



# Use of common carp waste for pigment production by *Monascus purpureus*

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## Abstract

Fish processing worldwide generates a large amount of waste that has no further use. In this work, we tested the possible biotechnological use of two waste materials from common carp – waste scales and residues from filleting process. Both sources were subjected to alkaline hydrolysis and subsequently used as a source of nitrogen, phosphorus and other elements for the cultivation of the fungus *Monascus purpureus*, which is known for the production of pigments with potential in pharmaceuticals and other sectors of industry. After 14 day-long cultivation of *Monascus purpureus* in a medium containing hydrolysate as the sole source of all nutrients except carbon, the extraction of pigments and their further analysis were carried out. It was shown that a simple as well as inexpensive alkaline hydrolysis can be used to prepare a complex culture medium suitable for pigment production using fish waste. Moreover, there is a big potential that this medium would also be suitable for many more biotechnological applications.

**Keywords:** *Monascus purpureus*; pigments; common carp; waste substrate; scales; cultivation

## 1 Introduction

*Monascus purpureus* is a species of microscopic, filamentous and heterotrophic fungus that is known to produce pigments as well as other bioactive substances. This genus is still relatively unknown in western countries, but it is very well known in oriental countries like China, Japan or Korea. In these countries, this fungus was used for the production of red fermented rice, so-called “Red-Yeast Rice”, many centuries ago (Patáková, 2013; Patrovský et al., 2019). Red-Yeast Rice is a traditional product used in Eastern medicine and food processing. It is prepared by inoculating rice with spores of the fungus *Monascus purpureus*, or *Monascus ruber*, followed by fermentation. The *Monascus* fungus was used in the past and is still used for the preparation of long-lasting fermented foods and also for dyeing of food or textile (Patáková, 2013; Yang et al., 2018; Zhu et al., 2019).

Pigments represent the longest known metabolites produced by the *Monascus* genus. Strains belonging to the species *Monascus purpureus* generally produce six major pigments, which can be classified according to their colour: yellow pigments – monascin and ankaflavin; orange pigments – rubropunctatin and monascorubrin; and red pigments – rubropunctamine and monascorubramine (Feng et al., 2012; Patáková, 2013; Zhu et al., 2019). As for other produced biologically active substances, monacolin K is probably the most important. This is a substance that belongs to a group of statins. The main benefit of monacolin K lies in the interruption of the enzymatic pathway of cholesterol biosynthesis, which leads to the reduction in its formation in the body, as well as the overall reduction in the concentration of cholesterol in the blood. Moreover, monacolin K has other, non-specific, overall strengthening effects on the entire cardiovascular system (Patáková et al., 2015; Zhu et al., 2019). In addition to substances that have

obvious positive effects on human health, unfortunately, *Monascus purpureus* is also capable to produce substances with negative effects, such as mycotoxin citrinin and others (Blanc et al., 1995; Zhu et al., 2019). The citrinin production is species/strain specific and there are known strains, such as *Monascus* sp. DBM 4361 (Husáková et al., 2021) which do not produce citrinin. However, if a strain produces citrinin, its production can only be influenced by culture conditions, such as lowering citrinin production by low pH (Patrovský et al., 2019), but it cannot be totally eliminated. Factors affecting pigments production by *Monascus* fungi, such as nitrogen or carbon sources, temperature, light, pH, oxygen and others have recently been reviewed (He et al., 2021). The unpredictable production of mycotoxins is one of the reasons why the use of red fermented rice, or other products, is not yet widespread in Europe.

*Monascus purpureus*, like most members of the genus *Monascus*, is relatively undemanding to cultivation conditions. It can be cultured on conventional media designed for fungus cultivation (PDA, Sabouraud, etc.). The ideal temperature for cultivation is between 25 and 30 °C and humidity between 65 and 85% (Zhu et al., 2019). All representatives of the genus *Monascus* are aerobic organisms. The production of pigments and other valuable secondary metabolites is reduced in absence of oxygen. In the past it was shown that the used nitrogen source, together with the initial pH, are very important factors and fundamentally affect pigment production in submerged cultivation (Ajdari et al., 2011; Patrovský et al., 2019). The classical nitrogen source used in most experiments is yeast extract. Other nitrogen sources that have been tested with varying results for pigment production have been e.g.  $\text{NaNO}_3$ ,  $(\text{NH}_4)_2\text{SO}_4$ , or enzymatically cleaved peptone from casein or meat (Patrovský et al., 2019). In addition to the effect on pigment production, the nitrogen source used also has a major effect on production of bioactive compounds such as monacolin K or citrinin.

In this work, the use of an alkaline hydrolysate prepared from wastes generated during processing of freshwater fish was tested as a nitrogen source for the cultivation of the fungus *Monascus purpureus*.

## 2 Materials and methods

### 2.1 Microbial strain and its storage

The microbial strain *Monascus purpureus* DBM 4360 (DBM is the culture collection of the Department of Biochemistry and Microbiology, University of Chemistry and Technology in Prague) was used in this work. The strain was stored on Sabouraud's slant agar (glucose 40 g/l, yeast extract 10 g/l, agar 15 g/l; pH 5.6) at 4 °C for a long time.

### 2.2 Preparation of hydrolysates and production liquid medium

Waste scales from the de-scaling machine and filleting residues of common carp (*Cyprinus carpio*) were obtained from the Secondary School of Fishery and Water Management in Třeboň, Czech Republic. In all cases, the fish were caught in Czech Republic in the area of the Třeboň ponds.

As for the preparation of the hydrolysates, the methodology described in Branska et al. (2020) was adapted for the fish residues hydrolysis. Samples were first ground into smaller pieces using a chopper. A KOH solution (1.2% w/v for scales samples; 0.6% w/v for filleting residues) was then added to the samples in weight ratio 1:2 (sample:KOH solution). Following that flasks were manually mixed and hydrolysed at 80 °C for 30 h. After hydrolysis, the hydrolysate was mixed with an equal volume of demineralized water and sucrose was added to a final concentration of 40 g/l. The pH was adjusted to 5.5 using  $\text{H}_3\text{PO}_4$ .

### 2.3 Cultivation and pigment production

Spores of *Monascus purpureus* were first inoculated onto solid Sabouraud agar; cultivation was carried out for 4 days at 30 °C. The flasks containing 250 ml of the production liquid medium were inoculated with one colony, which was excised from Sabouraud agar. Cultivation was then carried out on a shaker at 150 rpm and 30 °C for 14 days. All experiments were carried out at least in triplicate; the data published in this work are the average of these values.

### 2.4 Pigment extraction and analysis

When cultivation was completed, the production medium with the mycelium was filtered through filter paper (Whatman no. 1). The mycelium was quantitatively transferred from the filter paper back into a clean flask and a 70% v/v water solution of ethanol was added to the total volume of 40 ml. Subsequently, the flasks were placed on a shaker for 1 hour at 100 rpm and 30 °C. The extracts were then filtered again and the finished extracts were stored in dark vials at -20 °C.

For spectrophotometry, the samples of pigment extracts were diluted with a 70% v/v ethanol solution so that the  $A_{500}$  was in the range of 0.3–1. Samples were further analysed using a spectrometer (ONDA; Spectrophotometer UV-20) measuring the continuous spectrum in the wavelength range of 300–800 nm. The amount of yellow, orange and red pigments was monitored at their absorption maxima, i.e. 390, 470 and 500 nm.

Quantitative analysis of pigments content was performed using HPLC method (Husáková et al., 2021). Samples were centrifuged on a benchtop centrifuge and

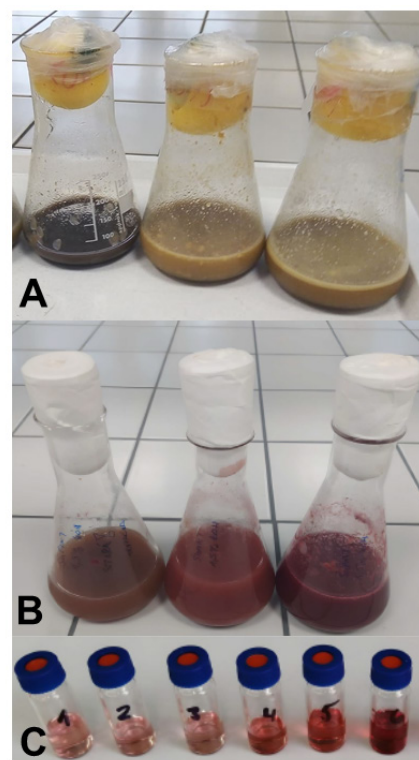
filtered through a microfilter (PTFE, 0.2  $\mu\text{m}$  sieve). An Agilent 1260 Infinity II with DAD and FLD detector was used for analysis under the following conditions: isocratic elution using 2 mobile phases (A 0.025%  $\text{H}_3\text{PO}_4$  in demineralized water; B 100% acetonitrile) in a volume ratio of 40:60; C18 polar column, sample injection volume 5  $\mu\text{l}$ , mobile phase flow rate 1.2 ml/min, analysis time 17 min. The eluted pigments were detected using DAD detector at wavelengths of 390, 470 and 500 nm. Content of mycotoxin citrinin was determined using fluorescence detector at 331 nm excitation and 500 nm emission wavelengths.

### 3 Results and discussion

The pigments produced by the fungus *Monascus purpureus* can be a valuable raw material for e.g. food processing and dyeing, textile dyeing or pharmaceutical applications. The most widely used method of cultivation is on a solid substrate, namely rice. In this work, submerged cultivation was used. This option allows for instance a better control of the cultivation process, possible use of different waste substrates as alternative sources of carbon or nitrogen, etc. As the most common source of nitrogen in submerged cultures, yeast extract or other enzymatically cleaved complex proteins are used for the cultivation of the fungus *Monascus purpureus* (see e.g. Carels et al., 1977; Broder and Koehler, 2006; Patrovský et al., 2019; Zhu et al., 2019) as well as in microbiology and biotechnology in general. The disadvantage of these complex sources may be a relatively high cost or the inability to precisely define the composition of these mixtures. In this work, the use of fish waste hydrolysate as an alternative source of nitrogen and other elements for cultivation and pigment production by the fungus *Monascus purpureus* was tested.

Scales generally consist mainly of bone and keratin, which is a complex protein. The other components are mainly mucilage, containing other proteins such as mucins, etc. Without enzymatic or hydrolytic action, scales do not degrade in solution in any way and they are not useful to micro-organisms as a source of nitrogen and carbon for cultivation. In global, fish scales have not had any biotechnological applications yet and there are only a few papers mentioning their possible hydrolysis and use, e.g. Zhang et al., 2019. Filleting residues are very complex materials containing meat, bone, cartilage and other parts in varying proportions. These residues are already useful for the cultivation of micro-organisms in the form of simple broth, but hydrolysis can supply micro-organisms with a much larger proportion of the nutrients contained.

The main advantage is the low cost of this substrate, which currently has no practical use. Another advantage is the relatively simple and inexpensive process of the hydrolysis, without the use of expensive enzymes or other chemicals. Similar method is successfully used, for example, for lignocellulose pre-treatment or hydrolysis of other biological substrates (Han et al., 2012; Camesasca et al., 2015; Stiborová et al., 2016; Branská et al., 2020). On the basis of our previous experience with hydrolysis of various biological substrates, a method using 0.6% KOH or NaOH solution was first tested for the alkaline hydrolysis of fish scales and filleting residues. However, when hydroxides of this concentration were used, there was no satisfactory hydrolysis of the scales (see above), and even after 30 hours of hydrolysis, a non-negligible solid fraction remained in the flasks. For waste scales, the concentration was therefore changed to 1.2% KOH in the course of the pilot experiments. At this concentration, hydrolysis was already proceeding satisfactorily for scales. Hydrolysis was in the case of use of NaOH generally slower and imperfect. The resulting hydrolysates (see Figure 1) still contained small portion of solid, undissolved particles after 30 hours of alkaline hydrolysis. On the other hand, these were only a negligible proportion of the inserted material. Red colonies of *Monascus purpureus* grown on



**Figure 1** Waste fish material after alkaline hydrolysis – scales on the left, filleting residues on the right (A); mycelium of the fungus *Monascus purpureus* grown on production medium (B); extracted pigments from the production medium ready for analysis (C).

Sabouraud agar were used to inoculate the production medium. The production cultivation of *Monascus purpureus* was carried out for 14 days on a laboratory shaker to ensure better access of oxygen to the medium. After about 7–10 days, the production of colour pigments became evident, which was manifested first by pink and later red colour of the media (see Figure 1).

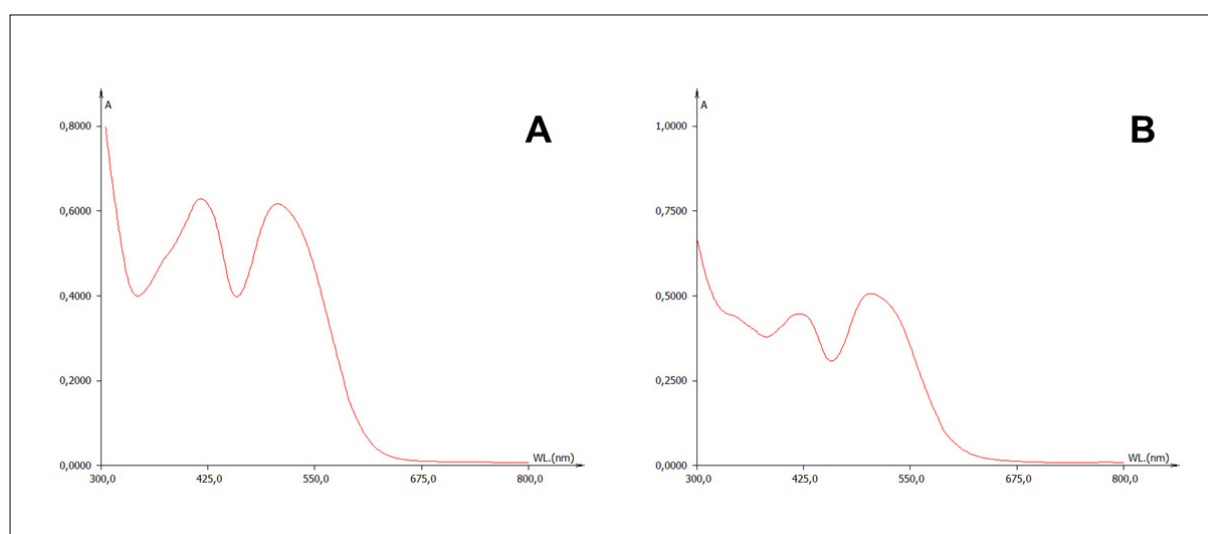
Extraction was performed with 70% ethanol. The ethanol extracts (see Figure 1) had varying levels of colouration from orange-red to deep red. The mycelia of the fungus also showed the same colouration. After one hour of extraction with ethanol, there was no noticeable discoloration of the mycelia despite a clear transition of pigments to extract. The efficiency of the extraction could be further improved, for example, by using a higher temperature ethanol solution, as previously shown for example by Wu et al. (2011) in their work where extraction efficiency was investigated. However, at 30 °C, the extraction efficiency should still be sufficient and at the same time not so demanding for energy consumption. As described previously, mycelium can also be extracted repeatedly with decreasing pigment yields (Wu et al., 2011). Due to the fact that ethanol was used for extraction in this work without any pH adjustment, the chemical reorganization of the orange pigments to red due to their reaction with the amino group has occurred (Liu et al., 2018). If an acidic ethanol with a pH of about 2 was used, the structure of the orange pigments would be protected and they would most likely be detectable in the samples.

For qualitative analysis of the content of yellow, orange and red pigments in the extracts the spectrophotometric method was used. The peaks corresponding to the yellow, orange and red pigments are known at about the absorption maxima of 390, 470 and 500 nm, re-

spectively. In all samples, peaks for yellow and red pigments were detected. Examples of absorption spectra in a sample using scale hydrolysate and filleting residues are shown in Figure 2.

For the quantification and determination of individual pigments present in the extracts, the HPLC-DAD method was employed. A total of 4 different yellow and two red pigments were detected in the samples. Among the yellow pigments, monascin, ankaflavin and two other yellow pigments were identified in the samples. These two pigments have not yet been precisely identified and were therefore designated as unknown pigments 1 and 2 (see Figure 3). It can be assumed that these are derivatives of monascin or ankaflavin with modification of the attached chemical groups. In any case, it would be advisable to carry out a closer structural analysis in the future. The production of other pigment analogues on different media and especially nitrogen sources has been described in the past as well (Liu et al., 2018; Zhu et al., 2019). Also, the formation of analogues is in particular strongly dependent on the composition of the culture medium. Among the red pigments, monascorubramine and rubropunctamine have been identified (see Figure 3). The concentration of pigments is expressed in  $\mu\text{mol/l}$  of the ethanol extract prepared as above. In future work, it would also be very useful to measure biomass growth and overall weight to get a better overview of pigment productivity. The mycotoxin citrinin was not detected in any of the samples.

The concentrations of pigments in the extract achieved in this work can be considered relatively low. On the other hand, high pigment yields were not the aim of this work. In the case of the scales sample, the highest concentration of monascin in the extract was observed, about 3.5  $\mu\text{mol/l}$ . The average concentra-



**Figure 2** Examples of absorption spectra of a sample using scale hydrolysate (A) and filleting residues (B). The Y-axis shows the absorbance, the X-axis the wavelength.

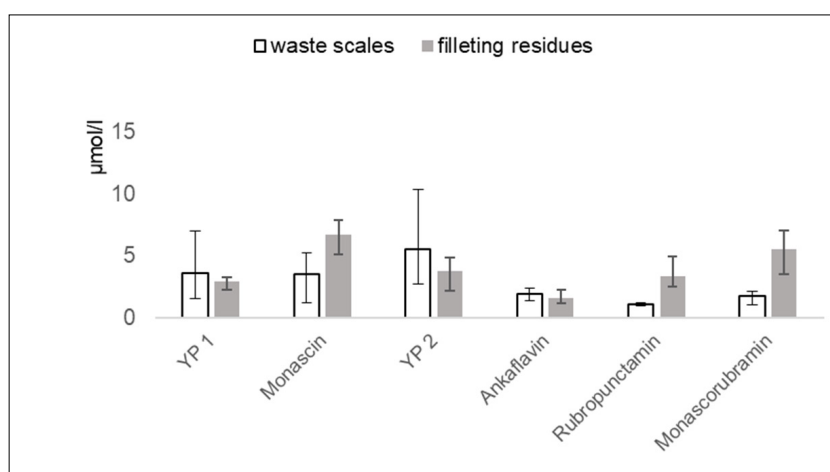
tions of ankaflavin, monascorubramine and rubropunctamine were 2.0, 1.7 and 1.1  $\mu\text{mol/l}$ , respectively. For the sample of filleting residues, the values of monascin, ankaflavin, monascorubramine and rubropunctamine were 6.7, 1.6, 1.5 and 3.4  $\mu\text{mol/l}$  respectively (see Figure 3). Since different works use different units like including plain AU (see above), it is not easy to compare pigment production with each other. Overall, it can be stated that the highest productions of pigments achieved were 10 to 100 times higher than the production obtained in this work (Pattangul et al., 2007; Zhu et al., 2019). However, this was only a pilot experiment. It is highly likely that the production could be significantly increased in several ways: for instance a simple extension the cultivation time, adjusting the composition of the culture medium ratios or other optimization. In conclusion, the biotechnological use of fish waste may have great potential due to the nature of the waste substrate, its composition, availability and ease of processing.

#### 4 Conclusion

It has been shown that waste products from common carp processing can be an interesting substrate for the preparation of culture media. In this work the possibility of cultivating the fungus *Monascus purpureus* and the production of colour pigments using this fungus was tested. The waste substrate was successfully hydrolysed to produce a suitable culture medium for pigment production. A total of 6 different colour pigments were produced, including 2 pigments with a previously undescribed structure. The method described in this work could be used in the future for the evaluation of fish processing residues, as most of the residues have no other use at the moment. Furthermore, the resulting hydrolysate could very likely be used for many other biotechnological applications.

#### 5 Acknowledgement

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**Figure 3** Pigments detected by HPLC and their concentrations in extracts after 14 days of cultivation. Yellow pigments with unknown structure are designated as YP 1 (yellow pigment 1) and YP 2 (yellow pigment 2). The error bars represent the highest and lowest values achieved.

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