lowest MIC, indicating that ethyl acetate-extracted MFSE exhibited the highest antibacterial activity against both *S. aureus* and *E. coli*. Meanwhile, ethanol and water-extracted MFSE showed lower antibacterial activity, with water-extracted displaying the lowest. Most of *Monascus* pigments are slightly polar, making them more soluble in organic solvents like ethanol than in water (Carvalho et al., 2007; Qian and Wu, 2010; Bai et al., 2022). As a result, the amount of *Monascus* pigments that are extracted by ethyl acetate and ethanol is larger (Table 3.). This larger quantity of extracted pigments is responsible for the observed antibacterial activity, thus making ethyl acetate and ethanol-extracted MFSE to have lower MIC than water-extracted MFSE.

In addition to *Monascus* pigments, *Monascus*-fermented products are known to contain significant amounts of phenolic compounds (Srianta et al., 2013; Razak et al., 2015). Ethanol-extracted MFSE contain the highest total phenolic content (Table 3.). As per Haminiuk et al. (2014), phenolic compounds also exhibit higher solubility in organic solvents that are less polar than water. Razak et al. (2015) found that phenolic acid present in *Monascus*-fermented rice bran extract are ρ-coumaric, ferulic, sinapic, vanillic, caffeic, and syringic acid. All of those phenolic acid exhibited antimicrobial properties (Liu et al., 2020). Amoxicillin, a common antibiotic medication against Gram-positive and Gram-negative bacteria, was used as a reference. The MIC values of all three MFSEs remain higher than amoxicillin which exhibited a MIC value of 3.125 µg/ml against *S. aureus* and 12.5 µg/ml against *E. coli***.**

Table 3. Pigment and total phenolic contents of MFSE

Solvent	Pigment content (AU/g)			Total phenolic content (mg/mL)
	Yellow	Orange	Red	
Ethyl acetate	177.3378	64.7206	52.4588	1.6974
Ethanol	153.8563	59.0424	66.1631	9.8037
Water	47.4565	24.5389	20.1564	2.3995

Several studies have explored the antibacterial properties of red, orange, and yellow *Monascus* pigment. Gökmen et al. (2021) discovered that *Monascus* red pigments had a MIC value of 128 mg/mL against *S. aureus* and >128 mg/mL against *E. coli*. They observed that the effect was stronger compared to using a commercial red pigment which had a MIC value of 256 mg/mL against *S. aureus* and >256 mg/mL against *E. coli*. Feng et al. (2019) that found orange pigments demonstrated antibacterial activity against *S. aureus*, displaying inhibition zone diameters ranging from 5-34 mm for orange pigment concentration ranging from 0-10 mg/mL. Kim et al. (2006) discovered that amino acid derivatives of *Monascus* pigment

showed a high antimicrobial activity. Derivatives containing L-Phe, D-Phe, L-Asp, D-Asp, L-Tyr, D-Tyr, L-Cys, and Nisin exhibited high activities against *S. aureus* (Gram-positive bacteria) with MIC values ranging from 8-16 µg/mL, while the control red pigment showed a MIC value of 64 µg/mL. For *E. coli* (Gram-negative bacteria), the same derivatives exhibited lower activities with MIC values ranging from 8-64 µg/mL, and the control red pigment with MIC value of >128 µg/mL. Kim and Ku (2018) reviewed several studies regarding the antimicrobial effects of *Monascus* pigments and conclude that Gram-positive bacteria were more prone to inhibition compared to Gram-negative bacteria.

Some studies suggested that the mechanism might involve interaction of *Monascus* pigment with enzymes in the germinated spores and vegetative cells, restricting the use of iron and affect cell membrane permeability. This could result in reduced transportation of nutrients, oxygen and metabolites (Kim et al. 2006; Xu, 2011). Feng et al. (2019) also observed that orange pigment cause *S. aureus* cells to wrinkle, damaging the membrane and causing protein leakage of bacteria cells. Meanwhile, amino acid derivatives of *Monascus* pigment depends on the hydrophobicity of the pigment and the amount of pigment adsorbed to the cell surface. The more hydrophobic, the higher the adsorption, which leads to limitation of oxygen uptake (Kim et al., 2006).

4. Conclusion

Ethyl acetate, ethanol, and water-extracted MFSE demonstrated antibacterial activity against *S. aureus* and *E. coli*. Ethyl acetate exhibited the highest activity, followed by ethanol and water extracts. All of the MFSE exhibited stronger activity on Gram-positive bacteria.

Conflict of interest - Disclose any potential conflict of interest appropriately.

The authors declare no conflict of interest.

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Tables and Figures – 1 PAGE 1 TABLE/FIGURE. PLACE ALL TABLES AND FIGURES AT THE END OF THE MANUSCRIPT BODY AFTER THE REFERENCES. ARRANGE THE TABLES AND FIGURES ACCORDING TO THEIR APPEARANCE IN THE TEXT.

Table 1. Moisture content, color and biomass content of MFS

Moisture content (%)	Color					Biomass (mg/g)
	L* value	a* value	b*value	C	°h	
8.72	46.5	23.6	13.8	27.4	3.3	825.786

Table 2. MIC of amoxicillin and MFSE against *S. aureus* and *E. coli*

Bacteria	Amoxicillin (µg/ml)	Ethyl acetate extract (mg/ml)	Ethanol extract (mg/ml)	Water extract (mg/ml)
Staphylococcus aureus ATCC 25920	3.125	3.125	12.5	100
Escherichia coli ATCC 25927	12.5	6.25	50	200

329 Table 3. Pigment and total phenolic contents of MFSE

Solvent	Pigment content (AU/g)			Total phenolic content (mg/mL)
	Yellow	Orange	Red	
Ethyl acetate	177.3378	64.7206	52.4588	1.6974
Ethanol	153.8563	59.0424	66.1631	9.8037
Water	47.4565	24.5389	20.1564	2.3995

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December 11, 2023

Professor Dr. Son Radu
Chief Editor
Food Research
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Dear Professor Dr. Son Radu,

I am writing to submit our manuscript titled "Antibacterial Activity of *Monascus*-fermented Sorghum Extracts against *Staphylococcus aureus* and *Escherichia coli*" for consideration in Food Research journal. We believe our research aligns with the scope and interests of your esteemed publication.

This manuscript represents original work and has not been previously published nor is it under consideration elsewhere. Our study presents significant findings of the antibacterial activity exhibited by the extract of *Monascus*-fermented sorghum.

In the recent few years, there has been a growing preference of the utilization of natural compounds over synthetic ones. The demonstrated strong antibacterial activity of the *Monascus*-fermented sorghum extract could cater to this growing preference, offering a compelling avenue for researchers seeking novel antimicrobial solutions.

Our work is the first to examine the antibacterial properties of a *Monascus*-fermented product utilizing sorghum seed as its substrate. Additionally, pigment and total phenolic content of the extracts were also investigated. This exploration builds upon previous research in the field and contributes significantly to the investigation of natural antibacterial compounds derived from the product of the fermentation.

We believe that our manuscript will be of interest to the journal readers given its novel insights and its contribution as a continuum of prior investigations.

We suggest Mrs. Netty Kusumawati (Microbiology, netty@ukwms.ac.id), Mrs. Martha Ervina (Phytochemistry, marthaervina@gmail.com), and Mrs. Sri Satya Antarlina (Food fermentation, ssantarlina@gmail.com) as reviewers for the manuscript.

Lastly, we have no conflict of interest to disclose. Please address all correspondence regarding this manuscript to me (srianta@ukwms.ac.id). Thank you for considering our submission.

Sincerely,

Ignatius Srianta

Author

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Widya Mandala Catholic University Surabaya, Jalan Dinoyo 42-44 Surabaya, Indonesia 60265

2

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Authors	Srianta, I., Sutedja, A.M. and Nugerahani, I.
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Antibacterial activity of *Monascus*-fermented sorghum extracts against *Staphylococcus aureus* and *Escherichia coli*

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Abstract

This research was aimed to investigate the antibacterial activity of *Monascus*-fermented sorghum extract (MFSE). Three different solvents were used for extraction: ethyl acetate, ethanol, and water. Antibacterial activity was assessed by determining minimum inhibitory concentration (MIC). Broth microdilution method was used to determine the MIC of the extract against *Staphylococcus aureus* and *Escherichia coli*. Additionally, moisture content, color, and biomass of *Monascus*-fermented sorghum (MFS) were analyzed, along with the pigment and total phenolic content of MFSE. Antibacterial activity was observed in MFSE extracted with all three solvents, displaying MIC values of 0.018, 0.216, and 0.794 mg/L against *S. aureus* and 0.996, 1.205, and 1.138 mg/L against *E. coli* for ethyl acetate, ethanol, and water extract, respectively. The MFSE extracted with ethyl acetate exhibited lowest MIC, indicating highest antibacterial activity.

Keywords: *Monascus*, sorghum, extract, antibacterial, MIC.

1. Introduction

In recent years, there has been a growing preference for the development and utilization of natural compounds over synthetic ones (Gökmen et al., 2021). *Monascus purpureus* is one of the few types of edible fungi. Through solid-state fermentation, *M. purpureus* produces pigments and compounds known for their abundant bioactivity, such as antioxidants, anti-inflammation, anticancer, antidiabetic, antiobesity, and antimicrobial (Hong et al., 2005; Kim et al., 2006; Kim et al., 2007; Kim et al., 2010; Lee et al., 2011; Hsu and Pan, 2012; Srianta et al., 2017; Feng et al., 2019; Choe et al., 2020; Gökmen et al., 2021; Ding et al., 2022). Commonly, rice is used to produce red-yeast rice (angkak), which has been used as natural food colorant, preservative, food supplement and traditional medicine in Asia. However, any other cereal grains containing sufficient fermentable carbohydrates for *M. purpureus* can also be considered as suitable substrates.

Sorghum (*Sorghum bicolor* (L.) Moench) is the fifth most important cereal globally, following maize, rice, wheat, and barley. It requires less water and shows higher resilience to significant shifts in climate compared to other cereals (Balakrishna et al., 2019). In spite of that, sorghum is still underutilized. Sorghum has a composition similar to multiple cereal grains. For every 100 grams, sorghum, rice, and maize contain carbohydrates at 73g, 79g, and 72g respectively, protein at 11g, 7g, and 9g, fat at 3.3g, 0.7g, and 4.5g, fiber at 2.3g, 1.0g, and 2.7g, calcium at 28mg, 6mg, and 9mg, phosphorus at 287mg, 147mg, and 380mg, and iron at 4.4mg, 0.8mg, and 4.6mg (Sirappa, 2003). Srianta and Harijono (2015) had successfully fermented sorghum using *M. purpureus*. The fermentation process yielded yellow, orange and red pigments at concentration of 61.00, 29.10, 35.60 AU/g respectively, for ethanol extract and 16.30, 12.40, 13.30 AU/g for water extract.

Monascus pigments have been reported to possess antibacterial properties (Kim et al., 2006; Feng et al., 2019; Gökmen et al., 2021). *M. purpureus* produces 6 pigments, classified into three groups: orange pigments [monascorubrin ($C_{23}H_{30}O_5$) and rubropunctanin ($C_{21}H_{22}O_5$)], yellow pigments [ankaflavin ($C_{23}H_{30}O_5$) and monascin ($C_{21}H_{26}O_5$)], and red pigments [monascorubramin ($C_{23}H_{27}NO_4$) and rubropunctamine ($C_{21}H_{23}NO_4$)] (Feng et al., 2012). Antibacterial activity was observed on *Monascus* red pigment (Gökmen et al., 2021), orange pigment (Feng et al., 2019), and amino acid derivatives of *Monascus* pigment (Kim et al., 2006). Antibacterial activity was investigated against Gram-positive bacteria *Staphylococcus aureus* and Gram-negative bacteria *Escherichia coli*. *S. aureus* and *E. coli* were pathogens causing various human infection and foodborne diseases (Tong et al., 2015; Bintsis, 2017; Braz et al., 2020)

This research was aimed to investigate the antibacterial activity of MFSE against *Staphylococcus aureus* and *Escherichia coli*. It is the first to examine antibacterial activity of *Monascus*-fermented product that utilizes sorghum as substrate.

2. Materials and methods

2.1 Microorganism, substrate, and chemical

M. purpureus M9 culture was used for solid-state fermentation of sorghum. It was isolated from commercial *Monascus*-fermented rice in Surabaya, Indonesia and identified as *M. purpureus* M9 (NCBI Accesion Number: HM188425.1). *Staphylococcus aureus* ATCC 25920 and *Escherichia coli* ATCC 25927 were used for antibacterial assay. The bacteria were suspended in DMSO 20%. The sorghum used was commercial dehulled sorghum. It was vacuum packed until used. All chemical and media used in the analysis was analytical grade.

2.2 Starter culture preparation

M. purpureus M9 was monthly cultured on potato dextrose agar (PDA) slant. Starter culture was prepared with inoculating 8 loops of the culture scrabbed from the PDA slant, then incubated at 30^{o}C for 7 days. Before use, the culture was suspended in 10 mL of sterile distilled water for 1 hour and homogenized with a vortex.

2.3 Sorghum solid-state fermentation

The fermentation process was carried out according to Srianta et al. (2016) with modification. Dehulled sorghum was washed and steamed at 90^{o}C for 60 minutes. Fifty g of steamed sorghum was weighed and put inside an Erlenmeyer flask, then sterilized at 121^{o}C for 20 minutes. Solid state fermentation was carried out by inoculating 5 mL of *M. purpureus* M9 starter culture containing 5 x

10^5 spores/mL into each flask containing sterilized substrate. It was then incubated at 30°C for 14 days with daily shaking of the flask to ensure thorough fermentation. The fermented material was then dried at 45°C for 24 hours, grounded into powder, and sieved (size 45 mesh). The MFS powder was analyzed for moisture content, color and biomass.

2.4 Moisture content, color and biomass analysis of MFS

Moisture content of MFS was analyzed using oven drying method (AOAC, 2005). The sample underwent drying for a specific duration at a constant temperature. The loss in weight due to moisture evaporation was then used to calculate the moisture content of the sample. The color of MFS was measured using color reader (Konica Minolta, 2015). The MFS was packed inside a transparent plastic and measured for L*, a*, b*, C, and °h value. These measurements were repeated in triplicate.

Biomass analysis was conducted according to Srianta et al. (2016). The fungal biomass was estimated by determining the amount of N-acetyl glucosamine released through the acid hydrolysis of chitin, a component within the cell wall of the mycelia (Babitha et al., 2006). Chitin hydrolysis was carried out by using 10 M HCl and autoclaving at 130°C for 2 hours. The hydrolysate was neutralized to pH 7.0, mixed with acetyl acetone reagent and followed by Ehrlich reagent. Optical density was measured at 530 nm against the reagent blank. N-acetyl glucosamine (Sigma Aldrich Co. LLC) was used as a standard.

2.5 MFS extraction

The MFS was extracted with three different solvents: ethyl acetate, ethanol, and water. One gram of the powder was weighed and extracted with ethyl acetate/ethanol/water with ratios of 1:50 (w/v) (one gram in 50 mL solvent) with Soxhlet apparatus at 80°C for 2 hours. The extract obtained was evaporated at 200 mbar, 50 rpm for 30 minutes using a rotary evaporator, producing a concentrated extract. The MFSE was analyzed for antibacterial activity assay, pigment and total phenolic contents.

2.6 Antibacterial activity assay

Broth microdilution test was carried out according to Klančnik et al. (2010). Fifty µL of *S. aureus* and *E. coli* suspension in Müeller Hinton Broth (MHB) medium was added to the wells of a sterile 96-well microtitre plate containing 50 µL of two-fold serially diluted MFSE or amoxicillin. The final volume in each well was 100 µL. Control wells were prepared with culture medium, bacterial suspension only, MFSE only and DMSO 20% in amounts corresponding to the highest quantity present. The contents of each well were mixed on a microplate shaker at 900 rpm for 1 minute prior to 24-hour incubation. The MIC was the lowest concentration where no viability was observed after 24 hours on the basis of metabolic activity (Mourey and Canillac, 2002). Viability could be indicated by the presence of respiratory and ATP activity. To indicate respiratory activity, the presence of colorr was determined after adding 10 µL/well of TTC (2,3,5-triphenyltetrazoliumchloride) dissolved in water (TTC 20 mg/mL). The microplate was then incubated under appropriate cultivation conditions for 30 minutes in the dark (Ellof, 1998). To determine the ATP activity, the bioluminescence signal was measured by a microplate reader after adding 100 µL/well of BacTiter-Glo™ reagent and after 5 min incubation in the dark (Klančnik et al., 2009). Positive controls were wells with a bacterial suspension in an appropriate growth medium and a bacterial suspension in an appropriate growth medium with DMSO 20% in amounts corresponding to the highest quantity present in the broth microdilution assay. Negative controls were wells with growth medium and MFSE or amoxicilin. All measurements of MIC values were repeated in triplicate.

2.7 Pigment content analysis

Pigment content of MFSE analysis was carried out according to Srianta et al. (2016). The MFSE was dissolved in respective solvent and the absorbance was measured with UV-vis spectrophotometer at λ 400 nm, 470 nm, and 500 nm for yellow, orange, and red pigment, respectively. The pigment content was express as absorbance (nm) at the wavelength per gram of dry matter (AU/g).

2.8 Total phenolic content

The total phenolic content of MFSE was analyzed according to Srianta et al. (2014). Folin-Ciocalteu was used as a reagent and Gallic acid was used as a standard. A total of 0.1 mL of extract was put into a 10 mL volumetric flask and 0.5 mL of Folin-Ciocalteu reagent was added. The samples were left at room temperature for 5 minutes then added with 1.5 mL 20% (w/v) Na_2CO_3. The mixture was added with distilled water until the volume reached 10 mL. After 30 minutes at room temperature, absorbance was measured at a wavelength of 765 nm versus blank using a spectrophotometer (Shimadzu UV 1800, Japan). Total phenol content was expressed in mg GAE/g.

3. Results and discussion/Results

3.1 Moisture content, color and biomass content of MFS

The MFS powder had a moisture content of 8.72% (Table 1.). Moisture content of food powder below 10% has a high stability for storage (Zambrano et al., 2019). The lightness (L*), redness (a*), yellowness (b*), chroma (C), and hue (^{o}h) values of the MFS powder presented in Table 1. The positive a* and b* values of all the fermented-products reflected that the powder color is combination of red and yellow. Hue indicate a red color and chroma value indicate a dull-brownish red color. L* value below 50 indicate low lightness, resulting in a dark red color.

Fungal biomass, branched fibrous mycelium of *Monascus*, was form during the fermentation. Generally, biomass comprise of protein, lipids, polysaccharides, chitin (or chitosan), and various inorganic salts in different proportions (Isaza-Pérez et al., 2020). The biomass yield of MFS was recorded at 825.786 mg/g or 0.825 g/mL. It was higher than the biomass obtained from *M. purpureus* fermentation on potato waste, which ranged from 0.046 to 0.262 g/mL (inoculum concentration 7×10^5 spores/mL) (Abdel-Raheam et al., 2022). High biomass implies that sorghum was a suitable substrate for *M. purpureus* fermentation. Additionally, the high biomass could have resulted from the assistance of the steaming process (90°C for 60 minutes), which helped soften the sorghum and facilitated easier utilization for the growth of *M. purpureus* (Zhao et al., 2018).

3.2 Antibacterial activity

MIC value is defined as the lowest concentration of the assayed antimicrobial agent that inhibits the visible growth of the microorganism tested (Balouiri et al., 2016). Low MIC value indicates a high antibacterial activity. The extraction using ethyl acetate produced MFSE with the lowest MIC, indicating that ethyl acetate-extracted MFSE exhibited the highest antibacterial activity against both *S. aureus* and *E. coli*. Meanwhile, ethanol and water-extracted MFSE showed lower antibacterial activity, with water-extracted displaying the lowest. Most of *Monascus* pigments are slightly polar, making them more soluble in organic solvents like ethanol than in water (Carvalho et al., 2007; Qian and Wu, 2010; Bai et al., 2022). As a result, the amount of *Monascus* pigments that are extracted by ethyl acetate and ethanol is larger

(Table 3.). This larger quantity of extracted pigments is responsible for the observed antibacterial activity, thus making ethyl acetate and ethanol-extracted MFSE to have lower MIC than water-extracted MFSE.

In addition to *Monascus* pigments, *Monascus*-fermented products are known to contain significant amounts of phenolic compounds (Srianta et al., 2013; Razak et al., 2015). Ethanol-extracted MFSE contain the highest total phenolic content (Table 3.). As per Haminiuk et al. (2014), phenolic compounds also exhibit higher solubility in organic solvents that are less polar than water. Razak et al. (2015) found that phenolic acid present in *Monascus*-fermented rice bran extract are ρ-coumaric, ferulic, sinapic, vanillic, caffeic, and syringic acid. All of those phenolic acid exhibited antimicrobial properties (Liu et al., 2020). Amoxicillin, a common antibiotic medication against Gram-positive and Gram-negative bacteria, was used as a reference. The MIC values of all three MFSEs remain higher than amoxicillin which exhibited a MIC value of 3.125 µg/ml against *S. aureus* and 12.5 µg/ml against *E. coli***.**

Several studies have explored the antibacterial properties of red, orange, and yellow *Monascus* pigment. Gökmen et al. (2021) discovered that *Monascus* red pigments had a MIC value of 128 mg/mL against *S. aureus* and >128 mg/mL against *E. coli*. They observed that the effect was stronger compared to using a commercial red pigment which had a MIC value of 256 mg/mL against *S. aureus* and >256 mg/mL against *E. coli*. Feng et al. (2019) that found orange pigments demonstrated antibacterial activity against *S. aureus*, displaying inhibition zone diameters ranging from 5-34 mm for orange pigment concentration ranging from 0-10 mg/mL. Kim et al. (2006) discovered that amino acid derivatives of *Monascus* pigment showed a high antimicrobial activity. Derivatives containing L-Phe, D-Phe, L-Asp, D-Asp, L-Tyr, D-Tyr, L-Cys, and Nisin exhibited high activities against *S. aureus* (Gram-positive bacteria) with MIC values ranging from 8-16 µg/mL, while the control red pigment showed a MIC value of 64 µg/mL. For *E. coli* (Gram-negative bacteria), the same derivatives exhibited lower activities with MIC values ranging from 8-64 µg/mL, and the control red pigment with MIC value of >128 µg/mL. Kim and Ku (2018) reviewed several studies regarding the antimicrobial effects of *Monascus* pigments and conclude that Gram-positive bacteria were more prone to inhibition compared to Gram-negative bacteria.

Some studies suggested that the mechanism might involve interaction of *Monascus* pigment with enzymes in the germinated spores and vegetative cells, restricting the use of iron and affect cell membrane permeability. This could result in reduced transportation of nutrients, oxygen and metabolites (Kim et al., 2006; Xu, 2011). Feng et al. (2019) also observed that orange pigment cause *S. aureus* cells to wrinkle, damaging the membrane and causing protein leakage of bacteria cells. Meanwhile, amino acid derivatives of *Monascus* pigment depends on the hydrophobicity of the pigment and the amount of pigment adsorbed to the cell surface. The more hydrophobic, the higher the adsorption, which leads to limitation of oxygen uptake (Kim et al., 2006).

4. Conclusion

Ethyl acetate, ethanol, and water-extracted MFSE demonstrated antibacterial activity against *S. aureus* and *E. coli*. Ethyl acetate exhibited the highest activity, followed by ethanol and water extracts. All of the MFSE exhibited stronger activity on Gram-positive bacteria.

Conflict of interest - Disclose any potential conflict of interest appropriately.

The authors declare no conflict of interest.

Acknowledgements

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Tables and Figures – 1 PAGE 1 TABLE/FIGURE. PLACE ALL TABLES AND FIGURES AT THE END OF THE MANUSCRIPT BODY AFTER THE REFERENCES. ARRANGE THE TABLES AND FIGURES ACCORDING TO THEIR APPEARANCE IN THE TEXT.

Table 1. Moisture content, color and biomass content of MFS

Moisture content (%)	Color					Biomass (mg/g)
	L* value	a* value	b*value	C	°h	
8.72	46.5	23.6	13.8	27.4	3.3	825.786

Table 2. MIC of amoxicillin and MFSE against *S. aureus* and *E. coli*

Bacteria	Amoxicillin (µg/ml)	Ethyl acetate extract (mg/ml)	Ethanol extract (mg/ml)	Water extract (mg/ml)
Staphylococcus aureus ATCC 25920	3.125	3.125	12.5	100
Escherichia coli ATCC 25927	12.5	6.25	50	200

Table 3. Pigment and total phenolic contents of MFSE

Solvent	Pigment content (AU/g)			Total phenolic content (mg/mL)
	Yellow	Orange	Red	
Ethyl acetate	177.3378	64.7206	52.4588	1.6974
Ethanol	153.8563	59.0424	66.1631	9.8037
Water	47.4565	24.5389	20.1564	2.3995

December 11, 2023

Professor Dr. Son Radu
Chief Editor
Food Research
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Dear Professor Dr. Son Radu,

I am writing to submit our manuscript titled "Antibacterial Activity of *Monascus*-fermented Sorghum Extracts against *Staphylococcus aureus* and *Escherichia coli*" for consideration in Food Research journal. We believe our research aligns with the scope and interests of your esteemed publication.

This manuscript represents original work and has not been previously published nor is it under consideration elsewhere. Our study presents significant findings of the antibacterial activity exhibited by the extract of *Monascus*-fermented sorghum.

In the recent few years, there has been a growing preference of the utilization of natural compounds over synthetic ones. The demonstrated strong antibacterial activity of the *Monascus*-fermented sorghum extract could cater to this growing preference, offering a compelling avenue for researchers seeking novel antimicrobial solutions.

Our work is the first to examine the antibacterial properties of a *Monascus*-fermented product utilizing sorghum seed as its substrate. Additionally, pigment and total phenolic content of the extracts were also investigated. This exploration builds upon previous research in the field and contributes significantly to the investigation of natural antibacterial compounds derived from the product of the fermentation.

We believe that our manuscript will be of interest to the journal readers given its novel insights and its contribution as a continuum of prior investigations.

We suggest Mrs. Netty Kusumawati (Microbiology, netty@ukwms.ac.id), Mrs. Martha Ervina (Phytochemistry, marthaervina@gmail.com), and Mrs. Sri Satya Antarlina (Food fermentation, ssantarlina@gmail.com) as reviewers for the manuscript.

Lastly, we have no conflict of interest to disclose. Please address all correspondence regarding this manuscript to me (srianta@ukwms.ac.id). Thank you for considering our submission.

Sincerely,

Ignatius Srianta

Author

Department of Food Technology, Faculty of Agricultural Technology
Widya Mandala Catholic University Surabaya, Jalan Dinoyo 42-44 Surabaya, Indonesia 60265

4

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Antibacterial activity of *Monascus*-fermented sorghum extracts against *Staphylococcus aureus* and *Escherichia coli*

Abstract

This research was aimed to investigate the antibacterial activity of *Monascus*-fermented sorghum extract (MFSE). Three different solvents were used for extraction: ethyl acetate, ethanol, and water. Antibacterial activity was assessed by determining minimum inhibitory concentration (MIC). Broth microdilution method was used to determine the MIC of the extract against *Staphylococcus aureus* and *Escherichia coli*. Additionally, moisture content, color, and biomass of *Monascus*-fermented sorghum (MFS) were analyzed, along with the pigment and total phenolic content of MFSE. Antibacterial activity was observed in MFSE extracted with all three solvents, displaying MIC values of 0.018, 0.216, and 0.794 mg/L against *S. aureus* and 0.996, 1.205, and 1.138 mg/L against *E. coli* for ethyl acetate, ethanol, and water extract, respectively. The MFSE extracted with ethyl acetate exhibited lowest MIC, indicating highest antibacterial activity.

Keywords: *Monascus*, sorghum, extract, antibacterial, MIC.

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1. Introduction

In recent years, there has been a growing preference for the development and utilization of natural compounds over synthetic ones (Gökmen et al., 2021). *Monascus purpureus* is one of the few types of edible fungi. Through solid-state fermentation, *M. purpureus* produces pigments and compounds known for their abundant bioactivity, such as antioxidants, anti-inflammation, anticancer, antidiabetic, antiobesity, and antimicrobial (Hong et al., 2005; Kim et al., 2006; Kim et al., 2007; Kim et al., 2010; Lee et al., 2011; Hsu and Pan, 2012; Srianta et al., 2017; Feng et al., 2019; Choe et al., 2020; Gökmen et al., 2021; Ding et al., 2022). Commonly, rice is used to produce red-yeast rice (angkak), which has been used as natural food colorant, preservative, food supplement and traditional medicine in Asia. However, any other cereal grains containing sufficient fermentable carbohydrates for *M. purpureus* can also be considered as suitable substrates.

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Sorghum (*Sorghum bicolor* (L.) Moench) is the fifth most important cereal globally, following maize, rice, wheat, and barley. It requires less water and shows higher resilience to significant shifts in climate compared to other cereals (Balakrishna et al., 2019). In spite of that, sorghum is still underutilized. Sorghum has a composition similar to multiple cereal grains. For every 100 grams, sorghum, rice, and maize contain carbohydrates at 73g, 79g, and 72g respectively, protein at 11g, 7g, and 9g, fat at 3.3g, 0.7g, and 4.5g, fiber at 2.3g, 1.0g, and 2.7g, calcium at 28mg, 6mg, and 9mg, phosphorus at 287mg, 147mg, and 380mg, and iron at 4.4mg, 0.8mg, and 4.6mg (Sirappa, 2003). Srianta and Harijono (2015) had successfully fermented sorghum using *M. purpureus*. The fermentation process yielded yellow, orange and red pigments at concentration of 61.00, 29.10, 35.60 AU/g respectively, for ethanol extract and 16.30, 12.40, 13.30 AU/g for water extract.

Monascus pigments have been reported to possess antibacterial properties (Kim et al., 2006; Feng et al., 2019; Gökmen et al., 2021). *M. purpureus* produces 6 pigments, classified into three groups: orange

pigments [monascorubrin ($C_{23}H_{30}O_5$) and rubropunctanin ($C_{21}H_{22}O_5$)], yellow pigments [ankaflavin ($C_{23}H_{30}O_5$) and monascin ($C_{21}H_{26}O_5$)], and red pigments [monascorubramin ($C_{23}H_{27}NO_4$) and rubropunctamine ($C_{21}H_{23}NO_4$)] (Feng et al., 2012). Antibacterial activity was observed on *Monascus* red pigment (Gökmen et al., 2021), orange pigment (Feng et al., 2019), and amino acid derivatives of *Monascus* pigment (Kim et al., 2006). Antibacterial activity was investigated against Gram-positive bacteria *Staphylococcus aureus* and Gram-negative bacteria *Escherichia coli*. *S. aureus* and *E. coli* were pathogens causing various human infection and foodborne diseases (Tong et al., 2015; Bintsis, 2017; Braz et al., 2020)

This research was aimed to investigate the antibacterial activity of MFSE against *Staphylococcus aureus* and *Escherichia coli*. It is the first to examine antibacterial activity of *Monascus*-fermented product that utilizes sorghum as substrate.

2. Materials and methods

2.1 Microorganism, substrate, and chemical

M. purpureus M9 culture was used for solid-state fermentation of sorghum. It was isolated from commercial *Monascus*-fermented rice in Surabaya, Indonesia and identified as *M. purpureus* M9 (NCBI Accesion Number: HM188425.1). *Staphylococcus aureus* ATCC 25920 and *Escherichia coli* ATCC 25927 were used for antibacterial assay. The bacteria were suspended in DMSO 20%. The sorghum used was commercial dehulled sorghum. It was vacuum packed until used. All chemical and media used in the analysis was analytical grade.

2.2 Starter culture preparation

M. purpureus M9 was monthly cultured on potato dextrose agar (PDA) slant. Starter culture was prepared with inoculating 8 loops of the culture scrabbed from the PDA slant, then incubated at 30°C for 7 days. Before use, the culture was suspended in 10 mL of sterile distilled water for 1 hour and homogenized with a vortex.

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2.3 Sorghum solid-state fermentation

The fermentation process was carried out according to Srianta et al. (2016) with modification. Dehulled sorghum was washed and steamed at 90°C for 60 minutes. Fifty g of steamed sorghum was weighed and put inside an Erlenmeyer flask, then sterilized at 121°C for 20 minutes. Solid state fermentation was carried out by inoculating 5 mL of *M. purpureus* M9 starter culture containing 5 x 10^5 spores/mL into each flask containing sterilized substrate. It was then incubated at 30°C for 14 days with daily shaking of the flask to ensure thorough fermentation. The fermented material was then dried at 45°C for 24 hours, grounded into powder, and sieved (size 45 mesh). The MFS powder was analyzed for moisture content, color and biomass.

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Commented [Ma9]: Please explain the drying method?

2.4 Moisture content, color and biomass analysis of MFS

Moisture content of MFS was analyzed using oven drying method (AOAC, 2005). The sample underwent drying for a specific duration at a constant temperature. The loss in weight due to moisture evaporation was then used to calculate the moisture content of the sample. The color of MFS was measured using color reader (Konica Minolta, 2015). The MFS was packed inside a transparent plastic and measured for L*, a*, b*, C, and °h value. These measurements were repeated in triplicate.

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Biomass analysis was conducted according to Srianta et al. (2016). The fungal biomass was estimated by determining the amount of N-acetyl glucosamine released through the acid hydrolysis of chitin, a component within the cell wall of the mycelia (Babitha et al., 2006). Chitin hydrolysis was

carried out by using 10 M HCl and autoclaving at 130°C for 2 hours. The hydrolysate was neutralized to pH 7.0, mixed with acetyl acetone reagent and followed by Ehrlich reagent. Optical density was measured at 530 nm against the reagent blank. N-acetyl glucosamine (Sigma Aldrich Co. LLC) was used as a standard.

2.5 MFS extraction

The MFS was extracted with three different solvents: ethyl acetate, ethanol, and water. One gram of the powder was weighed and extracted with ethyl acetate/ethanol/water with ratios of 1:50 (w/v) (one gram in 50 mL solvent) with Soxhlet apparatus at 80°C for 2 hours. The extract obtained was evaporated at 200 mbar, 50 rpm for 30 minutes using a rotary evaporator, producing a concentrated extract. The MFSE was analyzed for antibacterial activity assay, pigment and total phenolic contents.

2.6 Antibacterial activity assay

Broth microdilution test was carried out according to Klančnik et al. (2010). Fifty µL of *S. aureus* and *E. coli* suspension in Müeller Hinton Broth (MHB) medium was added to the wells of a sterile 96-well microtitre plate containing 50 µL of two-fold serially diluted MFSE or amoxicillin. The final volume in each well was 100 µL. Control wells were prepared with culture medium, bacterial suspension only, MFSE only and DMSO 20% in amounts corresponding to the highest quantity present. The contents of each well were mixed on a microplate shaker at 900 rpm for 1 minute prior to 24-hour incubation. The MIC was the lowest concentration where no viability was observed after 24 hours on the basis of metabolic activity (Mourey and Canillac, 2002). Viability could be indicated by the presence of respiratory and ATP activity. To indicate respiratory activity, the presence of colorr was determined after adding 10 µL/well of TTC (2,3,5-triphenyltetrazoliumchloride) dissolved in water (TTC 20 mg/mL). The microplate was then incubated under appropriate cultivation conditions for 30 minutes in the dark (Ellof, 1998). To determine the ATP activity, the bioluminescence signal was measured by a microplate reader after adding 100 µL/well of BacTiter-Glo™ reagent and after 5 min incubation in the dark (Klančnik et al., 2009). Positive controls were wells with a bacterial suspension in an appropriate growth medium and a bacterial suspension in an appropriate growth medium with DMSO 20% in amounts corresponding to the highest quantity present in the broth microdilution assay. Negative controls were wells with growth medium and MFSE or amoxicilin. All measurements of MIC values were repeated in triplicate.

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2.7 Pigment content analysis

Pigment content of MFSE analysis was carried out according to Srianta et al. (2016). The MFSE was dissolved in respective solvent and the absorbance was measured with UV-vis spectrophotometer at λ 400 nm, 470 nm, and 500 nm for yellow, orange, and red pigment, respectively. The pigment content was express as absorbance (nm) at the wavelength per gram of dry matter (AU/g).

2.8 Total phenolic content

The total phenolic content of MFSE was analyzed according to Srianta et al. (2014). Folin-Ciocalteu was used as a reagent and Gallic acid was used as a standard. A total of 0.1 mL of extract was put into a 10 mL volumetric flask and 0.5 mL of Folin-Ciocalteu reagent was added. The samples were left at room temperature for 5 minutes then added with 1.5 mL 20% (w/v) Na_2CO_3. The mixture was added with distilled water until the volume reached 10 mL. After 30 minutes at room temperature,

absorbance was measured at a wavelength of 765 nm versus blank using a spectrophotometer (Shimadzu UV 1800, Japan). Total phenol content was expressed in mg GAE/g.

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3. Results and discussion/Results

3.1 Moisture content, color and biomass content of MFS

The MFS powder had a moisture content of 8.72% (Table 1.). Moisture content of food powder below 10% has a high stability for storage (Zambrano et al., 2019). The lightness (L*), redness (a*), yellowness (b*), chroma (C), and hue (°h) values of the MFS powder presented in Table 1. The positive a* and b* values of all the fermented-products reflected that the powder color is combination of red and yellow. Hue indicate a red color and chroma value indicate a dull-brownish red color. L* value below 50 indicate low lightness, resulting in a dark red color.

Fungal biomass, branched fibrous mycelium of *Monascus*, was form during the fermentation. Generally, biomass comprise of protein, lipids, polysaccharides, chitin (or chitosan), and various inorganic salts in different proportions (Isaza-Pérez et al., 2020). The biomass yield of MFS was recorded at 825.786 mg/g or 0.825 g/mL. It was higher than the biomass obtained from *M. purpureus* fermentation on potato waste, which ranged from 0.046 to 0.262 g/mL (inoculum concentration 7×10^5 spores/mL) (Abdel-Raheam et al., 2022). High biomass implies that sorghum was a suitable substrate for *M. purpureus* fermentation. Additionally, the high biomass could have resulted from the assistance of the steaming process (90°C for 60 minutes), which helped soften the sorghum and facilitated easier utilization for the growth of *M. purpureus* (Zhao et al., 2018).

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3.2 Antibacterial activity

MIC value is defined as the lowest concentration of the assayed antimicrobial agent that inhibits the visible growth of the microorganism tested (Balouiri et al., 2016). Low MIC value indicates a high antibacterial activity. The extraction using ethyl acetate produced MFSE with the lowest MIC, indicating that ethyl acetate-extracted MFSE exhibited the highest antibacterial activity against both *S. aureus* and *E. coli*. Meanwhile, ethanol and water-extracted MFSE showed lower antibacterial activity, with water-extracted displaying the lowest. Most of *Monascus* pigments are slightly polar, making them more soluble in organic solvents like ethanol than in water (Carvalho et al., 2007; Qian and Wu, 2010; Bai et al., 2022). As a result, the amount of *Monascus* pigments that are extracted by ethyl acetate and ethanol is larger (Table 3.). This larger quantity of extracted pigments is responsible for the observed antibacterial activity, thus making ethyl acetate and ethanol-extracted MFSE to have lower MIC than water-extracted MFSE.

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In addition to *Monascus* pigments, *Monascus*-fermented products are known to contain significant amounts of phenolic compounds (Srianta et al., 2013; Razak et al., 2015). Ethanol-extracted MFSE contain the highest total phenolic content (Table 3.). As per Haminiuk et al. (2014), phenolic compounds also exhibit higher solubility in organic solvents that are less polar than water. Razak et al. (2015) found that phenolic acid present in *Monascus*-fermented rice bran extract are ρ-coumaric, ferulic, sinapic, vanillic, caffeic, and syringic acid. All of those phenolic acid exhibited antimicrobial properties (Liu et al., 2020). Amoxicillin, a common antibiotic medication against Gram-positive and Gram-negative bacteria, was used as a reference. The MIC values of all three MFSEs remain higher than amoxicillin which exhibited a MIC value of 3.125 µg/ml against *S. aureus* and 12.5 µg/ml against *E. coli*.

Several studies have explored the antibacterial properties of red, orange, and yellow *Monascus* pigment. Gökmen et al. (2021) discovered that *Monascus* red pigments had a MIC value of 128 mg/mL against *S. aureus* and >128 mg/mL against *E. coli*. They observed that the effect was stronger compared to using a commercial red pigment which had a MIC value of 256 mg/mL against *S. aureus* and >256 mg/mL against *E. coli*. Feng et al. (2019) that found orange pigments demonstrated antibacterial activity against *S. aureus*, displaying inhibition zone diameters ranging from 5-34 mm for orange pigment concentration ranging from 0-10 mg/mL. Kim et al. (2006) discovered that amino acid derivatives of *Monascus* pigment showed a high antimicrobial activity. Derivatives containing L-Phe, D-Phe, L-Asp, D-Asp, L-Tyr, D-Tyr, L-Cys, and Nisin exhibited high activities against *S. aureus* (Gram-positive bacteria) with MIC values ranging from 8-16 µg/mL, while the control red pigment showed a MIC value of 64 µg/mL. For *E. coli* (Gram-negative bacteria), the same derivatives exhibited lower activities with MIC values ranging from 8-64 µg/mL, and the control red pigment with MIC value of >128 µg/mL. Kim and Ku (2018) reviewed several studies regarding the antimicrobial effects of *Monascus* pigments and conclude that Gram-positive bacteria were more prone to inhibition compared to Gram-negative bacteria.

Some studies suggested that the mechanism might involve interaction of *Monascus* pigment with enzymes in the germinated spores and vegetative cells, restricting the use of iron and affect cell membrane permeability. This could result in reduced transportation of nutrients, oxygen and metabolites (Kim et al., 2006; Xu, 2011). Feng et al. (2019) also observed that orange pigment cause *S. aureus* cells to wrinkle, damaging the membrane and causing protein leakage of bacteria cells. Meanwhile, amino acid derivatives of *Monascus* pigment depends on the hydrophobicity of the pigment and the amount of pigment adsorbed to the cell surface. The more hydrophobic, the higher the adsorption, which leads to limitation of oxygen uptake (Kim et al., 2006).

4. Conclusion

Ethyl acetate, ethanol, and water-extracted MFSE demonstrated antibacterial activity against *S. aureus* and *E. coli*. Ethyl acetate exhibited the highest activity, followed by ethanol and water extracts. All of the MFSE exhibited stronger activity on Gram-positive bacteria.

Conflict of interest - Disclose any potential conflict of interest appropriately.

The authors declare no conflict of interest.

Acknowledgements

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Tables and Figures – 1 PAGE 1 TABLE/FIGURE. PLACE ALL TABLES AND FIGURES AT THE END OF THE MANUSCRIPT BODY AFTER THE REFERENCES. ARRANGE THE TABLES AND FIGURES ACCORDING TO THEIR APPEARANCE IN THE TEXT.

Table 1. Moisture content, color and biomass content of MFS

Moisture content (%)	Color					Biomass (mg/g)
	L* value	a* value	b*value	C	°h	
8.72	46.5	23.6	13.8	27.4	3.3	825.786

Table 2. MIC of amoxicillin and MFSE against *S. aureus* and *E. coli*

Bacteria	Amoxicillin (µg/ml)	Ethyl acetate extract (mg/ml)	Ethanol extract (mg/ml)	Water extract (mg/ml)
Staphylococcus aureus ATCC 25920	3.125	3.125	12.5	100
Escherichia coli ATCC 25927	12.5	6.25	50	200

Table 3. Pigment and total phenolic contents of MFSE

Solvent	Pigment content (AU/g)			Total phenolic content (mg/mL)
	Yellow	Orange	Red	
Ethyl acetate	177.3378	64.7206	52.4588	1.6974
Ethanol	153.8563	59.0424	66.1631	9.8037
Water	47.4565	24.5389	20.1564	2.3995

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Antibacterial activity of *Monascus*-fermented sorghum extracts against *Staphylococcus aureus* and *Escherichia coli*

Abstract

This research was aimed to investigate the antibacterial activity of *Monascus*-fermented sorghum extract (MFSE). Three different solvents were used for extraction: ethyl acetate, ethanol, and water. Antibacterial activity was assessed by determining minimum inhibitory concentration (MIC). Broth microdilution method was used to determine the MIC of the extract against *Staphylococcus aureus* and *Escherichia coli*. Additionally, moisture content, color, and biomass of *Monascus*-fermented sorghum (MFS) were analyzed, along with the pigment and total phenolic content of MFSE. All obtained data were calculated for the mean. Antibacterial activity was observed in MFSE extracted with all three solvents, displaying MIC values of 0.018, 0.216, and 0.794 mg/L against *S. aureus* and 0.996, 1.205, and 1.138 mg/L against *E. coli* for ethyl acetate, ethanol, and water extract, respectively. The MFSE extracted with ethyl acetate exhibited lowest MIC, indicating highest antibacterial activity.

Keywords: *Monascus*, sorghum, extract, various solvents, antibacterial, MIC.

1. Introduction

In recent years, there has been a growing preference for the development and utilization of natural compounds over synthetic ones (Gökmen *et al*., 2021). *Monascus purpureus* is one of the few types of edible fungi. Through solid-state fermentation, *M. purpureus* produces pigments and compounds known for their abundant bioactivity, such as antioxidants, anti-inflammation, anticancer, antidiabetic, antiobesity, and antimicrobial (Hong *et al*., 2008; Kim *et al*., 2006; Kim *et al*., 2007; Kim *et al*., 2010; Lee *et al*., 2011; Hsu and Pan, 2012; Srianta *et al*., 2017; Feng *et al*., 2019; Choe *et al*., 2020; Gökmen *et al*., 2021; Ding *et al*., 2022). Commonly, rice is used to produce red-yeast rice (angkak), which has been used as natural food colorant, preservative, food supplement and traditional medicine in Asia. However, any other cereal grains containing sufficient fermentable carbohydrates for *M. purpureus* can also be considered as suitable substrates.

Sorghum (*Sorghum bicolor* (L.) Moench) is the fifth most important cereal globally, following maize, rice, wheat, and barley. It requires less water and shows higher resilience to significant shifts in climate compared to other cereals (Balakrishna *et al*., 2019). In spite of that, sorghum is still underutilized. Sorghum has a composition similar to multiple cereal grains. For every 100 grams, sorghum, rice, and maize contain carbohydrates at 73g, 79g, and 72g respectively, protein at 11g, 7g, and 9g, fat at 3.3g, 0.7g, and 4.5g, fiber at 2.3g, 1.0g, and 2.7g, calcium at 28mg, 6mg, and 9mg, phosphorus at 287mg, 147mg, and 380mg, and iron at 4.4mg, 0.8mg, and 4.6mg (Sirappa, 2003). Srianta and Harijono (2015) had successfully fermented sorghum using *M. purpureus*. The fermentation process yielded yellow, orange and red pigments at concentration of 61.00, 29.10, 35.60 AU/g respectively, for ethanol extract and 16.30, 12.40, 13.30 AU/g for water extract.

Monascus pigments have been reported to possess antibacterial properties (Kim *et al*., 2006; Feng *et al*., 2019; Gökmen *et al*., 2021). *M. purpureus* produces 6 pigments, classified into three groups: orange

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pigments [monascorubrin ($C_{23}H_{30}O_5$) and rubropunctanin ($C_{21}H_{22}O_5$)], yellow pigments [ankaflavin ($C_{23}H_{30}O_5$) and monascin ($C_{21}H_{26}O_5$)], and red pigments [monascorubramin ($C_{23}H_{27}NO_4$) and rubropunctamine ($C_{21}H_{23}NO_4$)] (Feng *et al*., 2012). Antibacterial activity was observed on *Monascus* red pigment (Gökmen *et al*., 2021), orange pigment (Feng *et al*., 2019), and amino acid derivatives of *Monascus* pigment (Kim *et al*., 2006). Antibacterial activity was investigated against Gram-positive bacteria *Staphylococcus aureus* and Gram-negative bacteria *Escherichia coli*. *S. aureus* and *E. coli* were pathogens causing various human infection and foodborne diseases (Tong *et al*., 2015; Bintsis, 2017; Braz *et al*., 2020)

This research was aimed to investigate the antibacterial activity of MFSE against *Staphylococcus aureus* and *Escherichia coli*. It is the first to examine antibacterial activity of *Monascus*-fermented product that utilizes sorghum as substrate.

2. Materials and methods

2.1 Microorganism, substrate, and chemical

M. purpureus M9 culture was used for solid-state fermentation of sorghum. It was isolated from commercial *Monascus*-fermented rice in Surabaya, Indonesia and identified as *M. purpureus* M9 (NCBI Accesion Number: HM188425.1). *Staphylococcus aureus* ATCC 25920 and *Escherichia coli* ATCC 25927 were used for antibacterial assay. The bacteria were suspended in DMSO 20%. The sorghum used was commercial dehulled sorghum. It was vacuum packed until used. All chemical and media used in the analysis was analytical grade.

2.2 Starter culture preparation

M. purpureus M9 was monthly cultured on potato dextrose agar (PDA) slant. Starter culture was prepared with inoculating 8 loops of the culture (8 pieces of colony) scrabbed from the PDA slant, then incubated at 30°C for 7 days. Before use, the culture was suspended in 10 mL of sterile distilled water for 1 hour and homogenized with a vortex.

2.3 Sorghum solid-state fermentation

The fermentation process was carried out according to Srianta *et al*. (2016) with modification to the dehulled sorghum pre-treatment. Dehulled sorghum was washed and steamed first at 90°C for 60 min. Fifty g of steamed sorghum was weighed and put inside an Erlenmeyer flask, then sterilized at 121°C for 20 min. Solid state fermentation was carried out by inoculating 5 mL of *M. purpureus* M9 starter culture that was adjusted using a haemocytometer and containing 5 x 10^5 spores/mL into each flask containing sterilized substrate. It was then incubated at 30°C for 14 days with daily shaking of the flask to ensure thorough fermentation. The fermented material was then dried in an oven at 45°C for 24 hours, grounded into powder, and sieved (size 45 mesh). The MFS powder was analyzed for moisture content, color and biomass.

2.4 Moisture content, color and biomass analysis of MFS

Moisture content of MFS was analyzed using oven drying method (AOAC, 2005). The sample underwent drying at 105°C for 3 hours and followed by weighing every 30 min until a constant weight was obtained. The loss in weight due to moisture evaporation was then used to calculate the moisture content of the sample. The color of MFS was measured using color reader (Konica Minolta, 2015). The MFS was packed inside a transparent plastic and measured for L*, a*, b*, C, and °h value. These measurements were repeated in triplicate.

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Biomass analysis was conducted according to Srianta *et al*. (2016). The fungal biomass was estimated by determining the amount of N-acetyl glucosamine released through the acid hydrolysis of chitin, a component within the cell wall of the mycelia (Babitha *et al*., 2006). Chitin hydrolysis was carried out by using 10 M HCl and autoclaving at 130°C for 2 hours. The hydrolysate was neutralized to pH 7.0, mixed with acetyl acetone reagent and followed by Ehrlich reagent. Optical density was measured at 530 nm against the reagent blank. N-acetyl glucosamine (Sigma Aldrich Co. LLC) was used as a standard.

2.5 MFS extraction

The MFS was extracted with three different solvents: ethyl acetate, ethanol, and water. One gram of the powder was weighed and extracted with ethyl acetate/ethanol/water with ratios of 1:50 (w/v) (one gram in 50 mL solvent) with Soxhlet apparatus at 80°C for 2 hours. The extract obtained was evaporated at 200 mbar, 50 rpm for 30 min using a rotary evaporator, producing a concentrated extract. The MFSE was analyzed for antibacterial activity assay, pigment and total phenolic contents.

2.6 Antibacterial activity assay

Broth microdilution test was carried out according to Klančnik *et al*. (2010). Fifty µL of *S. aureus* and *E. coli* suspension in Müeller Hinton Broth (MHB) medium was added to the wells of a sterile 96-well microtitre plate containing 50 µL of two-fold serially diluted MFSE or amoxicillin. The final volume in each well was 100 µL. Control wells were prepared with culture medium, bacterial suspension only, MFSE only and DMSO 20% in amounts corresponding to the highest quantity present. The contents of each well were mixed on a microplate shaker at 900 rpm for 1 minute prior to 24 hour incubation. The MIC was the lowest concentration where no viability was observed after 24 hours on the basis of metabolic activity (Mourey and Canillac, 2002). Viability could be indicated by the presence of respiratory and ATP activity. To indicate respiratory activity, the presence of color was determined after adding 10 µL/well of TTC (2,3,5-triphenyltetrazoliumchloride) dissolved in water (TTC 20 mg/mL). The microplate was then incubated under appropriate cultivation conditions for 30 min in the dark (Ellof, 1998). To determine the ATP activity, the bioluminescence signal was measured by a microplate reader after adding 100 µL/well of BacTiter-Glo™ reagent and after 5 min incubation in the dark (Klančnik *et al*., 2009). Positive controls were wells with a bacterial suspension in MHB and a bacterial suspension in MHB with DMSO 20% in amounts corresponding to the highest quantity present in the broth microdilution assay. Negative controls were wells with MHB and MFSE or amoxicilin. All measurements of MIC values were repeated in triplicate.

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2.7 Pigment content analysis

Pigment content of MFSE analysis was carried out according to Srianta *et al*. (2016). The MFSE was dissolved in respective solvent and the absorbance was measured with UV-vis spectrophotometer at λ 400 nm, 470 nm, and 500 nm for yellow, orange, and red pigment, respectively. The pigment content was express as absorbance (nm) at the wavelength per gram of dry matter (AU/g).

2.8 Total phenolic content

The total phenolic content of MFSE was analyzed according to Srianta *et al*. (2014). Folin-Ciocalteu was used as a reagent and Gallic acid was used as a standard. A total of 0.1 mL of extract was put into a 10 mL volumetric flask and 0.5 mL of Folin-Ciocalteu reagent was added. The samples were left at room temperature for 5 min then added with 1.5 mL 20% (w/v) Na_2CO_3. The mixture was added with

distilled water until the volume reached 10 mL. After 30 min at room temperature, absorbance was measured at a wavelength of 765 nm versus blank using a spectrophotometer (Shimadzu UV 1800, Japan). Total phenolic content was expressed in mg GAE/g.

3. Results and discussion/Results

3.1 Moisture content, color and biomass content of MFS

The MFS powder had a moisture content of 8.72% (Table 1.). Moisture content of food powder below 10% has a high stability for storage (Zambrano *et al*., 2019). The lightness (L*), redness (a*), yellowness (b*), chroma (C), and hue (°h) values of the MFS powder presented in Table 1. The positive a* and b* values of all the fermented-products reflected that the powder color is combination of red and yellow. Hue indicate a red color and chroma value indicate a dull-brownish red color. L* value below 50 indicate low lightness, resulting in a dark red color.

Fungal biomass, branched fibrous mycelium of *Monascus*, was form during the fermentation. Generally, biomass comprise of protein, lipids, polysaccharides, chitin (or chitosan), and various inorganic salts in different proportions (Isaza-Pérez *et al*., 2020). The biomass yield of MFS was recorded at 825.786 mg/g or 0.825 g/mL. It was higher than the biomass obtained from *M. purpureus* fermentation on potato waste, which ranged from 0.046 to 0.262 g/mL (inoculum concentration 7×10^5 spores/mL) (Abdel-Raheam *et al*., 2022). High biomass implies that sorghum was a suitable substrate for *M. purpureus* fermentation. Additionally, the high biomass could have resulted from the assistance of the steaming process (90°C for 60 min), which helped soften the sorghum and facilitated easier utilization for the growth of *M. purpureus* (Zhao *et al*., 2018). Based on our previous research (Srianta and Harijono, 2015), the biomass of unsteamed dehulled sorghum only ranged from 26.64 to 36.70 mg/g (inoculum concentration 5×10^5 spores /mL).

3.2 Antibacterial activity

MIC value is defined as the lowest concentration of the assayed antimicrobial agent that inhibits the visible growth of the microorganism tested (Balouiri *et al*., 2016). Low MIC value indicates a high antibacterial activity. The extraction using ethyl acetate produced MFSE with the lowest MIC, indicating that ethyl acetate-extracted MFSE exhibited the highest antibacterial activity against both *S. aureus* and *E. coli*. Meanwhile, ethanol and water-extracted MFSE showed lower antibacterial activity, with water-extracted displaying the lowest. Most of *Monascus* pigments are slightly polar, making them more soluble in organic solvents like ethanol than in water (Carvalho *et al*., 2007; Qian and Wu, 2010; Bai *et al*., 2022). As a result, the amount of *Monascus* pigments that are extracted by ethyl acetate and ethanol is larger (Table 3.). This larger quantity of extracted pigments is responsible for the observed antibacterial activity, thus making ethyl acetate and ethanol-extracted MFSE to have lower MIC than water-extracted MFSE.

In addition to *Monascus* pigments, *Monascus*-fermented products are known to contain significant amounts of phenolic compounds (Srianta *et al*., 2013; Razak *et al*., 2015). Ethanol-extracted MFSE contain the highest total phenolic content (Table 3.). As per Haminiuk *et al*. (2014), phenolic compounds also exhibit higher solubility in organic solvents that are less polar than water. Razak *et al*. (2015) found that phenolic acid present in *Monascus*-fermented rice bran extract are ρ-coumaric, ferulic, sinapic, vanillic, caffeic, and syringic acid. All of those phenolic acid exhibited antimicrobial properties (Liu *et al*., 2020).

Commented [Ma19]: Consistensy: phenolic or phenol?

Commented [AW20R19]: Done: phenolic

Commented [Ma21]: It is better to provide Monascus growth data on unsteamed sorghum to support this statement.

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Commented [Ma23]: Monascus pigment consists of several compounds with varying degrees of polarity and antimicrobial activity. Isn't there a possibility that the antimicrobial activity is influenced by the type of dominant pigment extracted by the different solvents used?

Commented [AW24R23]: Yes, there is a possibility, but our main focus in this study is to investigate the antibacterial activity of MFSE (pigments) and not specific to which pigment is dominant in terms of its extractability and antimicrobial activity.

Amoxicillin, a common antibiotic medication against Gram-positive and Gram-negative bacteria, was used as a reference. The MIC values of all three MFSEs remain higher than amoxicillin which exhibited a MIC value of 3.125 µg/ml against *S. aureus* and 12.5 µg/ml against *E. coli*.

Several studies have explored the antibacterial properties of red, orange, and yellow *Monascus* pigment. Gökmen *et al*. (2021) discovered that *Monascus* red pigments had a MIC value of 128 mg/mL against *S. aureus* and >128 mg/mL against *E. coli*. They observed that the effect was stronger compared to using a commercial red pigment which had a MIC value of 256 mg/mL against *S. aureus* and >256 mg/mL against *E. coli*. Feng *et al*. (2019) that found orange pigments demonstrated antibacterial activity against *S. aureus*, displaying inhibition zone diameters ranging from 5-34 mm for orange pigment concentration ranging from 0-10 mg/mL. Kim *et al*. (2006) discovered that amino acid derivatives of *Monascus* pigment showed a high antimicrobial activity. Derivatives containing L-Phe, D-Phe, L-Asp, D-Asp, L-Tyr, D-Tyr, L-Cys, and Nisin exhibited high activities against *S. aureus* (Gram-positive bacteria) with MIC values ranging from 8-16 µg/mL, while the control red pigment showed a MIC value of 64 µg/mL. For *E. coli* (Gram-negative bacteria), the same derivatives exhibited lower activities with MIC values ranging from 8-64 µg/mL, and the control red pigment with MIC value of >128 µg/mL. Kim and Ku (2018) reviewed several studies regarding the antimicrobial effects of *Monascus* pigments and conclude that Gram-positive bacteria were more prone to inhibition compared to Gram-negative bacteria.

Some studies suggested that the mechanism might involve interaction of *Monascus* pigment with enzymes in the germinated spores and vegetative cells, restricting the use of iron and affect cell membrane permeability. This could result in reduced transportation of nutrients, oxygen and metabolites (Kim *et al*., 2006; Xu, 2011). Feng *et al*. (2019) also observed that orange pigment cause *S. aureus* cells to wrinkle, damaging the membrane and causing protein leakage of bacteria cells. Meanwhile, amino acid derivatives of *Monascus* pigment depends on the hydrophobicity of the pigment and the amount of pigment adsorbed to the cell surface. The more hydrophobic, the higher the adsorption, which leads to limitation of oxygen uptake (Kim *et al*., 2006).

4. Conclusion

Sorghum is a suitable substrate to produce *Monascus*-fermented product whose extract has antibacterial activity. Ethyl acetate, ethanol, and water-extracted MFSE demonstrated antibacterial activity against *S. aureus* and *E. coli*. Ethyl acetate exhibited the highest activity, followed by ethanol and water extracts. All of the MFSE exhibited stronger activity on Gram-positive bacteria.

Conflict of interest - Disclose any potential conflict of interest appropriately.

The authors declare no conflict of interest.

Acknowledgements

Thanks to Widya Mandala Surabaya Catholic University for financial support through Penelitian Internal UKWMS.

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Tables and Figures – 1 PAGE 1 TABLE/FIGURE. PLACE ALL TABLES AND FIGURES AT THE END OF THE MANUSCRIPT BODY AFTER THE REFERENCES. ARRANGE THE TABLES AND FIGURES ACCORDING TO THEIR APPEARANCE IN THE TEXT.

Table 1. Moisture content, color and biomass content of MFS

Moisture content (%)	Color					Biomass (mg/g)
	L* value	a* value	b*value	C	°h	
8.72	46.5	23.6	13.8	27.4	3.3	825.786

Table 2. MIC of amoxicillin and MFSE against *S. aureus* and *E. coli*

Bacteria	Amoxicillin (µg/ml)	Ethyl acetate extract (mg/ml)	Ethanol extract (mg/ml)	Water extract (mg/ml)
Staphylococcus aureus ATCC 25920	3.125	3.125	12.5	100
Escherichia coli ATCC 25927	12.5	6.25	50	200

Table 3. Pigment and total phenolic contents of MFSE

Solvent	Pigment content (AU/g)			Total phenolic content (mg/mL)
	Yellow	Orange	Red	
Ethyl acetate	177.3378	64.7206	52.4588	1.6974
Ethanol	153.8563	59.0424	66.1631	9.8037
Water	47.4565	24.5389	20.1564	2.3995

7

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Antibacterial activity of *Monascus*-fermented sorghum extracts against *Staphylococcus aureus* and *Escherichia coli*

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Abstract

Monascus purpureus is one of the few types of edible fungi known for their abundant bioactivity. This research was aimed to investigate the antibacterial activity of *Monascus*-fermented sorghum extract (MFSE). Three different solvents were used for extraction: ethyl acetate, ethanol, and water. Antibacterial activity was assessed by determining minimum inhibitory concentration (MIC). The broth microdilution method was used to determine the MIC of the extract against *Staphylococcus aureus* and *Escherichia coli*. Additionally, moisture content, color, and biomass of *Monascus*-fermented sorghum (MFS) were analyzed, along with the pigment and total phenolic content of MFSE. All obtained data were calculated for the mean. Antibacterial activity was observed in MFSE extracted with all three solvents, displaying MIC values of

0.018, 0.216, and 0.794 mg/L against *S. aureus* and 0.996, 1.205, and 1.138 mg/L against *E. coli* for ethyl acetate, ethanol, and water extract, respectively. The MFSE extracted with ethyl acetate exhibited the lowest MIC, indicating the highest antibacterial activity.

Keywords: *Monascus*, sorghum, extract, various solvents, antibacterial, MIC.

1. Introduction

In recent years, there has been a growing preference for the development and utilization of natural compounds over synthetic ones (Gökmen *et al*., 2021). *Monascus purpureus* is one of the few types of edible fungi. Through solid-state fermentation, *M. purpureus* produces pigments and compounds known for their abundant bioactivity, such as antioxidants, anti-inflammation, anticancer, antidiabetic, antiobesity, and antimicrobial (Kim *et al*., 2006; Kim *et al*., 2007; Hong *et a*l., 2008; Kim *et al*., 2010; Lee *et al*., 2011; Hsu and Pan, 2012; Srianta *et al*., 2017; Feng *et al*., 2019; Choe *et al*., 2020; Gökmen *et al*., 2021; Ding *et al*., 2022). Commonly, rice is used to produce red-yeast rice (angkak), which has been used as a natural food colorant, preservative, food supplement and traditional medicine in Asia. However, any other cereal grains containing sufficient fermentable carbohydrates for *M. purpureus* can also be considered suitable substrates.

Sorghum (*Sorghum bicolor* (L.) Moench) is the fifth most important cereal globally, following maize, rice, wheat, and barley. It requires less water and shows higher resilience to significant shifts in climate compared to other cereals (Balakrishna *et al*., 2019). In spite of that, sorghum is still underutilized. Sorghum has a composition similar to multiple cereal grains. For every 100 grams, sorghum, rice, and maize contain carbohydrates at 73 g, 79 g, and 72 g respectively, protein at 11 g, 7 g, and 9 g, fat at 3.3 g, 0.7 g, and 4.5 g, fiber at 2.3 g, 1.0 g, and 2.7 g, calcium at 28 mg, 6 mg, and 9 mg, phosphorus at 287 mg, 147 mg, and 380 mg, and iron at 4.4 mg, 0.8 mg, and 4.6 mg (Sirappa, 2003). Srianta and Harijono (2015) had successfully fermented sorghum using *M. purpureus*. The fermentation process yielded yellow, orange and red pigments at concentrations of 61.00, 29.10, 35.60 AU/g respectively, for ethanol extract and 16.30, 12.40, 13.30 AU/g for water extract.

Monascus pigments have been reported to possess antibacterial properties (Kim *et al*., 2006; Feng *et al*., 2019; Gökmen *et al*., 2021). *M. purpureus* produces 6 pigments, classified into three groups: orange pigments [monascorubrin ($C_{23}H_{30}O_5$) and rubropunctanin ($C_{21}H_{22}O_5$)], yellow pigments [ankaflavin ($C_{23}H_{30}O_5$) and monascin ($C_{21}H_{26}O_5$)], and red pigments [monascorubramin ($C_{23}H_{27}NO_4$) and

rubropunctamine ($C_{21}H_{23}NO_4$)] (Feng *et al*., 2012). Antibacterial activity was observed in *Monascus* red pigment (Gökmen *et al*., 2021), orange pigment (Feng *et al*., 2019), and amino acid derivatives of *Monascus* pigment (Kim *et al*., 2006). Antibacterial activity was investigated against Gram-positive bacteria *Staphylococcus aureus* and Gram-negative bacteria *E. coli*. *Staphylococcus aureus* and *E. coli* were pathogens causing various human infections and foodborne diseases (Tong *et al*., 2015; Bintsis, 2017; Braz *et al*., 2020)

This research aimed to investigate the antibacterial activity of MFSE against *Staphylococcus aureus* and *Escherichia coli*. It is the first to examine the antibacterial activity of a *Monascus*-fermented product that utilizes sorghum as a substrate.

2. Materials and methods

2.1 Microorganism, substrate, and chemical

Monascus purpureus M9 culture was used for solid-state fermentation of sorghum. It was isolated from commercial *Monascus*-fermented rice in Surabaya, Indonesia and identified as *M. purpureus* M9 (NCBI Accession Number: HM188425.1). *Staphylococcus aureus* ATCC 25920 and *Escherichia coli* ATCC 25927 were used for antibacterial assay. The bacteria were suspended in DMSO 20%. The sorghum used was commercial dehulled sorghum. It was vacuum-packed until used. All chemical and media used in the analysis was analytical grade.

2.2 Starter culture preparation

Monascus purpureus M9 was monthly cultured on a potato dextrose agar (PDA) slant. The starter culture was prepared with inoculating 8 loops of the culture (8 pieces of colony) scrabbed from the PDA slant, then incubated at 30°C for 7 days. Before use, the culture was suspended in 10 mL of sterile distilled water for 1 hr and homogenized with a vortex.

2.3 Sorghum solid-state fermentation

The fermentation process was carried out according to Srianta *et al*. (2016) with modification to the dehulled sorghum pre-treatment. Dehulled sorghum was washed and steamed first at 90°C for 60 mins. Fifty g of steamed sorghum was weighed and put inside an Erlenmeyer flask, then sterilized at 121°C for 20 min. Solid-state fermentation was carried out by inoculating 5 mL of *M. purpureus* M9 starter culture that was adjusted using a haemocytometer and containing 5 x 10^5 spores/mL into each flask containing sterilized substrate. It was then incubated at 30°C for 14 days with daily shaking of the flask to ensure

thorough fermentation. The fermented material was then dried in an oven at 45°C for 24 hrs, ground into powder, and sieved (size 45 mesh). The MFS powder was analyzed for moisture content, color and biomass.

2.4 Moisture content, color and biomass analysis of Monascus-fermented sorghum

The moisture content of MFS was analyzed using oven drying method (AOAC, 2005). The sample underwent drying at 105°C for 3 hrs and followed by weighing every 30 min until a constant weight was obtained. The loss in weight due to moisture evaporation was then used to calculate the moisture content of the sample. The color of MFS was measured using color reader (Konica Minolta, 2015). The MFS was packed inside a transparent plastic and measured for L*, a*, b*, C, and °h values. These measurements were repeated in triplicate.

Biomass analysis was conducted according to Srianta *et al*. (2016). The fungal biomass was estimated by determining the amount of N-acetyl glucosamine released through the acid hydrolysis of chitin, a component within the cell wall of the mycelia (Babitha *et al*., 2006). Chitin hydrolysis was carried out by using 10 M HCl and autoclaving at 130°C for 2 hrs. The hydrolysate was neutralized to pH 7.0, mixed with acetylacetone reagent and followed by Ehrlich reagent. Optical density was measured at 530 nm against the reagent blank. N-acetyl glucosamine (Sigma Aldrich Co. LLC) was used as a standard.

2.5 MFS extraction

The MFS was extracted with three different solvents: ethyl acetate, ethanol, and water. One gram of the powder was weighed and extracted with ethyl acetate/ethanol/water with ratios of 1:50 (w/v) (one gram in 50 mL solvent) with Soxhlet apparatus at 80°C for 2 hrs. The extract obtained was evaporated at 200 mbar, 50 rpm for 30 min using a rotary evaporator, producing a concentrated extract. The MFSE was analyzed for antibacterial activity assay, pigment and total phenolic contents.

2.6 Antibacterial activity assay

Broth microdilution test was carried out according to Klančnik *et al*. (2010). Approximately 50 µL of *S. aureus* and *E. coli* suspension in Müeller Hinton Broth (MHB) medium was added to the wells of a sterile 96-well microtitre plate containing 50 µL of two-fold serially diluted MFSE or amoxicillin. The final volume in each well was 100 µL. Control wells were prepared with culture medium, bacterial suspension only, MFSE only and DMSO 20% in amounts corresponding to the highest quantity present.

The contents of each well were mixed on a microplate shaker at 900 rpm for 1 min prior to 24 hrs incubation. The MIC was the lowest concentration where no viability was observed after 24 hrs on the basis of metabolic activity (Mourey and Canillac, 2002). Viability could be indicated by the presence of respiratory and ATP activity. To indicate respiratory activity, the presence of color was determined after adding 10 µL/well of TTC (2,3,5-triphenyltetrazoliumchloride) dissolved in water (TTC 20 mg/mL). The microplate was then incubated under appropriate cultivation conditions for 30 mins in the dark (Eloff, 1998). To determine the ATP activity, the bioluminescence signal was measured by a microplate reader after adding 100 µL/well of BacTiter-Glo™ reagent and after 5 min incubation in the dark (Klančnik *et al.*, 2009). Positive controls were wells with a bacterial suspension in MHB and a bacterial suspension in MHB with DMSO 20% in amounts corresponding to the highest quantity present in the broth microdilution assay. Negative controls were wells with MHB and MFSE or amoxicillin. All measurements of MIC values were repeated in triplicate.

2.7 Pigment content analysis

The pigment content of MFSE analysis was carried out according to Srianta *et al*. (2016). The MFSE was dissolved in respective solvent and the absorbance was measured with UV-vis spectrophotometer at λ 400 nm, 470 nm, and 500 nm for yellow, orange, and red pigment, respectively. The pigment content was expressed as absorbance (nm) at the wavelength per gram of dry matter (AU/g).

2.8 Total phenolic content

The total phenolic content of MFSE was analyzed according to Srianta *et al*. (2014). Folin-Ciocalteu was used as a reagent and Gallic acid was used as a standard. A total of 0.1 mL of extract was put into a 10 mL volumetric flask and 0.5 mL of Folin-Ciocalteu reagent was added. The samples were left at room temperature for 5 min then added with 1.5 mL 20% (w/v) Na_2CO_3. The mixture was added with distilled water until the volume reached 10 mL. After 30 mins at room temperature, absorbance was measured at a wavelength of 765 nm versus blank using a spectrophotometer (Shimadzu UV 1800, Japan). Total phenolic content was expressed in mg GAE/g.

3. Results and discussion

3.1 Moisture content, color and biomass content of Monascus-fermented sorghum

The MFS powder had a moisture content of 8.72% (Table 1). The moisture content of food powder below 10% has a high stability for storage (Zambrano *et al*., 2019). The lightness (L*), redness (a*), yellowness (b*), chroma (C), and hue (°h) values of the MFS powder are presented in Table 1. The positive a* and b* values of all the fermented products reflected that the powder color is a combination of red and yellow. Hue indicates a red color and chroma value indicates a dull-brownish red color. L* value below 50 indicates low lightness, resulting in a dark red color.

Fungal biomass, branched fibrous mycelium of *Monascus*, was formed during the fermentation. Generally, biomass comprises protein, lipids, polysaccharides, chitin (or chitosan), and various inorganic salts in different proportions (Isaza-Pérez *et al*., 2020). The biomass yield of MFS was recorded at 825.786 mg/g or 0.825 g/mL. It was higher than the biomass obtained from *M. purpureus* fermentation on potato waste, which ranged from 0.046 to 0.262 g/mL (inoculum concentration 7×10^5 spores/mL) (Abdel-Raheam *et al*., 2022). High biomass implies that sorghum was a suitable substrate for *M. purpureus* fermentation. Additionally, the high biomass could have resulted from the assistance of the steaming process (90°C for 60 min), which helped soften the sorghum and facilitated easier utilization for the growth of *M. purpureus* (Zhao *et al*., 2018). Based on our previous research (Srianta and Harijono, 2015), the biomass of unsteamed dehulled sorghum only ranged from 26.64 to 36.70 mg/g (inoculum concentration 5×10^5 spores /mL).

3.2 Antibacterial activity

MIC value is defined as the lowest concentration of the assayed antimicrobial agent that inhibits the visible growth of the microorganism tested (Balouiri *et al*., 2016). A low MIC value indicates a high antibacterial activity. The extraction using ethyl acetate produced MFSE with the lowest MIC, indicating that ethyl acetate-extracted MFSE exhibited the highest antibacterial activity against both *S. aureus* and *E. coli*. Meanwhile, ethanol and water-extracted MFSE showed lower antibacterial activity, with water-extracted displaying the lowest. Most *Monascus* pigments are slightly polar, making them more soluble in organic solvents like ethanol than in water (Carvalho *et al*., 2007; Qian and Wu, 2010; Bai *et al*., 2022). As a result, the amount of *Monascus* pigments that are extracted by ethyl acetate and ethanol is larger (Table 3.). This larger quantity of extracted pigments is responsible for the observed antibacterial activity, thus making ethyl acetate and ethanol-extracted MFSE have lower MIC than water-extracted MFSE.

In addition to *Monascus* pigments, *Monascus*-fermented products are known to contain significant amounts of phenolic compounds (Srianta *et al*., 2013; Razak *et al*., 2015). Ethanol-extracted MFSE contains the highest total phenolic content (Table 3.). As per Haminiuk *et al*. (2014), phenolic compounds also exhibit

higher solubility in organic solvents that are less polar than water. Razak *et al*. (2015) found that phenolic acid present in *Monascus*-fermented rice bran extract is ρ-coumaric, ferulic, sinapic, vanillic, caffeic, and syringic acid. All of those phenolic acids exhibited antimicrobial properties (Liu *et al*., 2020). Amoxicillin, a common antibiotic medication against Gram-positive and Gram-negative bacteria, was used as a reference. The MIC values of all three MFSEs remain higher than amoxicillin which exhibited a MIC value of 3.125 µg/ml against *S. aureus* and 12.5 µg/ml against *E. coli***.**

Several studies have explored the antibacterial properties of red, orange, and yellow *Monascus* pigment. Gökmen *et al*. (2021) discovered that *Monascus* red pigments had a MIC value of 128 mg/mL against *S. aureus* and >128 mg/mL against *E. coli*. They observed that the effect was stronger compared to using a commercial red pigment which had a MIC value of 256 mg/mL against *S. aureus* and >256 mg/mL against *E. coli*. Feng *et al*. (2019) that found orange pigments demonstrated antibacterial activity against *S. aureus*, displaying inhibition zone diameters ranging from 5-34 mm for orange pigment concentrations ranging from 0-10 mg/mL. Kim *et al*. (2006) discovered that amino acid derivatives of *Monascus* pigment showed a high antimicrobial activity. Derivatives containing L-Phe, D-Phe, L-Asp, D-Asp, L-Tyr, D-Tyr, L-Cys, and Nisin exhibited high activities against *S. aureus* (Gram-positive bacteria) with MIC values ranging from 8-16 µg/mL, while the control red pigment showed a MIC value of 64 µg/mL. For *E. coli* (Gram-negative bacteria), the same derivatives exhibited lower activities with MIC values ranging from 8-64 µg/mL, and the control red pigment with MIC value of >128 µg/mL. Kim and Ku (2018) reviewed several studies regarding the antimicrobial effects of *Monascus* pigments and concluded that Gram-positive bacteria were more prone to inhibition compared to Gram-negative bacteria.

Some studies suggested that the mechanism might involve the interaction of *Monascus* pigment with enzymes in the germinated spores and vegetative cells, restricting the use of iron and affecting cell membrane permeability. This could result in reduced transportation of nutrients, oxygen and metabolites (Kim *et al*., 2006; Xu, 2011). Feng *et al*. (2019) also observed that orange pigment causes *S. aureus* cells to wrinkle, damaging the membrane and causing protein leakage of bacteria cells. Meanwhile, amino acid derivatives of *Monascus* pigment depend on the hydrophobicity of the pigment and the amount of pigment adsorbed to the cell surface. The more hydrophobic, the higher the adsorption, which leads to limitation of oxygen uptake (Kim *et al*., 2006).

4. Conclusion

Sorghum is a suitable substrate to produce *Monascus*-fermented products whose extract has antibacterial activity. Ethyl acetate, ethanol, and water-extracted MFSE demonstrated antibacterial activity

against *S. aureus* and *E. coli*. Ethyl acetate exhibited the highest activity, followed by ethanol and water extracts. All of the MFSE exhibited stronger activity on Gram-positive bacteria.

Conflict of interest

The authors declare no conflict of interest.

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Table 1. Moisture content, color and biomass content of MFS.

Moisture content (%)	Color					Biomass (mg/g)
	L* value	a* value	b*value	C	°h	
8.72	46.5	23.6	13.8	27.4	3.3	825.786

Table 2. MIC of amoxicillin and MFSE against *S. aureus* and *E. coli.*

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Bacteria	Amoxicillin (µg/mL)	Ethyl acetate extract (mg/mL)	Ethanol extract (mg/mL)	Water extract (mg/mL)
Staphylococcus aureus ATCC 25920	3.125	3.125	12.5	100
Escherichia coli ATCC 25927	12.5	6.25	50	200

Table 3. Pigment and total phenolic contents of MFSE.

Solvent	Pigment content (AU/g)			Total phenolic content (mg/mL)
	Yellow	Orange	Red	
Ethyl acetate	177.3378	64.7206	52.4588	1.6974
Ethanol	153.8563	59.0424	66.1631	9.8037
Water	47.4565	24.5389	20.1564	2.3995

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Konfirmasi Respon Atas Permintaan Klarifikasi Beberapa Poin di Artikel Sebelum Masuk Tahap Produksi

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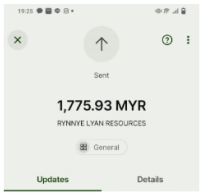

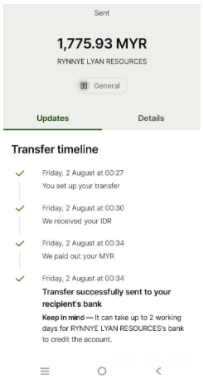

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RESEARCH PAPER

Antibacterial activity of *Monascus*-fermented sorghum extracts against *Staphylococcus aureus* and *Escherichia coli*

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Abstract

Monascus purpureus is one of the few types of edible fungi known for their abundant bioactivity. This research was aimed to investigate the antibacterial activity of *Monascus*-fermented sorghum extract (MFSE). Three different solvents were used for extraction: ethyl acetate, ethanol, and water. Antibacterial activity was assessed by determining minimum inhibitory concentration (MIC). The broth microdilution method was used to determine the MIC of the extract against *Staphylococcus aureus* and *Escherichia coli*. Additionally, moisture content, color, and biomass of *Monascus*-fermented sorghum (MFS) were analyzed, along with the pigment and total phenolic content of MFSE. All obtained data were calculated for the mean. Antibacterial activity was observed in MFSE extracted with all three solvents, displaying MIC values of 0.018, 0.216, and 0.794 mg/L against *S. aureus* and 0.996, 1.205, and 1.138 mg/L against *E. coli* for ethyl acetate, ethanol, and water extract, respectively. The MFSE extracted with ethyl acetate exhibited the lowest MIC, indicating the highest antibacterial activity.

1. Introduction

In recent years, there has been a growing preference for the development and utilization of natural compounds over synthetic ones (Gökmen *et al*., 2021). *Monascus purpureus* is one of the few types of edible fungi. Through solid-state fermentation, *M. purpureus* produces pigments and compounds known for their abundant bioactivity, such as antioxidants, anti-inflammation, anticancer, antidiabetic, antiobesity, and antimicrobial (Kim *et al*., 2006; Kim *et al*., 2007; Hong *et al*., 2008; Kim *et al*., 2010; Lee *et al*., 2011; Hsu and Pan, 2012; Srianta *et al*., 2017; Feng *et al*., 2019; Choe *et al*., 2020; Gökmen *et al*., 2021; Ding *et al*., 2022). Commonly, rice is used to produce red-yeast rice (angkak), which has been used as a natural food colorant, preservative, food supplement and traditional medicine in Asia. However, any other cereal grains containing sufficient fermentable carbohydrates for *M. purpureus* can also be considered suitable substrates.

Sorghum (*Sorghum bicolor* (L.) Moench) is the fifth most important cereal globally, following maize, rice, wheat, and barley. It requires less water and shows higher resilience to significant shifts in climate compared to other cereals (Balakrishna *et al*., 2019). In spite of that, sorghum is still underutilized. Sorghum has a composition similar to multiple cereal grains. For every 100 g, sorghum, rice, and maize contain carbohydrates at 73 g, 79 g, and 72 g respectively, protein at 11 g, 7 g, and 9 g, fat at 3.3 g, 0.7 g, and 4.5 g, fiber at 2.3 g, 1.0 g, and 2.7 g, calcium at 28 mg, 6 mg, and 9 mg, phosphorus at 287 mg, 147 mg, and 380 mg, and iron at 4.4 mg, 0.8 mg, and 4.6 mg (Sirappa, 2003). Srianta and Harijono (2015) had successfully fermented sorghum using *M. purpureus*. The fermentation process yielded yellow, orange and red pigments at concentrations of 61.00, 29.10, 35.60 AU/g respectively, for ethanol extract and 16.30, 12.40, 13.30 AU/g for water extract.

Monascus pigments have been reported to possess antibacterial properties (Kim *et al*., 2006; Feng *et al*., 2019; Gökmen *et al*., 2021). *Monascus purpureus* produces 6 pigments, classified into three groups: orange pigments [monascorubrin ($C_{23}H_{26}O_5$) and rubropunctanin ($C_{21}H_{22}O_5$)], yellow pigments [ankaflavin ($C_{23}H_{30}O_5$) and monascin ($C_{21}H_{26}O_5$)], and red pigments [monascorubramin ($C_{23}H_{27}NO_4$) and rubropunctamine ($C_{21}H_{23}NO_4$)] (Feng *et al*., 2012). Antibacterial activity was observed in *Monascus* red pigment (Gökmen *et al*., 2021), orange pigment (Feng *et al*., 2019), and amino acid derivatives of *Monascus* pigment (Kim *et al*., 2006). Antibacterial activity was investigated against Gram-positive bacteria *Staphylococcus aureus* and Gram-negative bacteria *E. coli*. *Staphylococcus aureus* and *E.*

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coli were pathogens causing various human infections and foodborne diseases (Tong *et al*., 2015; Bintsis, 2017; Braz *et al*., 2020)

This research aimed to investigate the antibacterial activity of MFSE against *S. aureus* and *E. coli*. It is the first to examine the antibacterial activity of a *Monascus*-fermented product that utilizes sorghum as a substrate.

2. Materials and methods

2.1 Microorganism, substrate, and chemical

Monascus purpureus M9 culture was used for solid-state fermentation of sorghum. It was isolated from commercial *Monascus*-fermented rice in Surabaya, Indonesia and identified as *M. purpureus* M9 (NCBI Accession Number: HM188425.1). *Staphylococcus aureus* ATCC 25920 and *Escherichia coli* ATCC 25927 were used for antibacterial assay. The bacteria were suspended in DMSO 20%. The sorghum used was commercial dehulled sorghum. It was vacuum-packed until used. All chemical and media used in the analysis was analytical grade.

2.2 Starter culture preparation

Monascus purpureus M9 was monthly cultured on a potato dextrose agar (PDA) slant. The starter culture was prepared with inoculating 8 loops of the culture (8 pieces of colony) scrabbed from the PDA slant, then incubated at 30°C for 7 days. Before use, the culture was suspended in 10 mL of sterile distilled water for 1 hr and homogenized with a vortex.

2.3 Sorghum solid-state fermentation

The fermentation process was carried out according to Srianta *et al*. (2016) with modification to the dehulled sorghum pre-treatment. Dehulled sorghum was washed and steamed first at 90°C for 60 mins. Fifty g of steamed sorghum was weighed and put inside an Erlenmeyer flask, then sterilized at 121°C for 20 mins. Solid-state fermentation was carried out by inoculating 5 mL of *M. purpureus* M9 starter culture that was adjusted using a haemocytometer and containing 5×10^5 spores/mL into each flask containing sterilized substrate. It was then incubated at 30°C for 14 days with daily shaking of the flask to ensure thorough fermentation. The fermented material was then dried in an oven at 45°C for 24 hrs, ground into powder, and sieved (size 45 mesh). The MFS powder was analyzed for moisture content, color and biomass.

2.4 Moisture content, color and biomass analysis of Monascus-fermented sorghum

The moisture content of MFS was analyzed using oven drying method (Association of the Official Analytical Collaboration (AOAC) International, 2005). The sample underwent drying at 105°C for 3 hrs and followed by weighing every 30 min until a constant weight was obtained. The loss in weight due to moisture evaporation was then used to calculate the moisture content of the sample. The color of MFS was measured using color reader (Konica Minolta, 2015). The MFS was packed inside a transparent plastic and measured for L*, a*, b*, C, and ^{o}h values. These measurements were repeated in triplicate.

Biomass analysis was conducted according to Srianta *et al*. (2016). The fungal biomass was estimated by determining the amount of N-acetyl glucosamine released through the acid hydrolysis of chitin, a component within the cell wall of the mycelia (Babitha *et al*., 2006). Chitin hydrolysis was carried out by using 10 M HCl and autoclaving at 130°C for 2 hrs. The hydrolysate was neutralized to pH 7.0, mixed with acetylacetone reagent and followed by Ehrlich reagent. Optical density was measured at 530 nm against the reagent blank. N-acetyl glucosamine (Sigma Aldrich Co. LLC) was used as a standard.

2.5 Monascus-fermented sorghum extraction

The MFS was extracted with three different solvents: ethyl acetate, ethanol, and water. One gram of the powder was weighed and extracted with ethyl acetate/ ethanol/water with ratios of 1:50 (w/v) (1 g in 50 mL solvent) with Soxhlet apparatus at 80°C for 2 hrs. The extract obtained was evaporated at 200 mbar, 50 rpm for 30 mins using a rotary evaporator, producing a concentrated extract. The MFSE was analyzed for antibacterial activity assay, pigment and total phenolic contents.

2.6 Antibacterial activity assay

Broth microdilution test was carried out according to Klančnik *et al*. (2010). Approximately 50 µL of *S. aureus* and *E. coli* suspension in Müeller Hinton Broth (MHB) medium was added to the wells of a sterile 96-well microtitre plate containing 50 µL of two-fold serially diluted MFSE or amoxicillin. The final volume in each well was 100 µL. Control wells were prepared with culture medium, bacterial suspension only, MFSE only and DMSO 20% in amounts corresponding to the highest quantity present. The contents of each well were mixed on a microplate shaker at 900 rpm for 1 min prior to 24 hrs incubation. The MIC was the lowest concentration where no viability was observed after 24 hrs on the basis of metabolic activity (Mourey and Canillac, 2002). Viability could be indicated by the

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presence of respiratory and ATP activity. To indicate respiratory activity, the presence of color was determined after adding 10 µL/well of TTC (2,3,5-triphenyltetrazoliumchloride) dissolved in water (TTC 20 mg/mL). The microplate was then incubated under appropriate cultivation conditions for 30 mins in the dark (Eloff, 1998). To determine the ATP activity, the bioluminescence signal was measured by a microplate reader after adding 100 µL/well of BacTiter-Glo™ reagent and after 5 min incubation in the dark (Klančnik *et al*., 2009). Positive controls were wells with a bacterial suspension in MHB and a bacterial suspension in MHB with DMSO 20% in amounts corresponding to the highest quantity present in the broth microdilution assay. Negative controls were wells with MHB and MFSE or amoxicillin. All measurements of MIC values were repeated in triplicate.

2.7 Pigment content analysis

The pigment content of MFSE analysis was carried out according to Srianta *et al*. (2016). The MFSE was dissolved in respective solvent and the absorbance was measured with UV-vis spectrophotometer at λ 400 nm, 470 nm, and 500 nm for yellow, orange, and red pigment, respectively. The pigment content was expressed as absorbance (nm) at the wavelength per gram of dry matter (AU/g).

2.8 Total phenolic content

The total phenolic content of MFSE was analyzed according to Srianta *et al*. (2014). Folin-Ciocalteu was used as a reagent and Gallic acid was used as a standard. A total of 0.1 mL of extract was put into a 10 mL volumetric flask and 0.5 mL of Folin-Ciocalteu reagent was added. The samples were left at room temperature for 5 min then added with 1.5 mL 20% (w/v) Na_2CO_3. The mixture was added with distilled water until the volume reached 10 mL. After 30 mins at room temperature, absorbance was measured at a wavelength of 765 nm versus blank using a spectrophotometer (Shimadzu UV 1800, Japan). Total phenolic content was expressed in mg GAE/g.

3. Results and discussion

3.1 Moisture content, color and biomass content of Monascus-fermented sorghum

The MFS powder had a moisture content of 8.72% (Table 1). The moisture content of food powder below 10% has a high stability for storage (Zambrano *et al*., 2019). The lightness (L*), redness (a*), yellowness (b*), chroma (C), and hue (^{o}h) values of the MFS powder are presented in Table 1. The positive a* and b* values of all the fermented products reflected that the powder color is a combination of red and yellow. Hue indicates a red color and chroma value indicates a dull-brownish red color. L* value below 50 indicates low lightness, resulting in a dark red color.

Fungal biomass, branched fibrous mycelium of *Monascus*, was formed during the fermentation. Generally, biomass comprises protein, lipids, polysaccharides, chitin (or chitosan), and various inorganic salts in different proportions (Isaza-Pérez *et al*., 2020). The biomass yield of MFS was recorded at 825.786 mg/g or 0.825 g/mL. It was higher than the biomass obtained from *M. purpureus* fermentation on potato waste, which ranged from 0.046 to 0.262 g/mL (inoculum concentration 7×10^5 spores/mL) (Abdel-Raheam *et al*., 2022). High biomass implies that sorghum was a suitable substrate for *M. purpureus* fermentation. Additionally, the high biomass could have resulted from the assistance of the steaming process (90°C for 60 mins), which helped soften the sorghum and facilitated easier utilization for the growth of *M. purpureus* (Zhao *et al*., 2018). Based on our previous research (Srianta and Harijono, 2015), the biomass of unsteamed dehulled sorghum only ranged from 26.64 to 36.70 mg/g (inoculum concentration 5×10^5 spores /mL).

3.2 Antibacterial activity

MIC value is defined as the lowest concentration of the assayed antimicrobial agent that inhibits the visible growth of the microorganism tested (Balouiri *et al*., 2016). A low MIC value indicates a high antibacterial activity. The MIC values of MFSE against *S. aureus* and *E. coli* are presented in Table 2. The extraction using ethyl acetate produced MFSE with the lowest MIC, indicating that ethyl acetate-extracted MFSE exhibited the highest antibacterial activity against both *S. aureus* and *E. coli*. Meanwhile, ethanol and water-extracted MFSE showed lower antibacterial activity, with water-extracted displaying the lowest. Most *Monascus* pigments are slightly polar, making them more soluble in organic solvents like ethanol than in water (Carvalho *et al*., 2007; Qian and Wu, 2010; Bai *et al*., 2022). As a result, the amount of *Monascus* pigments that are extracted by ethyl acetate and ethanol is larger (Table 3). This larger quantity of extracted pigments is responsible for the observed antibacterial activity, thus making ethyl acetate and ethanol-extracted MFSE have lower MIC than water-extracted MFSE.

In addition to *Monascus* pigments, *Monascus*-fermented products are known to contain significant amounts of phenolic compounds (Srianta *et al*., 2013; Razak *et al*., 2015). Ethanol-extracted MFSE contains the highest total phenolic content (Table 3). As per Haminiuk *et al*. (2014), phenolic compounds also exhibit

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Table 1. Moisture content, color and biomass content of MFS.

Moisture content (%)	Color					Biomass (mg/g)
	L* value	a* value	b*value	C	°h	
8.72	46.5	23.6	13.8	27.4	3.3	825.786

Table 2. MIC of amoxicillin and MFSE against *S. aureus* and *E. coli.*

Bacteria	Amoxicillin (µg/mL)	Ethyl acetate extract (mg/mL)	Ethanol extract (mg/mL)	Water extract (mg/mL)
Staphylococcus aureus ATCC 25920	3.125	3.125	12.5	100
Escherichia coli ATCC 25927	12.5	6.25	50	200

higher solubility in organic solvents that are less polar than water. Razak *et al*. (2015) found that phenolic acid present in *Monascus*-fermented rice bran extract is ρ-coumaric, ferulic, sinapic, vanillic, caffeic, and syringic acid. All of those phenolic acids exhibited antimicrobial properties (Liu *et al*., 2020). Amoxicillin, a common antibiotic medication against Gram-positive and Gram-negative bacteria, was used as a reference. The MIC values of all three MFSEs remain higher than amoxicillin which exhibited a MIC value of 3.125 µg/mL against *S. aureus* and 12.5 µg/mL against *E. coli*.

Several studies have explored the antibacterial properties of red, orange, and yellow *Monascus* pigment. Gökmen *et al*. (2021) discovered that *Monascus* red pigments had a MIC value of 128 mg/mL against *S. aureus* and >128 mg/mL against *E. coli*. They observed that the effect was stronger compared to using a commercial red pigment which had a MIC value of 256 mg/mL against *S. aureus* and >256 mg/mL against *E. coli*. Feng *et al*. (2019) that found orange pigments demonstrated antibacterial activity against *S. aureus*, displaying inhibition zone diameters ranging from 5-34 mm for orange pigment concentrations ranging from 0-10 mg/mL. Kim *et al*. (2006) discovered that amino acid derivatives of *Monascus* pigment showed a high antimicrobial activity. Derivatives containing L-Phe, D-Phe, L-Asp, D-Asp, L-Tyr, D-Tyr, L-Cys, and Nisin exhibited high activities against *S. aureus* (Gram-positive bacteria) with MIC values ranging from 8-16 µg/mL, while the control red pigment showed a MIC value of 64 µg/mL. For *E. coli* (Gram-negative bacteria), the same derivatives exhibited lower activities with MIC values ranging from 8-64 µg/mL, and the control red pigment with MIC value of >128 µg/mL. Kim and Ku (2018) reviewed several studies regarding the antimicrobial effects of *Monascus* pigments and concluded that Gram-positive bacteria were more prone to inhibition compared to Gram-negative bacteria.

Some studies suggested that the mechanism might involve the interaction of *Monascus* pigment with enzymes in the germinated spores and vegetative cells, restricting the use of iron and affecting cell membrane permeability. This could result in reduced transportation of nutrients, oxygen and metabolites (Kim *et al*., 2006; Xu, 2011). Feng *et al*. (2019) also observed that orange pigment causes *S. aureus* cells to wrinkle, damaging the membrane and causing protein leakage of bacteria cells. Meanwhile, amino acid derivatives of *Monascus* pigment depend on the hydrophobicity of the pigment and the amount of pigment adsorbed to the cell surface. The more hydrophobic, the higher the adsorption, which leads to limitation of oxygen uptake (Kim *et al*., 2006).

4. Conclusion

Sorghum is a suitable substrate to produce *Monascus* -fermented products whose extract has antibacterial activity. Ethyl acetate, ethanol, and water-extracted MFSE demonstrated antibacterial activity against *S. aureus* and *E. coli*. Ethyl acetate exhibited the highest activity, followed by ethanol and water extracts. All of the MFSE exhibited stronger activity on Gram-positive bacteria.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Table 3. Pigment and total phenolic contents of MFSE.

Solvent	Pigment content (AU/g)			Total phenolic content (mg/mL)
	Yellow	Orange	Red	
Ethyl acetate	177.3378	64.7206	52.4588	1.6974
Ethanol	153.8563	59.0424	66.1631	9.8037
Water	47.4565	24.5389	20.1564	2.3995

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