ANTIMICROBIAL PIGMENT FROM *FUSARIUM GRAMINEARUM*: OPTIMIZING CONDITIONS AND UTILIZING AGRO-INDUSTRIAL RESIDUES

Tugce DAG[1], Gulcan SAHAL[,1], Isil Seyis BILKAY[1]*

Address(es): Dr. Gulcan Sahal,
[1] Hacettepe University, Faculty of Sciences, Department of Biology (Biotechnology Section), 06800 Ankara, Turkey.

*Corresponding author: gulcanozbakir@gmail.com

ABSTRACT

Fusarium sp. produces various bioactive pigments widely used in pharmaceuticals, cosmetics, and the food industry. This study investigated the antimicrobial effect of the pigment produced from the highest pigment-producing *Fusarium* sp. strain and determined the optimum growth conditions and agro-industrial residues to obtain maximum pigment production. *Fusarium equiseti*, *Fusarium graminearum,* and *Fusarium poae* strains were tested in terms of their pigment production levels. Pigment quantification was assessed by a UV–Visible Spectrophotometer at 500 nm. Antimicrobial tests were determined by Disc Diffusion and Well Diffusion Methods. According to our results, the highest amount of pigment-producing strain was *F. graminearum* ($p<0.001$) and malt extract broth (MEB) was the optimum growth media ($p<0.001$). Extracted *F. graminearum* pigment was antimicrobial against *B. cereus* and *S. aureus* with a zone of inhibition diameters of 10.2 and 14.9 mm respectively. Initial pH levels of 8, 150 rpm rotation speed, 30 ºC temperature, and 9 days of incubation under the light condition in MEB media were determined as the optimum growth conditions for the highest reddish pigment production. Moreover, 10^{-2} times diluted molasses, Turkish feta cheese whey, and Turkish cheddar cheese whey were found as suitable low-cost growth media for reddish pigment production by *F. graminearum*. Our findings not only represent a pigment that might be used in the food industry as an antimicrobial bio-colorant but also show the potential use of molasses and whey as low-cost growth media for reddish pigment production by *F. graminearum*.

Keywords: Agro-industrial residues, antimicrobial bio-colorants, *F. graminearum*, food industry, microbial pigments

INTRODUCTION

Pigments are essential compounds that are widely used in industry, pharmacology, and medicine (**Delgado *et al.*, 2000; Malik *et al.*, 2012**) and are obtained both naturally and synthetically. Toxicity problems caused by synthetic pigments; carcinogenic properties of precursors used in the production of them and industrial byproducts of them on humans and the environment pose a danger in synthetical pigment production and use (**Boonyapranai *et al.*, 2008**). By this means, natural dyes and pigments in foodstuff, cosmetic and pharmaceutical manufacturing processes are regarded as important alternatives to potentially harmful synthetic dyes (**Velmurugan *et al.*, 2010**) and are becoming increasingly popular day by day (**Boonyapranai *et al.*, 2008; Velmurugan *et al.*, 2010**). Apart from this, since agro-industrial residues such as molasses and whey can also be used by various microorganisms as low-cost growth media, the production of pigments from microorganisms also provides conversion of industrial wastes into valuable bioproducts through biological processes (**Mehri *et al.*, 2021**). On the other hand, as a result of growing easily and quickly in a low-cost culture media and not being affected by the changes in climatic conditions, the production of pigments from microorganisms is an efficient and economical process compared to the production of pigments synthetically (**Boonyapranai *et al.*, 2008; Bouhri *et al.*, 2020; Velmurugan *et al.*, 2010; Waghela and Khan, 2018**). Moreover, the efficiency of pigment production can be easily improved by optimization of cultural parameters, mutation, and genetic engineering techniques when microbial sources are used (**Mapari *et al.*, 2005**). Microbial pigments are also reported to avoid food spoilage by displaying antimicrobial effects on various food pathogens (**Konuray and Erginkaya, 2015**). Considering the reasons for natural pigment usage in the food industry as; i) enhancing the present color of the food, ii) coloring the uncolored food, and iii) supplementing food with nutrients (**Konuray and Erginkaya, 2015**); antimicrobial natural pigments are preferable also to improve the shelf life of a particular foodstuff. *Fusarium* species are cosmopolitan, soilborne, and filamentous fungi known to cause a huge range of diseases on an extraordinary range of host plants (**Singha *et al.*, 2016**). Despite causing vascular wilt or root rot diseases in agricultural and ornamental crops throughout the world; many species belonging to *Fusarium* sp. are reported to produce various bioactive pigments widely used in pharmaceuticals, cosmetics, and the food industry (**Malini, 2018**). The objective of this study was to investigate the antimicrobial effect of the pigment produced by the highest pigment-producing *Fusarium* sp. strain, determine the optimal growth conditions for maximum pigment production, and assess the feasibility of conversion of agro-industrial wastes such as molasses and whey as low-cost growth media for pigment production by *Fusarium graminearum*. This study promotes the development of natural and antimicrobial pigment production, while also utilizing agro-industrial wastes as sustainable resources.

MATERIAL AND METHODS

Fusarium species

In this study, *Fusarium equiseti*, *Fusarium graminearum,* and *Fusarium poae* strains which were obtained from the Culture Collection of our laboratory in the Biology Department (Biotechnology Division) of Hacettepe University were used. These cultures were grown in Potatoe Dextrose Agar (PDA) (Merck) media for 7 days at 30 °C and stored at +4 °C for use in further experiments.

Inoculum preparation and growth conditions

For inoculum preparation, the fungal strains were first grown on a PDA medium for 7 days at 30 °C. Following incubation, 5 mm² plugs from the outer zone of the fungal colony were taken out with a sterile inoculation loop and transferred into a 10 mL sterile saline solution to obtain inoculums as fungal suspensions. 10 mL fungal suspensions were grown in a 250 mL flask containing 100 mL of Potato Dextrose Broth (PDB), Sabouraud Dextrose Broth (SDB), and Malt Extract Broth (MEB) (Merck), with an initial pH level of 5.5 for 7 days at 30 °C and 150 rpm rotation speed (**Pradeep and Pradeep, 2013**).

Dry cell weight and pigment quantification

Incubated fungal cultures were first filtered with a filter paper and the mycelia biomass yield was dried at 30 ºC for 48 h. Following this, filtered supernatants were centrifuged at 3220g for 10 min at 10 °C (Eppendorf 5810R, with an Eppendorf Swing-bucket rotor A-4-62, Hamburg, Germany). After centrifugation, supernatants were evaluated in terms of pigment production with the method described by **Pradeep *et al.*, (2013)**. Briefly, quantification of the fungal pigments was done by measuring the absorbance of the broth after centrifugation at 500 nm using a spectrophotometer (UV–Visible Spectrophotometer; Shimadzu UV-1700, Kyoto, Japan). This study evaluated the absorbance of PDB, SDB, and MEB

without any growth as negative control and set as zero in terms of pigment production.

Antimicrobial effect of the extracted pigment

In this part of the study, *E. coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella paratyphi* ATCC 9150, *Staphylococcus aureus* ATCC 25923 strains which were obtained from the Culture Collection of our laboratory in the Biology Department (Biotechnology) of Hacettepe University and *Bacillus cereus* isolate (Reference Strain: *Bacillus cereus* ATCC 14579) (**Ozyurek and Bilkay, 2017**) were used. The antimicrobial effect of the extracted *F. graminearum* pigment against these microbial strains was determined. These microbial strains were first grown in Nutrient Agar at 37 °C for 24 hours and stored at +4 °C for use in further experiments.

Pigment extraction

The pigment produced by *F. graminearum* was extracted by the solvent extraction method, described by **Velmurugan *et al.*, (2009)**. Briefly, 10 mL *F. graminearum* suspensions were grown in 100 mL of MEB (pH: 5.5) and incubated at 30 °C, 150 rpm rotation speed for 7 days. Following incubation, *F. graminearum* culture was filtered with a filter paper and the supernatant was centrifugated at 3220g for 10 min at 10°C (Eppendorf 5810R, with an Eppendorf Swing-bucket rotor A-4-62, Hamburg, Germany). After centrifugation, 10 mL of the centrifugated supernatant was mixed with 20 mL ethanol solution (90%) inside the tubes. Following this, these tubes were kept inside the shaking incubator at 200 rpm rotation speed for 1 hour. Then, the tubes were kept for 15 min at 25 °C. Then, the pigment-ethanol mixture was filtered with Whatman no:1 filter paper and the filtered mixture including fungal pigment was dried at 30 °C for 48 hours. The dry pigment (20 mg) was dissolved in 1 mL sterile distilled water and kept to be used for the antimicrobial tests.

Antimicrobial test

The antimicrobial effect of the extracted pigment against the selected microorganisms was determined by Disk Diffusion and Agar Well Diffusion Methods (**Bilen Ozyurek *et al.*, 2017; Hudzicki, 2012**). Briefly, single colonies of different bacterial strains were inoculated into 10 mL BHI broth (Lab M Ltd, Lancashire, UK) and incubated for 24 hours at 37 °C. After incubation, cells were harvested by centrifugation for 10 min at 10 °C (Eppendorf Centrifuge 5417C, Germany) washed 3 times with sterile saline solution, and were adjusted to 0.5 McFarland standard turbidity inside the sterile saline solution. 100 µL of a microbial suspension corresponding to a 0.5 McFarland standard turbidity was added to the Mueller-Hinton Agar medium and spread over the entire agar surface. After waiting for 15 min, pits with 1 cm diameter were prepared under sterile conditions on the agar plate. 20 µL of the extracted pigment, which was sterilized for an hour under UV light, was added to the previously prepared pits. All the plates were incubated for 24 h at 37°C and the diameters of zones of inhibitions (ZOI) were measured using a ruler. For the determination of the antimicrobial effect of the extracted pigment, 20 µL sterile distilled water was used as a negative control; whereas Vancomycin (30 µg.disk⁻¹) disk was used as a positive control.

Determination of optimum growth parameters for pigment production by *F. graminearum*

Effect of initial pH, temperature, different incubation conditions and periods were assessed to determine optimum growth parameters for pigment production by *F. graminearum* (**Boonyapranai *et al.*, 2008**).

Effect of initial pH

10 mL *F. graminearum* suspensions were grown in a 250 mL flask containing 100 mL of MEB with different pH levels covering the range from pH: 5 to 10 and incubated for 7 days at 150 rpm rotation speed, keeping the temperature constant at 30 °C for each group. Following incubation with different pH levels, dry cell weight and pigment quantification were assessed as previously described.

Effect of Temperature

10 mL *F. graminearum* suspensions were grown in a 250 mL flask containing 100 mL of MEB and incubated at three different temperatures as 25 °C, 30 °C, and 37 °C for 7 days at 150 rpm rotation speed, keeping pH constant at 5.5 for each group. Following incubation with different temperatures, dry cell weight and pigment quantification were assessed as previously described.

Effect of incubation under static/shaking conditions

10 mL *F. graminearum* suspensions were grown in a 250 mL flask containing 100 mL of MEB with a constant pH of 5.5 and incubated for 7 days, keeping the temperature also constant at 30 °C for each group under static and shaking (at 150 rpm rotation speed) conditions. Following incubation under static and shaking conditions, dry cell weight and pigment quantification were assessed as previously described.

Effect of incubation under light/dark conditions

10 mL *F. graminearum* suspensions were grown in a 250 mL flask containing 100 mL of MEB with a constant pH of 5.5 and incubated for 7 days, keeping the temperature also constant at 30 °C for each group under a light source (Cool White; Philips T8, Master TL-D, T8 51, Watt 840) and dark conditions at 150 rpm rotation speed. Following incubation under a light source and dark conditions, dry cell weight and pigment quantification were assessed as previously described.

Effect of incubation period in days

10 mL *F. graminearum* suspensions were grown in a 250 mL flask containing 100 mL of MEB and incubated for different incubation periods covering the range from 2 days to 10 days at 150 rpm rotation speed, keeping the temperature constant at 30 °C and the initial pH as 5.5 for each group. Following different incubation periods, pigment quantification was established as previously described. In this part of the study, pigment quantification was assessed every 24 hours from the same growing *F. graminearum* culture. Therefore, *F. graminearum* culture continued growing every day and dry cell weight was unable to be calculated for each day of incubation.

Pigment production from agro-industrial residues

Pigment production from agro-industrial residues was carried out using a modified version of the method described by **Lopes *et al.*, 2013**. In this part of the study, molasses, Turkish Cheddar Cheese Whey (TCCW), and Turkish Feta Cheese Whey (TFCW) as agro-industrial residues were used for pigment production. Molasses was obtained from one of the sugar factories in Ankara, Turkey; whereas TCCW and TFCW were obtained from One of the farm factories in Ankara, Turkey. Both deproteinized and non-deproteinized forms of TCCW and TFCW and their 10⁻² dilutions and 10⁻² times diluted molasses were tested in this study. To prepare deproteinized whey, TCCW and TFCW were boiled for 10 min. Afterward, they were centrifuged at 3220g for 10 min at 10°C (Eppendorf 5810R, with an Eppendorf Swing-bucket rotor A-4-62, Hamburg, Germany) and filtered through Whatman no.1 filter paper. The filtered supernatant was sterilized under autoclave at 110 °C, 1.5 atm for 15 min. For inoculum preparation, the fungal strains were first grown on a PDA medium for 7 days at 30 °C. Following incubation, 1 mm² plug from the outer zone of the fungal colony was taken out with a sterile inoculation loop and transferred into a 250 mL flask containing 100 mL MEB with an initial pH level of 5.5, and the flask was incubated for 7 days at 30 °C and 150 rpm rotation speed. For pigment production from agro-industrial residues, 5 mL *F. graminearum*, grown in 100 mL MEB was transferred into: i) 100 mL 10⁻² diluted molasses (sterilized under autoclave at 110 °C, 1.5 atm for 15 min.) ii) 100 mL 10⁻² diluted TFCW iii) 100 mL 10⁻² diluted TCCW, iv) 100 mL 10⁻² diluted deproteinized TFCW, v) 100 mL 10⁻² diluted deproteinized TCCW, vi) 100 mL TFCW, vii) 100 mL TCCW, viii) 100 mL deproteinized TFCW, viii) 100 mL deproteinized TCCW. Incubation was applied for 7 days at 30° C and 150 rpm rotation speed with an initial pH level of 8. Pigment quantification was assessed for *F. graminearum* grown in molasses by the method, described and the amounts of pigments, produced in whey were observed visually.

Statistical Analysis

The Shapiro–Wilk test was conducted to analyze the normality of the data. In case the results were found as normally distributed, a t-test was used for evaluation of our results. $P<0.05$ was considered statistically significant. Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) version 23 Software (IBM Corp, New York, USA).

RESULTS AND DISCUSSION

Colors have been widely used in various industries such as food, textile, cosmetics, and pharmaceuticals for many years (**Tuli *et al.*, 2015**). However, hyper allergenic, carcinogenic, and toxicological problems caused by most synthetic colorants have made it necessary to replace synthetic pigments with natural pigments and use natural colors obtained from pigment-producing organisms including plants, animals, and microorganisms as colorants (**Reyes et al., 1996; Tuli *et al.*, 2015**). Among all these organisms, microorganisms are generally considered as main sources of natural pigment production due to their availability, stability, and cost efficiency (**Tuli *et al.*, 2015**).

Growth and pigment production of *Fusarium equiseti*, *Fusarium graminearum*, and *Fusarium poae* species in different media

In the first part of this study, we tested growth and pigment production by *Fusarium graminearum*, *Fusarium poae*, and *Fusarium equiseti* species, in three different media as Malt Extract Broth (MEB), Potato Dextrose Broth (PDB), and

Sabouraud Dextrose Broth (SDB). Pigment production by all *Fusarium* species were found to be normally distributed and therefore t-test was applied revealing that pigment production statistically differs from each other. According to our results, the highest amount of pigment production by all *Fusarium* species was observed in MEB (Fig. 1). Since significantly more pigment production by *F. graminearum* in MEB media ($p<0.001$) was observed, both *F. graminearum*

and MEB media were selected to be used for further experiments. Our finding that shows significantly higher pigmentation of all the three tested *Fusarium* species in MEB ($p<0.001$), was in line with one of the studies, reporting lighter pigmentations in the media without organic nitrogen sources such as peptone, yeast extract, and malt extract (**Pradeep *et al.*, 2013**).
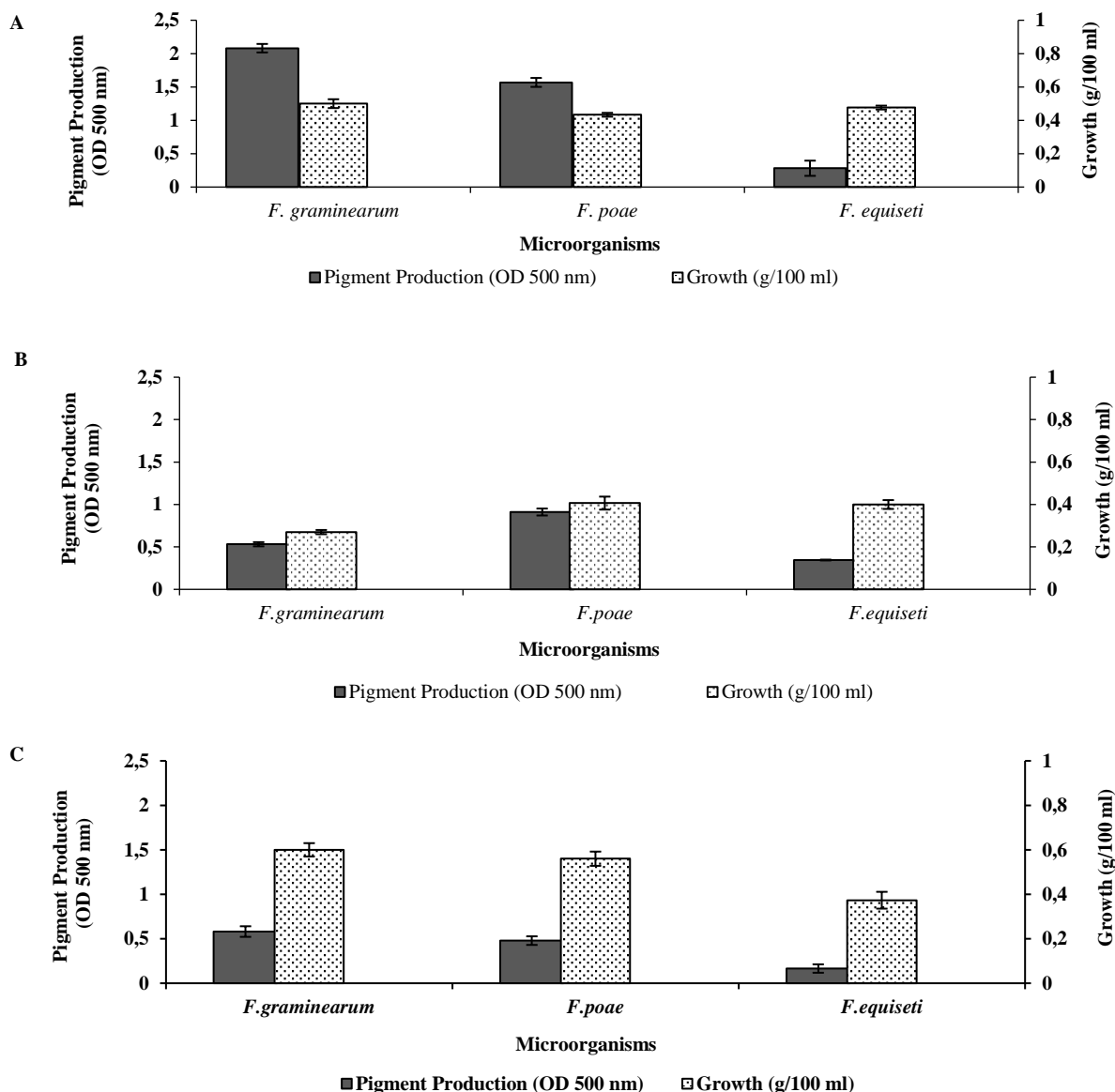

Figure 1 Pigment production and growth by *Fusarium graminearum*, *Fusarium poae,* and *Fusarium equiseti* strains in A) Malt Extract Broth, B) Potato Dextrose Broth, C) Sabouraud Dextrose Broth media. Error bars indicate standard deviations over triplicate experiments with separately grown fungal cells

Antimicrobial effect of the extracted *F. graminearum* pigment

F. graminearum colonizes on wheat plant surfaces with specialized unbranched hyphae called runner hyphae (RH) and is known as one of the most destructive plant pathogens worldwide, causing fusarium head blight (FHB) on cereals (**Mentges *et al.*, 2020**). Despite its destructive effect and being responsible for losses of over $2.7 billion in the United States between 1998 and 2000; *F. graminearum* is one of the most important fungal species by producing pigments such as aurofusarin, rubrofusarin, and culmorin (**Cambaza, 2018**). The reddish pigmentation of *F. graminearum* has been reported to be the result of aurofusarin

deposition in the cell walls (**Malz *et al.*, 2005**) in one of the previous studies. However recently, rubrofusarin synthesized by *F. graminearum* has been reported to be a red naphthoquinone pigment having antimicrobial, antiallergic, and phytotoxic properties (**Poorniammal *et al.*, 2021**). In this part of the study, the antimicrobial effect of the extracted *F. graminearum* pigment against 5 different bacterial species (*Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*) were tested. According to our results, extracted *F. graminearum* pigment exhibited an antimicrobial effect against *B. cereus* and *S. aureus* strains (Table 1 and Fig. 2).

Table 1 Antimicrobial effect of the extracted *F. graminearum* pigment against *Bacillus cereus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella paratyphi,* and *Staphylococcus aureus* strains by disc diffusion method.

Diameter Zone of Inhibition (mm)

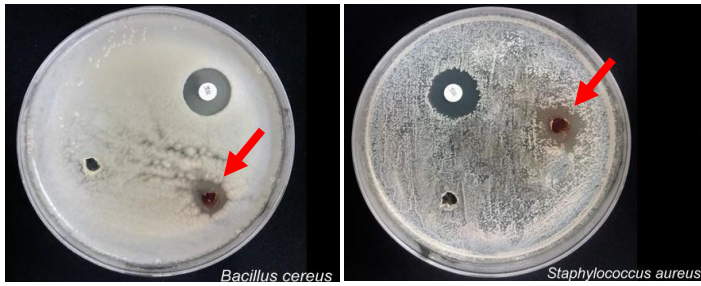| Tested Microorganisms	*B. cereus*	*E. coli*	*K. pneumoniae*	*P. aeruginosa*	*S. paratyphi*	*S. aureus*
Sterile Distilled Water (20 µL)	0.0	0.0	0.0	0.0	0.0	0.0
Pigment (20 µL)	10.2	0.0	0.0	0.0	0.0	14.9
Vancomycin (30 µg)	15.5	15.4	15.6	15.7	15.5	16.1

Figure 2 Images of antimicrobial effect of the extracted *F. graminearum* pigment against *B. cereus* and *S. aureus* strains (Red arrows present zone of inhibitions)

Among these *F. graminearum* pigment sensitive bacterial strains; *B. cereus* is known to be responsible for Food-Borne Diseases (FBD) such as diarrheal and an emetic illness that is caused by the ingestion of foods contaminated with *B. cereus* (**Griffiths *et al.*, 2017**). Apart from this, *S. aureus* is also reported as one of the significant causes of FBD, causing an estimated 241,000 illnesses per year in the United States (**Kadariya *et al.*, 2014**). By this means, according to our findings, the reddish pigment of *F. graminearum* that has been investigated in this study can be one of the most useful bio-colorants in the food industry not only because it is derived from a microbial source but also because it inhibits some food contaminants such as *B. cereus* and *S. aureus* that have been responsible for various FBD for years. In this respect, optimum incubation conditions to obtain the highest amount of pigment production by *F. graminearum* were aimed to be determined for the next part of this study.

Determination of optimum growth parameters for pigment production by *F. graminearum*

In this part of this study, the growth rate of *F. graminearum* was nearly the same when the pH values are adjusted to pH 5-8 (Fig. 3A). However, higher pigment production by *F. graminearum* was observed between pH levels of 6 and 8 (Fig. 3A). Among all the pH levels, the highest pigment production by *F. graminearum* was observed under the pH level of 8 by causing significantly higher pigment production compared pH levels of 5, 6 and 7 ($p<0.001$) (Fig. 3A). Following the increase in pH level (pH > 8) both the growth rate of and the pigment production by *F. graminearum* decreased (Fig. 3A). To determine the optimum temperature level for the reddish pigment production by *F. graminearum*; temperature levels of 25, 30 and 37 ºC were tested. Reddish pigment production and growth were observed at 25 ºC and 30 ºC (Fig. 3B). However, pigment production by *F. graminearum* at 30 ºC was significantly higher than the pigment production at 25 ºC and 30 ºC ($p<0.001$) (Fig. 3B). At 37 ºC, both the growth rate of and the pigment production by *F. graminearum* decreased (Fig. 3B). When the pigment production by *F. graminearum* is tested under static and shaking incubation conditions, reddish pigment production and growth was observed only in shaking incubation conditions (Fig. 3C). Apart from this, although there was growth under light and dark incubation conditions, reddish pigment production by *F. graminearum* was observed only in the light provided incubation conditions (Fig. 3D). Fig. 3E shows the effect of incubation periods in days on the reddish pigment production by *F. graminearum*. According to our results, reddish pigment production increasingly continued until the 9th day of incubation and a significant decrease was observed afterward ($p<0.05$). Therefore, 9 days of incubation was determined as the optimum incubation day for the reddish pigment production by *F. graminearum*. To sum up, optimum pigment production by *F. graminearum* was observed under light-provided conditions, with an initial pH level of 8, at 150 rpm shaking speed, 30 ºC temperature and for 9 days of incubation (Fig. 3).
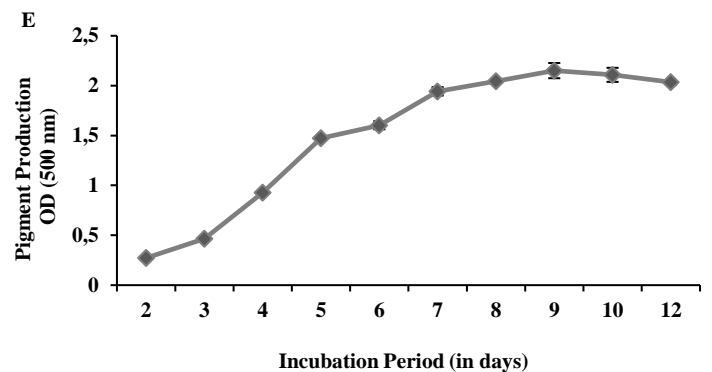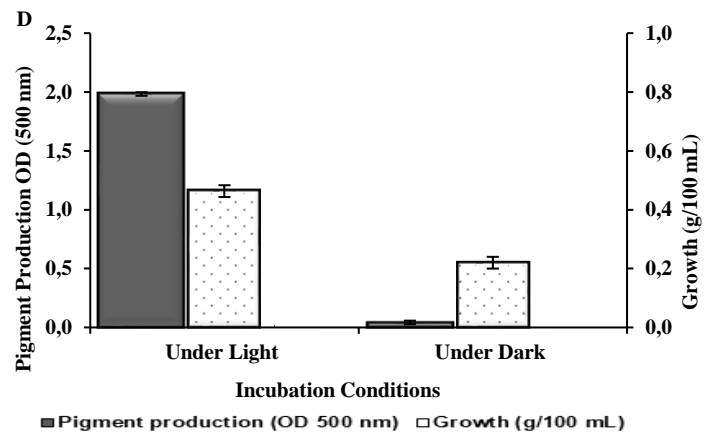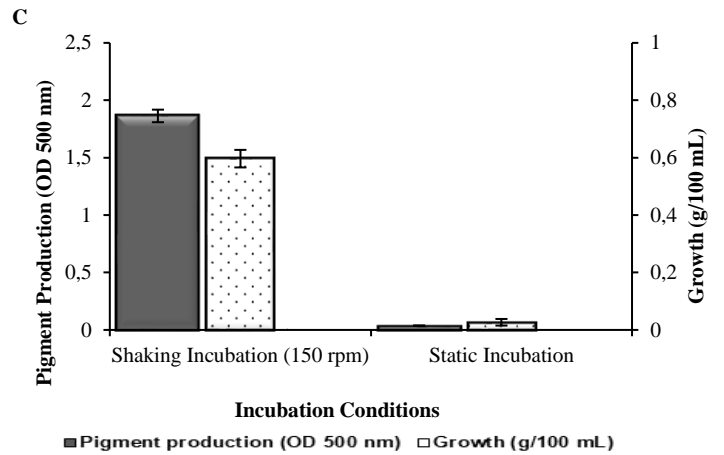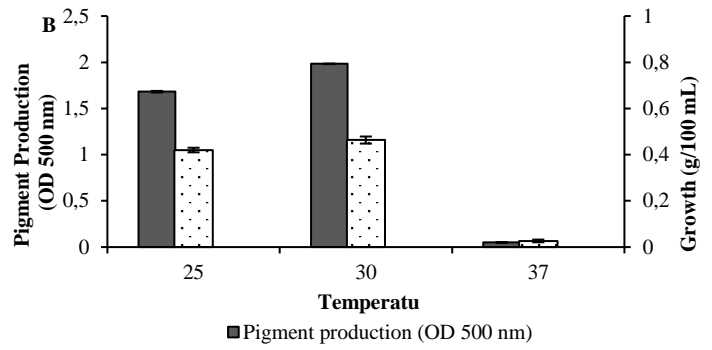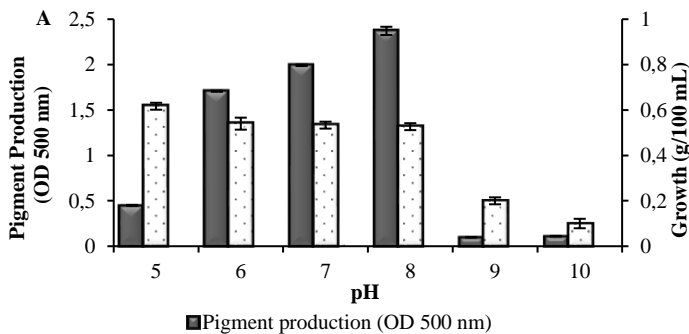

Figure 3 Pigment production and growth by *Fusarium graminearum* A) in different pH levels, B) in different temperatures (ºC), C) in static and shaking incubation conditions, D) under light and dark incubation conditions, E) pigment production by *Fusarium graminearum* under different incubation periods (in days). Error bars indicate standard deviations over triplicate experiments with separately grown fungal cells. (Detail information on growth conditions that are kept constant is given).

Similar to our findings, in the studies carried out on pigment production by *Fusarium* sp., the highest mycelial growth and pigment production have been reported to be seen at 28 ºC temperature for 8-10 days of incubation under shaking conditions (**Malini, 2019; Pradeep *et al.*, 2013**). However, pH levels of 5 and 5.5 have been reported as the optimum levels for the highest growth and pigment production by *F. graminearum* (**Malini, 2019; Pradeep *et al.*, 2013**). Therefore, the pH level has been kept constant at 5.5, during the determination of the optimum growth parameters for pigment production by *F. graminearum*. However, different from the other studies carried out on pigment production by *Fusarium* sp.; the highest mycelial growth and pigment production was seen at a pH level of 8 in our study ($p<0.001$).

Pigment production by *F. graminearum* from agro-industrial residues

Agro-industrial residues are known as many different wastes from the food and agriculture industry including multiple plant-based materials, such as straws, stems, stalks, leaves, husks, shells, peels, lint, seeds, pulps, stubbles, bagasse, spent coffee grounds, brewer's spent grains, and some animal byproducts, including feathers and whey which are also known to be rich in nutrient components, such as carbohydrates, proteins, fibers, minerals, and vitamins (**Lopes and Ligabue-Braum, 2021**). Nowadays, researchers have focused on various biotechnological approaches that aim to convert food waste into valuable bioproducts through various biological processes (**Mehri *et al.*, 2021**). In accordance with this aim, the effect of molasses and whey on the reddish pigment production by *F. graminearum* was tested in the last part of our study. According to our findings, 10^{-2} times diluted molasses was suitable for reddish pigment production by *F. graminearum* (Fig. 4). Apart from this, although the growth of *F. graminearum* was significantly lower in 10^{-2} diluted versions of TFCW and TCCW ($p<0.001$), more reddish pigment production by *F. graminearum* was visually observed in 10^{-2} diluted versions of TFCW and TCCW (Fig. 5). Additionally, an increase in reddish color was also visually observed after deproteinization of undiluted TFCW and TCCW (Fig. 5).
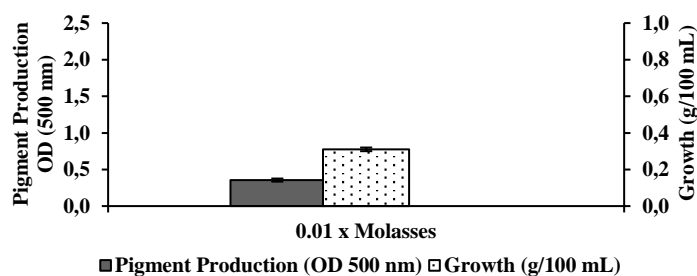

Figure 4 Pigment production and growth by *F. graminearum* after incubation in 10^{-2} diluted Molasses. Incubation was provided for 7 days under light conditions at 30 °C, 150 rpm rotation speed with an initial pH level of 8. Error bars indicate standard deviations over triplicate experiments with separately grown fungal cells

Figure 5 Growth of *F. graminearum* after incubation in different TFCW and TCCW conditions. Incubation was provided for 7 days under light conditions at 30 °C, 150 rpm rotation speed with an initial pH level of 8. Error bars indicate standard deviations over 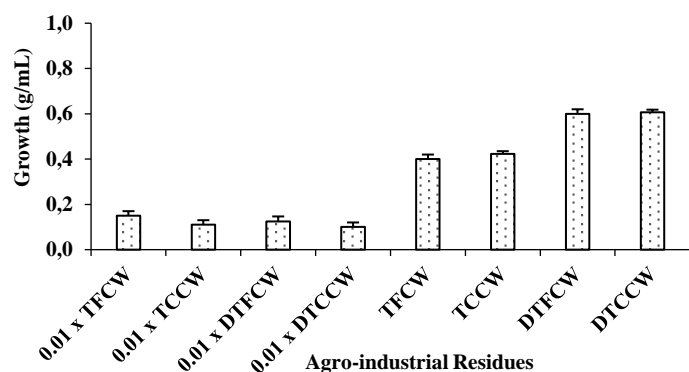triplicate experiments with separately grown fungal cells. *TFCW: Turkish Feta Cheese Whey, TCCW: Turkish Cheddar Cheese Whey, DTFCW: Deproteinized Turkish Feta Cheese Whey, DTCCW: Deproteinized Turkish Cheddar Cheese Whey*

In 2013, 250 million tons of agro-industrial wastes have been reported to be generated, globally per year, during processing of different plant crops, mainly cereals, starchy roots, fruits, and other vegetables (**Heredia-Guerrero *et al.*, 2017**). Because, the utilization of this waste eliminates both the disposal problems and the environment pollution (**Lopes and Ligabue-Braum, 2021**). The bio-colorants derived from agro-industrial sources offers various potential applications. Some of the possible agro-industrial applications of bio-colorants include their use in food products, beverages, cosmetics, pharmaceuticals, and textile dyeing (**Lopes *et al.*, 2013**). However, it is essential to consider the possible risk of mycotoxins associated with bio-colorants derived from fungal sources as well (**Lopes *et al.*, 2013; Pessôa *et al.*, 2017; Cambaza *et al.*, 2018**);
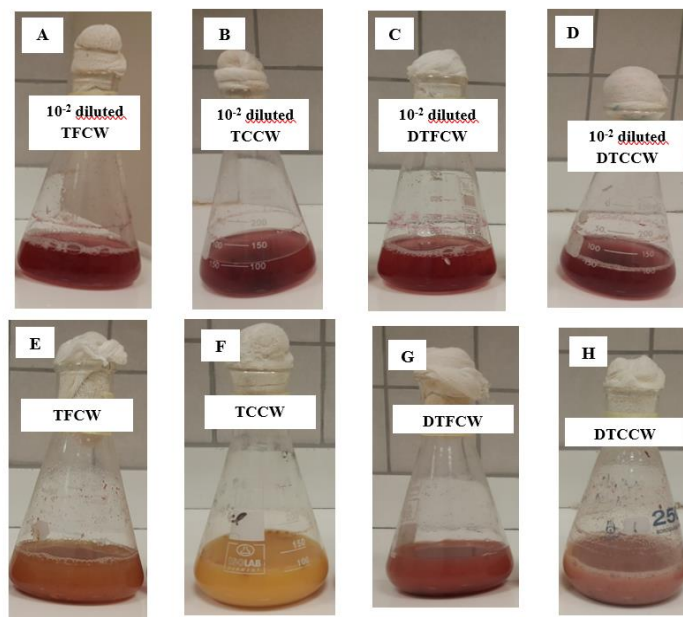

Figure 6 Pigment production by *F. graminearum* after incubation in A) 10^{-2} diluted TFCW, B) 10^{-2} diluted TCCW, C) 10^{-2} diluted deproteinized TFCW, D) 10^{-2} diluted deproteinized TCCW E) TFCW, F) TCCW, G) Deproteinized TFCW, H) Deproteinized TCCW. Incubation was performed for 7 days under light conditions at 30 °C and 150 rpm rotation speed with an initial pH level of 8. The reddish color indicates pigment production. **TFCW: Turkish Feta Cheese Whey, TCCW: Turkish Cheddar Cheese Whey, DTFCW: Deproteinized Turkish Feta Cheese Whey, DTCCW: Deproteinized Turkish Cheddar Cheese Whey*

Molasses is obtained from sugar cane as a byproduct of the white sugar production process and has been reported to contain polysaccharides, sucrose, glucose, fructose, amino acids, and some inorganic salts (**Akar and Canbaz, 2016; Qiang *et al.*, 2019**). By comprising complex lignocellulosic carbon-containing compounds, molasses are also reported as a low-cost energy source for microbial growth and the production of microbial metabolites under different fermentation conditions (**Hamano and Kilikian, 2006; Mustafa *et al.*, 2020**). In line with this information, using molasses instead of glucose as a supplementary material has been reported as more advantageous for the red pigment production by *Monascus Purpureus* AHK12 via solid state fermentation by offering a lower cost than using other sugars (**Nimnoi *et al.* 2015**). In our study, 10^{-2} times diluted molasses is also determined as a suitable growth media for reddish pigment production by *F. graminearum* (Fig. 4).

Whey is one of the major wastes generated by dairy industries and has been considered as an important environmental pollutant due to its high organic content for years (**Guven *et al.*, 2012; Hausjell *et al.*, 2019; Mehri *et al.*, 2021**). Since whey is rich in organic ingredients such as carbon and nitrogen, it is regarded as a low-cost, robust, and sustainable microbial pigment production medium. Additionally, the utilization of whey with bioprocesses reduces environmental pollution. In this respect, similar to one of the recent studies that investigate the production of red color pigment from whey by using *Monascus purpureus* (**Mehri *et al.*, 2021**), we aimed to test whether or not pigment production by *F. graminearum* is also possible when agro-industrial residues such as TFCW and TCCW are used as growth media in the last part of this study. According to our findings, although the growth of *F. graminearum* was significantly lower in 10^{-2} times diluted TFCW, TCCW, DTFCW, and DTCCW ($p<0.001$) (Fig. 5); more reddish pigment production by *F. graminearum* was visually observed in them, compared to their non-diluted versions (Fig. 6). Additionally, more pigment production by *F. graminearum* in deproteinized versions of non-diluted TFCW and TCCW were visually observed. Similar to our findings, the water-soluble yellow pigment has been reported to be produced from *Penicillium aculeatum* ATCC 10409 in submerged culture using a whey media (**Afshari *et al.*, 2015**). Additionally, a recent study with pigment-producing fungal isolates from soil (including *Fusarium solani*) has reported pigment production by these isolates on green waste and whey cocktail media using submerged and solid-state fermentation methods (**Molelekoa *et al.*, 2021**). To the best of our knowledge, this is the first report showing the reddish pigment production by *F. graminearum* using agro-industrial residues of molasses and whey. Fungal species, such as those belonging to the *Fusarium* genus, have been found to have the capability to

produce mycotoxins, which are toxic secondary metabolites (**Pessôa *et al.*, 2017**). Therefore, the detection of mycotoxins in bio-colorants is crucial to ensure consumer safety and compliance with regulatory standards.

CONCLUSION

In conclusion, this study demonstrates that the reddish *F. graminearum* pigment obtained in this study is antimicrobial against two important food pathogens of *B. cereus* and *S. aureus*. Additionally, an initial pH level of 8, 150 rpm rotation speed, 30 ºC temperature, and 9 days of incubation under the light condition in Malt Extract Broth (MEB) are the optimum growth conditions for reddish pigment production at maximum levels. Moreover, the utilization of agro-industrial residues such as molasses and whey as cost-effective growth media has been successfully determined. These findings highlight the potential of this reddish pigment as an antimicrobial bio-colorant for the food industry and the conversion of industrial residues into valuable bioproducts. However, it is important to conduct further research, including safety assessment, to explore the applicability of this pigment in diverse food applications.

Conflicts of Interest: The authors declare that there are no conflicts of interest.

Authors' Contribution

Conceptualization: TD and ISB and GS; Supervision: ISB; Investigation: TD; Data analysis: TD, GS, and ISB; Writing – first draft: TD and GS; Review and editing: GS and ISB. All authors have read and agreed to the published version of the manuscript.

REFERENCES

Afshari, M., Shahidi, F., Mortazavi, S.A., Tabatabai, F., & Eshaghi, Z. (2015). Investigating the influence of pH, temperature and agitation speed on yellow pigment production by *Penicillium aculeatum* ATCC 10409. *Natural Product Research*, 29 (14), 1300–1306. https://doi.org/10.1080/14786419.2014.999059

Akar, C., & Canbaz, M. (2016). Effect of molasses as an admixture on concrete durability. *Journal of Cleaner Production,* 112, 2374–2380. https://doi.org/10.1016/j.jclepro.2015.09.081

Bilen Ozyurek, S., Seyis Bilkay, I., & Diken Gur, S. (2017). Antimicrobial Activity of Thyme and Rosemary Oils against *Pseudomonas aeruginosa* Strains. *Hacettepe Journal of Biology and Chemistry*, 45, 435–442. https://doi.org/10.15671/hjbc.2018.185

Boonyapranai, K., Tungpradit, R., Lhieochaiphant, S., & Phutrakul, S. (2008). Optimization of submerged culture for the production of naphthoquinones pigment by *Fusarium verticillioides*. *Chiang Mai Journal of Science*, 35(3), 457–466.

Bouhri, Y., Askun, T., Tunca, B., Deniz, G., Aksoy, S.A., & Mutlu, M. (2020). The orange-red pigment from *Penicillium mallochii*: Pigment production, optimization, and pigment efficacy against Glioblastoma cell lines. *Biocatalysis and Agricultural Biotechnology*, 23. https://doi.org/10.1016/j.bcab.2019.101451

Cambaza, E. (2018). Comprehensive description of *Fusarium graminearum* pigments and related compounds. *Foods,* 7 (10). https://doi.org/10.3390/foods7100165

Delgado Vargas, F., Jiménez, A.R., Paredes López, O., & Francis, F.J. (2000). Natural pigments: Carotenoids, anthocyanins, and betalains - Characteristics, biosynthesis, processing, and stability. *In Critical Reviews in Food Science and Nutrition*, 40, 173-289. https://doi.org/10.1080/10408690091189257

Griffiths, M.W., & Schraft, H. (2017). *Bacillus cereus* Food Poisoning. In:Foodborne Diseases, 3rd edn. Elsevier Inc, Amsterdam, 395-405, https://doi.org/10.1016/B978-0-12-385007-2.00020-6

Guven, G., Perendeci, A., Ozdemir, K., & Tanyolaç, A. (2012). Specific energy consumption in electrochemical treatment of food industry wastewaters. *Journal of Chemical Technology and Biotechnology*, 87(4), 513–522. https://doi.org/10.1002/jctb.2739

Hamano, P.S. & Kilikian, B.V. (2006). Production of red pigments by *Monascus ruber* in culture media containing corn steep liquor. *Brazilian Journal of Chemical Engineering*, 23 (4), 443–449. https://doi.org/10.1590/S0104-66322006000400002

Hausjell, J., Miltner, M., Herzig, C., Limbeck, A., Saracevic, Z., Saracevic, E., Weissensteiner, J., Molitor, C., Halbwirth, H., & Spadiut, O. (2019). Valorisation of cheese whey as substrate and inducer for recombinant protein production in *E. coli* HMS174(DE3). *Bioresource Technology Reports,*8, 100340. https://doi.org/10.1016/j.biteb.2019.100340

Heredia-Guerrero, J. A., Heredia, A., Dominguez, E., Cingolani, R., Bayer, I. S.,

Athanassiou, A., et al. (2017). Cutin from agro-waste as a raw material for the production of bioplastics. *Journal of Experimental Botany*, 68, 5401–5410. https://doi.org/10.1093/jxb/erx272

Hudzicki, J. (2012). Kirby-Bauer Disk Diffusion Susceptibility Test Protocol Author Information. *American Society For Microbiology: chrome-extension://efaidnbmnnnibpcajpcglclefindmkaj/https://asm.org/getattachment/25 94ce26-bd44-47f6-8287-0657aa9185ad/Kirby-Bauer-Disk-Diffusion-Susceptibility-Test-Protocol-pdf.pdf*.

Kadariya, J., Smith, T. C., & Thapaliya, D. (2014). *Staphylococcus aureus* and Staphylococcal Food-Borne Disease: An Ongoing Challenge in Public Health. *BioMed Research International*, 2014. https://doi.org/10.1155/2014/827965

Konuray, G., & Erginkaya, Z. (2015). Antimicrobial and antioxidant properties of pigments synthesized from microorganisms. *The Battle Against Microbial Pathogens: Basic Science, Technological Advances and Educational Programs*, 17, 27–32.

Lopes, F.C., Tichota, D.M., Pereira, J.Q., Segalin, J., Rios Ade, O., & Brandelli, A. (2013). Pigment production by filamentous fungi on agro-industrial byproducts: an eco-friendly alternative. *Applied Biochemistry and Biotechnology*, 171(3), 616-25. https://doi.org/10.1007/s12010-013-0392-y

Lopes F.C., & Ligabue-Braum R. (2021). Agro-Industrial Residues: Eco-Friendly and Inexpensive Substrates for Microbial Pigments Production. *Frontiers in Sustainale Food Systems*, 5, 1-16. https://doi.org/10.3389/fsufs.2021.589414

Malik, K., Tokkas, J., & Goyal, S. (2012). Microbial Pigments: A review. *International Journal of Microbial Resource Technology*. 1, 361-1365.

Malini, H., & Razia, D. (2018). Optimization of Pigment Production in *Fusarium incarnatum*. *IJRAR* 5, 450-60.

Malz, S., Grell, M.N., Thrane, C., Maier, F.J., Rosager, P., Felk, A., Albertsen, K.S., Salomon, S., Bohn, L., Schäfer, W., & Giese, H. (2005). Identification of a gene cluster responsible for the biosynthesis of aurofusarin in the *Fusarium graminearum* species complex. *Fungal Genetics and Biology*, 42, 420–433. https://doi.org/10.1016/j.fgb.2005.01.010

Mapari, S.A.S., Nielsen, K.F., Larsenn, T.O., Frisvad, J.C., Meyer, A.S., & Thrane, U. (2005). Exploring fungal biodiversity for the production of water-soluble pigments as potential natural food colorants. *Current Opinion in Biotechnology*, 16(2), 231–238. https://doi.org/10.1016/j.copbio.2005.03.004

Mehri, D., Perendeci, N.A., & Goksungur, Y. (2021). Utilization of whey for red pigment production by *Monascus purpureus* in submerged fermentation. *Fermentation ,* 7, 1–17. https://doi.org/10.3390/fermentation7020075

Mentges, M., Glasenapp, A., Boenisch, M., Malz, S., Henrissat, B., Frandsen, R.J.N., Güldener, U., Münsterkötter, M., Bormann, J., Lebrun, M.H., Schäfer, W., & Martinez Rocha, A.L. (2020). Infection cushions of *Fusarium graminearum* are fungal arsenals for wheat infection. *Molecular Plant Pathology*, 21(8), 1070–1087. https://doi.org/10.1111/mpp.12960

Molelekoa, T.B.J., Regnier, T., Silva da, L.S., & Augustyn, W. (2021). Production of pigments by filamentous fungi cultured on agro-industrial by-products using submerged and solid-state fermentation methods. *Fermentation,* 7 (4). https://doi.org/10.3390/fermentation7040295

Mustafa, G., Arshad, M., Bano, I., & Abbas, M. (2020). Biotechnological applications of sugarcane bagasse and sugar beet molasses. *Biomass Conversion and Biorefinery*, 13, 1489–1501. https://doi.org/10.1007/s13399-020-01141-x

Nimnoi, P., Pongsilp, N., & Lumyong, S. (2015). Utilization of agro-industrial products for increasing red pigment production of *Monascus purpureus* AHK12. *Chiang Mai Journal Science*, 42(2), 331–338.

Ozyurek, S., & Bilkay, I.S. (2017). Determination of petroleum biodegradation by bacteria isolated from drilling fluid, waste mud pit, and crude oil. *Turkish Journal of Biochemistry*, 42, 609–616. https://doi.org/10.1515/tjb-2017-0087

Poorniammal, R., Prabhu, S., Dufossé, L., & Kannan, J. (2021). Safety evaluation of fungal pigments for food applications. *Journal of Fungi* 7(9), 15. https://doi.org/10.3390/jof7090692

Pradeep, F.S., Begam, M.S., Palaniswamy, M., & Pradeep, B.V. (2013). Influence of Culture Media on Growth and Pigment Production by *Fusarium moniliforme* KUMBF1201 Isolated from paddy field soil. *World Applied Science Journal*, 22, 70–77. https://doi.org/10.5829/idosi.wasj.2013.22.01.7265

Pradeep, S., & Pradeep, B.V. (2013). Optimization of pigment and biomass production from *Fusarium moniliforme* under submerged fermentation conditions. *International Journal of Pharmacy and Pharmaceutical Sciences*, 5, 526-535.

Qiang, X., Luo, J., Guo, S., Cao, W., Hang, X., Liu, J., & Wan, Y. (2019). A novel process for molasses utilization by membrane filtration and resin adsorption. *Journal of Cleaner Production,* 207, 432–443. https://doi.org/10.1016/j.jclepro.2018.10.005

Pessôa, M.G., Paulino, B.N., Mano, M.C.R., Neri-Numa, I.A., Molina, G., Pastore, G.M. (2017) *Fusarium* species-a promising tool box for industrial biotechnology. *Applied Microbiology and Biotechnology*, 101(9), 3493-3511. https://doi.org/10.1007/s00253-017-8255-z

Reyes, F.G.R., Valim, M.F.C.F.A., Vercesi, & A.E. (1996). Effect of organic synthetic food colors on mitochondrial respiration.*Food Additives Contaminants,*13(1), 5–11. https://doi.org/10.1080/02652039609374376

Singha, I.M., Kakoty, Y., Unni, B.G., Das, J., & Kalita, M.C. (2016). Identification and characterization of *Fusarium sp.* using ITS and RAPD causing Fusarium wilt of tomato isolated from Assam, North East India. *Journal of Genetic Engineering*

and Biotechnology, 14, 99–105. https://doi.org/10.1016/j.jgeb.2016.07.001

Stanly, Pradeep, F., & Pradeep, B.V. (2013). Optimization of pigment and biomass production from *Fusarium moniliforme* under submerged fermentation conditions. *International Journal Pharmacy and Pharmaceutical Sciences*, 5, 526–535.

Tuli, H.S., Chaudhary, P., Beniwal, V., & Sharma, A.K. (2015). Microbial pigments as natural color sources: current trends and future perspectives. *Journal Food Science Technology*, 52(8), 4669–4678. https://doi.org/10.1007/s13197-014-1601-6

Velmurugan, P., Lee, Y.H., Venil, C.K., Lakshmanaperumalsamy P., Chae, J.C., & Oh, B.T. (2010). Effect of light on growth, intracellular and extracellular pigment production by five pigment-producing filamentous fungi in synthetic medium. *Journal of Bioscience and Bioengineering,* 109(4), 346–350. https://doi.org/10.1016/j.jbiosc.2009.10.003

Waghela, M., & Khan, S. (2018). Isolation, characterization of pigment-producing bacteria from various food samples and testing of antimicrobial activity of bacterial pigments. *DAV International Journal of Science*, 7 (1), 1-9.