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Isolation and Characterisation of Pigments from Pigment-producing Microorganisms Isolated from Environment and Their Antibacterial Activity

***Goda Deveikaite, Renata Zvirdauskiene**
Kaunas University of Technology, 73 K. Donelaičio street, Kaunas, Lithuania

Abstract. In the food industry, there is always a demand for food products which are colourful, have an attractive appearance, and also have nutritional and health-enhancing properties to attract the attention of consumers. Synthetic pigments are widely used in the global market, however, they can cause many side effects such as hyperallergenicity, carcinogenicity and other toxicological problems. Recent studies have revealed that microorganisms are an abundant source of natural colours that allow the industrial production of safe, environmentally friendly biodegradable pigments. The aim of the work was to isolate pigmented microorganisms from environmental samples, select fermentation conditions, isolate pigments from microorganisms and check their antimicrobial activity. Pigments have been isolated from various sources such as soil, food waste, flour, etc. Growth parameters of pigment-producing microorganisms such as growth temperature, pH, tryptone and NaCl concentration in the medium were optimised to evaluate pigment production. After fermentation, five types of pigments were isolated by cell lysis with an ultrasonic bath and solvent extraction. The antimicrobial activity of the extracted pigments was investigated. During the study, the optimal conditions for the growth of microorganisms were determined: temperature of 30 °C, pH of 7, concentration of 3% tryptone and 6% NaCl in the culture medium. Glycerol was found as an additional carbon source, which had a positive effect on pigments production. The results of the antibacterial effect of the extracted pigments showed that *P. aeruginosa* was the most sensitive to the effect of the pigments. The pink-red pigment showed the highest antimicrobial activity against the tested pathogenic bacteria.
Key words: pigments, microorganisms, isolation, fermentation.

Introduction

Humans experience the world through sight, because the brain receives a large part of information through the eyes, so it is obvious that colours affect perception, mood and decisions. Synthetic dyes are used for dyeing food, textiles and cosmetics, which turns out to be not the best solution because they have negative effects and are not environmentally friendly, which is a very important issue at the moment. Inside the microorganisms, there are impressive metabolic processes that produce very valuable compounds, including pigments. This product of theirs not only has colour but also unique properties.

Microorganisms produce various biologically active pigments such as carotenoids, melanins, flavins, quinines, monascins, violacein, etc. Of the produced natural pigments, the most important in this field are bacteria and filamentous fungi, which can be isolated from various environmental sources such as water, soil, plants, food waste, insects and animals. Recent studies have revealed that microorganisms are a promising source of natural colourants and have attracted the attention of the industry, for the production of new, safe, easily degradable, environmentally friendly pigments with no adverse effects is becoming more and more relevant. The present study was carried out with an aim to isolate pigment-producing microorganisms from environment sources, select fermentation conditions, extract pigments from microorganisms and check antimicrobial activity.

Materials and Methods

Chemicals and media

Chemicals*:* HCl, NaOH, 99.8% methanol, NaCl, tryptone, distilled water, glycerine, 98% ethanol, gentian violet dye, Lugol's solution, 96% methanol, fuchsine.

* Corresponding Author's email: godadev@gmail.com

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Media*:* Nutrient agar, nutrient broth, plate count agar (Liofilchem, Italy).

Isolation of pigmented microorganisms

Microorganisms have been isolated from rotten fruit waste, soil, wheat and rice flour, etc. 10 g of each sample were taken, which was poured into 90 ml of physiological solution (0.85% NaCl w/v). This mixture was homogenised to form of a homogeneous suspension, followed by a serial dilution method to isolate various microorganisms, including pigment-producing ones. The isolates were further investigated using morphological, microscopic examination and chemical methods. They were incubated at 30 °C and pH 7 (Muneefa & Thirumalai, 2021).

Characterisation and identification of isolated pigmented microorganisms

Colonies of isolated microorganisms were characterised by determining their morphological features such as colour, margin profile, size, shape, transparency, etc. (Jackman, 2011) Also, the studied microorganisms were identified using the gram staining method using microscopic examination (Tripathi & Sapra, 2022).

Optimisation of growth parameters to maximize pigment production

The effects of various physiological parameters on the growth of pigment-producing microorganisms, such as temperature, pH, tryptone and salt concentrations, were tested to identify and evaluate the effect on pigment production. These studies were performed in test tubes on nutrient agar slants (Ratnakaran, Bhoir & Durve-Gupta, 2020).

For all determined physiological parameters tested, the results were evaluated in terms of microorganism growth and pigmentation. This characteristic was checked after 24 and 48 hours of incubation using a negative control (sterile simple nutrient agar with nutrients) and a positive control (sterile simple nutrient agar with test microorganism isolates) (Ratnakaran, Bhoir & Durve-Gupta, 2020).

Influence of temperature

Prepared tubes with nutrient agar were infected with different pigment-producing microorganisms and after that incubated at different temperatures. Incubation was carried out for 24–48 hours at 20 °C, 30 °C and 37 °C in order to study the influence of temperature on the growth and pigment production of the isolates (Bhat, Khan & Amin, 2013; Ratnakaran, Bhoir & Durve-Gupta, 2020).

Influence of pH

Sterile tubes with nutrient agar at different pH (5, 7, 9 and 12), which was adjusted before autoclaving, were inoculated with isolated pigmented microorganisms and kept at 37 °C for 24–48 hours to study the effect of pH on the selected microorganisms.

Effect of tryptone concentration

The effect of tryptone as a potential source of amino acids was determined by placing cultures on sterile nutrient agar containing 1%, 2% and 3% tryptone concentrations and incubated at 37 °C for 24-48 hours with further observation of pigment production and growth intensity of the cultures (Ratnakaran, Bhoir & Durve-Gupta, 2020).

Effect of salt concentration

NaCl was added to nutrient agar to study the effect of different concentrations of salt on microbial growth and pigment production. The salt concentration in the growth media was 2%, 4%, 6% and 8%. Tube media with different salt concentrations were infected with microbial isolates and incubated at 37 °C for 24–48 hours (Bhat, Khan & Amin, 2013; Ratnakaran, Bhoir & Durve-Gupta, 2020).

Pigment production and effect of glycerine on fermentation

Once the most suitable fermentation conditions have been determined, preparations for the fermentation process begin by initially preparing microorganism suspensions following the McFarland standard (Dalynn Biologicals, 2014). From the prepared suspensions, 1 ml was taken and poured into bottles with a sterile nutrient broth, in which 3% tryptone and 6% NaCl were additionally added, and another fermentation was carried out under the same conditions, but with the addition of 2% glycerol, which is believed to enhance the pigment bacteria. Both fermentations were incubated at 30 °C for 7 days in a rotary shaker at 180 rpm to achieve the maximal pigment production (Ratnakaran, Bhoir & Durve-Gupta, 2020). After evaluating the effect of glycerine on fermentation, the most suitable fermentation conditions were chosen according to the growth of biomass, and further carried out in 100 ml of broth in order to extract more biomass for pigment extraction in further procedures.

Extraction of pigment

The obtained biomass was separated from the broth by centrifugation for 15 min at room temperature and 6000 rpm. The pigments were extracted from the biomass by first dissolving in 99.8% methanol to form a homogeneous solution, followed by another round of centrifugation at the same parameters. It was observed that the pigments were not distributed in the solvent, indicating that the pigments were located inside the cells, known as intracellular pigments. Subsequently, the dissolved cells of the isolated microorganisms were lysed using ultrasound in a sonicator bath for 30 min at a temperature of 30 °C. After cell lysis, the solution was mixed well to dissolve the pigment in methanol. This solution was centrifuged at 6000 rpm at room temperature for 15 min to separate dissolved pigments

from cell debris. The cell sediments were further washed with 10 ml of 99.8% methanol and heated in a water bath at 45 °C. This process was repeated until the pellet became colourless. Coloured solutions with dissolved pigments were filtered through a membrane filter with a pore diameter of 0.22 µm to remove cell debris. Filtrates were poured into petri dishes and were dried for a day in a dryer at a temperature of 60 °C. After drying, the extracted crude pigment was stored in the dark at room temperature for further use (Chaudhari, Mehta & Shah, 2019; Padhan *et al*., 2021; Patel *et al*., 2020; Sinha *et al*., 2017).

Antimicrobial activity of pigments

The antibacterial activity of microorganism pigments was determined using the filter disc diffusion method (Balouiri *et al*., 2016). Eight bacteria strains were used in the study to evaluate the antibacterial properties of the investigated pigments: *Escherichia coli* ATCC 8739, *Listeria monocytogenes* ATCC 13932, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 13525, *Citrobacter freundii* ATCC 43864, *Micrococcus luteus* ATCC 9341, *Salmonella typhimurium* ATCC 14028, *Enterococcus faecalis* ATCC 19433.

Bacterial cell suspensions were introduced into dissolved PCA medium cooled to 47 °C (Andrews, 1992). Sterile 6 mm diameter filter discs (Hahnemühle, Germany) were placed on the surface of the solidified inoculated PCA medium. A 20 µl volume of ethanol solutions of microbial pigments (pigment concentration 100 µg/ml) was poured onto the filter discs using a pipette. The plates were then incubated at 37 °C for 24 h. Ethanol was used as a negative control. After incubation, the antibacterial activity was evaluated by measuring the diameter of the clear inhibitory zones in millimetres. Experiments were performed in triplicate (Piddock, 1990). Mean values ± standard deviations (STDEV) were calculated.

Results and Discussion

1. Characterisation of isolated pigmented microorganisms

Different colonies were obtained by serial dilution, among which pigment-producing microorganisms were also distributed. Pigment-producing colonies were picked and placed onto new sterile nutrient agar petri dishes to obtain pure cultures (Ratnakaran, Bhoir & Durve-Gupta, 2020). Six colonies were selected with the colours yellow-orange, light pink, light yellow, yellow, orange and pink (Fig. 1). Individual colonies of isolated pure cultures were characterised by morphological features and gram staining, as results are shown in Table 1. Compared to the results of other sources, such as Ratnakaran, Bhoir & Durve-Gupta (2020) and Sinha *et al*. (2017), the morphological characteristics of the colonies of pigment-producing microorganisms are very similar.

2. Optimisation of growth parameters to maximise pigment production

The growth of microorganisms can be regulated and enhanced by certain environmental parameters. Pigment production from microorganisms also depends on physiological constraints such as pH, temperature, mixing, aeration, carbon source, etc. Thus, finding the right factors is necessary to optimise bacterial growth. Appropriate growth conditions were determined by growth factor experiments (Qayyum *et al.*, 2020). In this study, the effects of temperature, pH, tryptone and NaCl on the growth of pigmented microorganisms were investigated.

Table 1

Gram-staining and morphological characterisation of colonies of pigment producing microorganisms

Culture	Colour	Size	Shape	Consistency	Elevation	Margin	Opacity	Gram characters
1	Yellow-orange	1 mm	Circular	Sticky	Convex	Entire	Opaque	Gram negative bacilli
2	Light pink	1 mm	Circular	Smooth	Convex	Entire	Opaque	Gram positive yeast
3	Light yellow	2 mm	Circular	Smooth	Convex	Entire	Opaque	Gram positive cocci
4	Yellow	1 mm	Circular	Smooth	Convex	Entire	Opaque	Gram negative bacilli
5	Orange	1 mm	Circular	Smooth	Convex	Entire	Opaque	Gram positive cocci
6	Pink	1 mm	Circular	Smooth	Convex	Entire	Opaque	Gram positive cocci

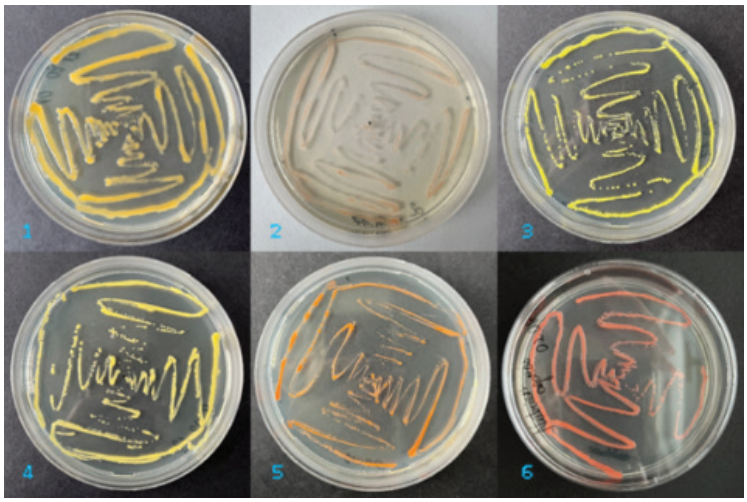


Figure 1. Isolated pigment–producing microorganisms.

Table 2

Influence of temperature on the growth of microorganisms

Culture	Temperature, °C					
	20		30		37	
	Incubation period, h					
	24	48	24	48	24	48
1	++	++	++	++	−/+	+
2	++	++	++	++	−/+	++
3	+	++	++	++	++	++
4	+	++	++	++	++	++
5	+	++	+	++	+	++
6	+	++	++	++	−	−
Key: − No growth, −/+ slight growth, + normal growth, ++ good growth						

2.1. Influence of temperature

According to the obtained results, which can be seen in the Table 2, the best pigment production and growth of all isolated microorganisms was at 30 °C, while at 37 °C, the growth and colony colours were pale, and the pink colour microorganisms did not grow. Comparing the temperatures tested in the research of Ratnakaran, Bhoir & Durve-Gupta (2020), good growth was observed at room temperature but also at 37 °C, indicating that different bacteria have different growth temperature ranges. Most of the microorganisms grew at these tested temperatures, but a decrease in pigment production was seen at higher temperatures, so the optimum temperature was found to be 30 °C.

2.2. Influence of pH

As it can be seen in the results shown in Table 3, growth was observed at all tested pH values, but the most suitable for evaluating pigment brightness and growth results for all tested microorganisms was pH 7. In the research of Ratnakaran, Bhoir & Durve-Gupta (2020), according to the results of the tests, this pH value was also the best for growth. However, different results were obtained at pH 5. According to the source results, microorganisms did not grow, but growth was recorded in this study.

2.3. Effect of tryptone concentration

All isolated microorganisms show growth and pigment production at all concentrations of tryptone. Table 4 shows that 3% tryptone concentration was best suited for all isolated microorganisms in the growth medium. Comparing these results with the research article of Ratnakaran, Bhoir & Durve-Gupta (2020), there tryptone concentrations did not have a significant effect on growth. The effect of tryptone shows that this supports cell growth as well as pigment production.

Table 3

Influence of pH on the growth of microorganisms

Culture	pH							
	5		7		9		12	
	Incubation period, h							
	24	48	24	48	24	48	24	48
1	++	++	++	++	++	++	+	+
2	++	++	++	++	++	++	+	++
3	++	++	++	++	++	++	+	++
4	++	++	++	++	++	++	+	+
5	++	++	++	++	+	++	+	++
6	−/+	+	++	++	−/+	−/+	−/+	−/+

Key: − No growth, −/+ slight growth, + normal growth, ++ good growth

Table 4

Effect of different concentrations of tryptone on microorganism growth

Culture	Tryptone concentration, %					
	1		2		3	
	Incubation period, h					
	24	48	24	48	24	48
1	++	++	++	++	++	++
2	+	++	++	++	++	++
3	++	++	+	++	++	++
4	++	++	++	++	++	++
5	+	++	+	++	+	++
6	+	++	+	++	+	++

Key: − No growth, −/+ slight growth, + normal growth, ++ good growth

Table 5

Effect of different concentrations of NaCl on microorganism growth

Culture	NaCl concentration, %							
	2		4		6		8	
	Incubation period, h							
	24	48	24	48	24	48	24	48
1	++	++	++	++	++	++	++	++
2	++	++	++	++	++	++	++	++
3	++	++	++	++	++	++	++	++
4	++	++	++	++	++	++	++	++
5	++	++	++	++	++	++	+	++
6	++	++	++	++	++	++	+	++

Key: − No growth, −/+ slight growth, + normal growth, ++ good growth

2.4. Effect of NaCl concentration

Most of the microorganisms tested showed good growth at all NaCl concentrations, but the best growth and pigment intensity were recorded in the medium with NaCl concentration of 6%; results are shown in Table 5. For the microorganisms studied in the research of Muneefa & Thirumalai (2021), the maximum growth and pigment production was observed at 2% NaCl concentration, with a gradual increase from 4% to 8%.

3. Pigment production and effect of glycerine on fermentation

In the fermentation broth containing glycerol, which was added as an additional carbon source, the biomass grew better than in the broth without it. In the article by Ratnakaran, Bhoir & Durve-Gupta (2020), both fermentation media gave good results. However, in this experiment, isolate 6 did not grow in the medium containing glycerol, but in the medium without this substance, it showed good growth. According to the reaction of microorganisms to glycerol, a 100 ml fermentation was carried out and pigments were extracted from the biomasses.

4. Extraction of pigments

The following methods were used to extract pigment from pigment-producing microorganisms: centrifugation, cell lysis, extraction using methanol as an organic solvent and filtration. From the six cultivated biomasses of microorganisms, five pigments were successfully extracted, which were light pink, light yellow, yellow, pink-red and pink (Fig. 2), and their antimicrobial activity was further investigated.

5. Antimicrobial activity of pigments

The results of the antibacterial effect of pigments showed that among the tested pathogenic bacteria, *P. aeruginosa* was the most sensitive to the influence of pigments. The pink-red pigment showed the highest antimicrobial activity against the tested pathogenic bacteria; the results are shown in Table 6. Comparing the results with the study of Sinha *et al*. (2017), they are different – the pigment that showed the highest antimicrobial activity was yellow and *Pseudomonas* was not affected by the pigments. Thus, the tested pigments showed a bacteriostatic effect on certain human pathogens, such as *P. aeruginosa* and *S. typhimurium* (Table 6).



Figure 2. Pigment extraction (a – biomass of pigmented microorganisms, b – extracted pigments, c – dried pigments).

Table 6

Antibacterial activity of microbial pigments expressed by growth inhibition zones in mm ±STDEV

Bacterial strain	Microbial pigment				
	Light pink	Light yellow	Yellow	Pink-red	Pink
	Radius of zone of inhibition, mm				
E. coli	0.00±0.00	0.00±0.00	0.00±0.00	4.63±0.50	0.00±0.00
E. faecalis	0.00±0.00	0.00±0.00	0.00±0.00	5.25±1.73	0.00±0.00
C. freundii	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
L. monocytogenes	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
M. luteus	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
P. aeruginosa	6.63±2.06	5.38±1.50	7.13±0.96	5.50±0.82	5.38±2.06
S. aureus	0.00±0.00	0.00±0.00	0.00±0.00	3.88±0.50	0.00±0.00
S. typhimurium	7.88±0.96	4.00±0.00	0.00±0.00	6.38±2.22	4.25±0.58

Conclusions

Pigments produced by microorganisms can be an excellent substitute for synthetic ones, as they are obtained from renewable sources, easily degradable, safe and have unique properties such as antimicrobial, antioxidant effects, etc. In this study, pigment-producing microorganisms were isolated from the substrates such as rotting fruit, flour and soil. During the research, it was found that the most optimal conditions for the growth of microorganisms were at the temperature of 30 °C, pH 7, 3% tryptone and 6% NaCl concentration in the growth medium, and with glycerol as an additional carbon source, which had a positive influence on the formation of pigments. Successful fermentation and pigment extraction from biomass has been achieved using various extraction methods. During the study, five types of pigments were extracted from the six investigated isolates. The results of the antibacterial effects of the isolated pigments showed that among the tested pathogenic bacteria, *P. aeruginosa* was the most sensitive to the effects of the pigments. The pink-red pigment showed the highest antimicrobial activity against the tested pathogenic bacteria.

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