Indian Journal of Biotechnology
Vol 6, April 2007, pp 194-199

Effect of substrates on the production of *Monascus* biopigments by solid-state fermentation and pigment extraction using different solvents

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Received 19 April 2005; revised 20 March 2006; accepted 25 June 2006

The aim of the present work was to study the potential of natural substrates for biopigment production using a strain of *Monascus* sp, and to compare different solvents for extracting these pigments. Rice was the best substrate for cultivation of the culture in SSF. However, some of the other substrates used also presented good biopigment production, especially corn, wheat and cassava. Cassava bagasse gave a low pigment yield, but it is an agro-industrial residue whose low price might compensate for its low yield. However, efficient pigment production with this substrate requires supplementation of the culture medium with nutrients. Optimum fermentation time for SSF in cassava bagasse was 10-11 d. The production of yellow pigments was superior to that of red pigments, but the ratio red/yellow (ABS 500/ABS 400) grew during the course of the process. The comparison of several solvents for extraction showed that methanol was the best solvent, closely followed by DMSO and ethanol. The results also indicated that a mixture of ethanol-water with *ca*. 60% ethanol (w/w) was more efficient than other ethanol concentrations. The solvent to substrate ratio had little influence in the extraction. Finally, temperature in the range of 0ºC to 60ºC showed no difference in the extraction of the biopigments under static condition.

Keywords: pigment, substrate, extraction, solvent, solid-state fermentation, *Monascus*
IPC Code: Int. Cl.[8] C07C403/24, C12N1/12

Introduction

Colour additives are essential in food industry, conferring attractive colour to foods, as in the case of several candies and beverages, or standardizing foods such as sausages or fruit juices. Recent doubts concerning the toxicity of several artificial colorants stimulated their substitution by natural colours[1]. Some artificial colours, such as azorubrin or tartrazin, may cause allergies[2]. Natural colour additives most often used[3] are of increasing interest since they are very diverse, ranging from fruit and vegetable juices to caramel.

Biopigments produced by fermentation present a great potential for food applications, because they are natural, produced quickly (when compared with vegetable or animal pigments) and at any time of the year. Commercial biopigments produced by fermentation include riboflavin (vitamin B_2, a yellow pigment with permitted use in most countries), biopigments from *Monascus* (red pigments, widely used in Asia, specially China, Japan, Thailand and Indonesia, but not yet regulated in several other countries), carotenoids (yellow to red pigments produced by several microorganisms, but as yet produced economically only using micro algae), and phycobiliproteins such as phycocyanin (a blue pigment used in food and cosmetics)[4].

Strains of *Monascus* species produce several biopigments with related polyketide structures[5-7]. The structure of the main pigments is shown in Fig. 1. Although six pigments are recognized as the most important, some new structures have been recently described[8]. According to some authors, there are more than 10 different biopigments, although only part of them had their structure elucidated[9]. The red biopigments produced by *Monascus* are the most important, since they are possible substitutes to synthetic pigments such as erythrosine (FD & C red no. 3)[10]. These biopigments are stable to temperature and *p*H (in the range of 2-10)[11]. They present a moderately polar structure, which affects their solubility in water and lipids and ultimately affects the production process and their applications.

In traditional production, *Monascus* is grown over steamed rice for several days. This fermented mass, known as *ang-kak*, is dried and ground, and the

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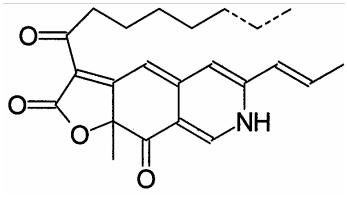

Fig. 1 — *Monascus* sp. red pigments structure. With 7 carbon atoms in the lateral chain: monascorubramine; with 5 carbons: rubropunctatine.

powder is used directly as a colouring agent. It is also possible to extract the pigments from the product by solvent extraction and evaporate the solvent from the solution in order to obtain it in a concentrated form.

Alternative culture media for *Monascus* biopigments production are very diverse, ranging from defined compositions to natural substrates. Being a common contaminant in grains, and having been isolated from several substrates with a high solids concentration, *Monascus* grows on a wide variety of natural substrates.

The cultivation of *Monascus* in solid-state fermentation (SSF) over steamed rice is very exuberant. However, there are many natural substrates that show similar or even higher quantities of carbohydrates and proteins, and are presumably good sources of C and N for the fungus development. There are several cheap by-products and residues of food processing that might be used as potential substrates for *Monascus* fermentation. Such substrates have shown promising results in the production of other metabolites in SSF[12]. There are reports describing some other raw materials used as substrate for *Monascus*, which include cassava starch[13-15], prickly pear juice[16], and dairy milk[17]. In some cases it is necessary to supplement these substrates with nutrients such as vitamins and organic nitrogen supplements. The components of the complex culture media include sugars (most commonly glucose), micronutrients and organic nitrogen sources (amino acids, peptones) or inorganic nitrogen (ammonium nitrates)[18].

The aim of the present work was to compare several potential natural substrates for *Monascus* cultivation for biopigment production, and also to compare the efficiency of different solvents for the extraction of red biopigments under different conditions, in order gather data for the development of an industrial bioprocess.

Materials and Methods

Microorganism and Inoculum

A wild isolate of *Monascus* sp., LPB-31, available in the Culture Bank of Bioprocesses Engineering and Biotechnology Division of UFPR was used in this study. The strain was cultivated on PDA medium at 30ºC for 10 d, preserved at 4-8°C and sub-cultured monthly. Inoculum was prepared by growing the culture in 250 mL Erlenmeyer flask containing 50 mL PDB medium at 30ºC for 7 d, under static conditions. The fermented medium+mycelium was used as inoculum.

Solid-state Fermentation

Eleven different natural substrates [broken rice, wheat, corn, soy, soy bran, two batches of textured soy protein (TSP A and B), cassava, two traditional kinds of cassava flour, cassava bagasse and potatoes] were used as substrate for SSF. The substrates were dried at 60°C overnight, ground and sieved. The fraction of 0.8 to 2.0 mm was used. Ten g of substrate was taken in 600 mL jars and mixed with a known quantity of distilled water to adjust the moisture at 56% (*p*H 6.5). The jars were then autoclaved at 121ºC for 15 min, cooled, inoculated with 1 mL of culture of *Monascus*, closed with filter membranes to avoid contamination but allow oxygen transfer, and incubated at 30-32°C for 8 d in a chamber with saturated moist air.

Extraction of Pigments

The fermented media were dried overnight at 55°C. Substrate-to-solvent amounts varied for different experiments, and these amounts are described accordingly in the Results section. The pigments were extracted with 95% ethanol for 12 h when using Soxhlet extraction, under partial vacuum at 65ºC. Static extractions were performed with 1 g of fermented substrate in 250 mL Erlenmeyer flasks with different solvents for 24 h at ambient temperature, except where indicated. Agitated extractions were performed in 250 mL Erlenmeyer flasks with 95% ethanol or ethanol-water mixtures at 110 rpm on a rotary shaker for 1 h at room temperature. In all cases, the extracts were centrifuged at 10,000 g for 15 min.

Solvents used for Extraction

Dimethyl sulfoxide (DMSO), hexane, ethyl ether, ethyl alcohol, methyl alcohol and acetonitrile (analysis grade) and water (deionized) were used for

the extraction. When necessary, water *p*H was adjusted with either HCl or NaOH.

Pigment Assay

Pigment produced was quantified by reading the absorbance with a spectrophotometer at 500 nm corresponding to red pigments in extracts. This was transformed in specific absorbance (in AU/g) relative to substrate dry mass, multiplying the absorbance by dilution factors and dividing by the substrate dry mass on fermentation medium. This method permits direct comparison between different processes, since it reflects the amount of coloured matter produced by fermentation.

Results and Discussion

To select the best natural substrate for biopigments production by *Monascus* sp, 11 different agricultural products were evaluated. Table 1 shows the approximate composition of these substrates[19]. The amount of pigment produced with different substrates (expressed as specific absorbance at 500 nm) varied to a great extent.

It was expected that cereals would be good substrates, since rice has been reported as the conventional substrate for this fermentation. This was partly true, but the performance of wheat and corn, although better than cassava starch or potato, for example, was lower in comparison to rice. It was also to be expected that substrates with a greater carbohydrate concentration, protein or phosphorus content could be better fermentation media for *Monascus* fermentation. This, however, was not the case, as the results recorded in Table 1 showed that wheat, corn or potato were similar to rice (concerning nutrients), but performed quite poorly comparing with the latter. Some of the substrates presented a reduced fungal growth (visual observation). It was postulated that the water content, always 56% was not adequate for all substrates. In fact, soy, cassava starch and potato agglutinated much more than the other substrates, even with free water on the flasks.

Although cassava bagasse presented a low pigment yield, this substrate was further used for subsequent moisture studies because it is a cheap agro-industrial residue, whose price might compensate the low yield, and also because the cassava bagasse, still having a 66% of starch, might be a potentially good substrate if properly supplemented. Experiments were conducted with different water contents, from 50-90%. At 80% or more, the culture medium became too wet with free water and agglutinated. The results of the study are shown in Fig. 2.

Table 1 — Biopigment production (AU/g dry substrate) and substrate composition

Substrate	Approximate composition (g/kg dry basis)			Average specific absorbance (AU/g dry substrate)
	Carbo-hydrate	Protein	Phos-phorus	
Rice	820	90	1.14	216
Wheat	770	140	3.63	79
Corn	780	130	3.19	60
Soy	330	400	6.00	13
Soy bran	400	480	7.00	22
TSP	-	-	-	12.6
Cassava	864	19	10.99	119.6
Cassava starch	900	20	3.3	38.5
Cassava flour	910	14	2.20	98.1
Cassava bagasse	660	11	-	15.7
Potato	800	100	9.6	4.7

(-) indicates data not known

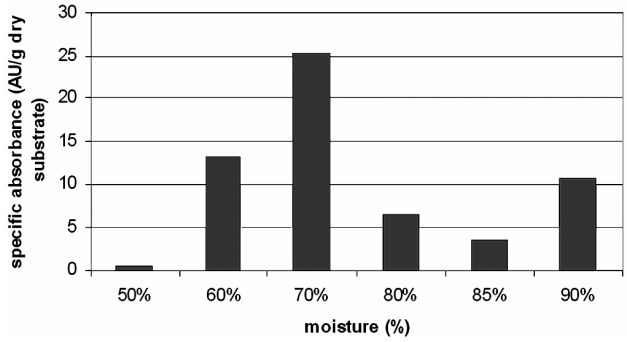

Fig. 2 — Specific absorbance (AU/g dry substrate) of red pigments extracted with 95% ethanol, produced in SSF with cassava bagasse with different water contents.

As expected, cassava bagasse (being more porous in nature and with higher fiber content than rice) showed optimum moisture of 70% m higher than that traditionally used for rice[10]. This is the moisture amount used for further cassava bagasse fermentations.

Growth Kinetics in Cassava Bagasse with 70% Water

In order to determine the growth kinetics for the culture on cassava bagasse, SSF was carried out for 24 d (Fig. 3). The maximum pigment production occurred at 10-11 d, which was a longer period than that for rice fermentation with the same inoculum (data not shown). This result could be due to higher availability of rice starch in comparison to cassava starch, or the lack of other nutrients in the latter.

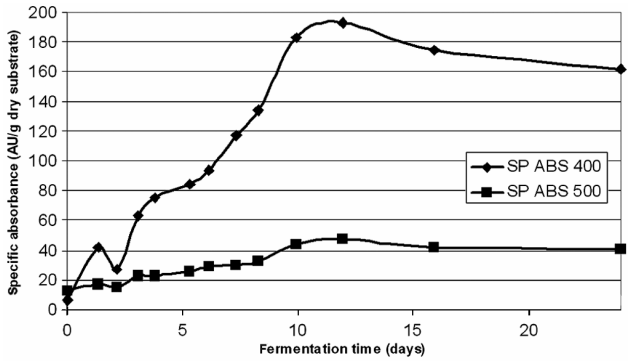

Fig. 3 — Production of *Monascus* sp. pigments in SSF of cassava bagasse, expressed as specific absorbance (AU/g dry substrate) as a function of time (in d).

Fig. 3 also shows that the absorbance of the pigment extract was higher at 400 nm, compared to absorbance at 500 nm. Many researchers use the value at 500 nm to estimate red pigment production, and absorbance at 400 nm to estimate yellow pigment production. Thus, it could be stated that the ratio of red/yellow pigments grew up during the course of the fermentation. However, red pigments also absorb light at 400 nm, so that such a comparison should be checked by chromatography assays.

In order to use cassava bagasse as a substrate for *Monascus* pigment production, the culture medium might require supplementation with nitrogen, phosphorus and a micronutrient source, which could improve pigment production and productivity. In order to test these modifications, however, it is necessary to guarantee that the pigment can be efficiently extracted. Also, it is important to define optimal conditions of extraction for industrial use. The following experiments were performed in order to define the best conditions for pigment extraction.

Extraction by Different Solvents

Chemical structures of the pigments produced by *Monascus* showed that the molecules were slightly polar. The solubility of red pigments in different solvents was estimated (Table 2). The results indicated that water was not a suitable solvent for *Monascus* pigments. There was a change in colour at *p*H 12.3 indicating that there could be changes in components of the substrate or in the structure of the pigments, which would need further investigation. Among the organic solvents, methanol is the best solvent, closely followed by DMSO and ethanol. Since ethanol is a cheaper, volatile, and non-toxic solvent, it would be the natural choice for an industrial process, and it was used for subsequent experiments.

Table 2 — Specific absorbance of red pigments extracted using different solvents (solvents are tabulated in decreasing order of polarity)

Solvent	Relative absorbance (% of maximum extraction)
Water, (*p*H 1)	2.1
Water, (*p*H 3)	5.9
Water, (*p*H 7)	5.3
Water, (*p*H 11)	3.6
Water, (*p*H 12.3)	18.7
DMSO	94.9
Acetonitrile	67.2
Ethanol	91.6
Methanol	100.0
Ethyl ether	72.8
Hexane	7.9

Solvent Amount

In order to determine whether there is saturation of the solvent with pigments or not, a series of agitated extractions was performed with different quantities of solvent (95% ethanol).

Table 3 shows that there was little difference in specific absorbance when using large amounts of solvent. This showed that in all the cases the solvent extracted nearly all the pigment that could be dissolved and absorbance was proportional to the mass of substrate used. The solvent ratio traditionally used by many authors was 5:1 g/g. These results indicated that although this ratio (5:1 g/g) worked in the present study also, less solvent (a 3:1 ratio) could be used with the same efficiency of extraction. This would lead to use of much less solvent in industrial extractions.

Effect of Temperature

Static extraction using 95% ethanol showed no significant dependence of efficiency to temperature with test values of 2, 22, 32, 39 and 58ºC.

Solvent Mixtures-Ethanol/Water

In order to study whether anhydrous ethanol, 70% ethanol or 95% ethanol were the best “alcohol” to be used, a test was performed with samples of 1 g fermented mass extracted with 10 g of different water-ethanol mixtures under static conditions.

Table 3 — Influence of solvent (95% ethanol) amount used in the extraction of red pigments of *Monascus*

Fermented mass (g)	Solvent mass (g)	Solvent/ Substrate ratio	Specific Abs_{500} (AU_{500}/g dry biomass)	AU extracted by 1 g solvent (AU/g)
5	15	3	16.2	5.40
5	20	4	15.5	3.87
5	28	6	15.0	2.65
10	90	9	16.6	1.85
5	95	19	16.0	0.84
5	95	19	18.1	0.95
4	96	24	16.6	0.69
3	97	32.3	16.3	0.50
2	98	49	16.0	0.33
2	98	49	17.5	0.36
1	99	99	15.2	0.15
0.8	99.2	124	15.3	0.12
0.4	99.6	249	15.8	0.06
0.2	99.8	499	18.3	0.04

Table 4 — Effect of extraction using mixtures of ethanol-water on specific absorbance of red pigments produced by *Monascus*

Ethanol (g)	Water (g)	Extraction efficiency (% of the maximum extract absorbance)
10	0	76.8
9	1	81.3
8	2	94.5
7	3	88.5
6	4	100.0
5	5	83.3
8	6	74.4
3	7	47.4
2	8	36.0
1	9	30.0
0	10	18
10 g of 95% ethanol, Soxhlet extraction		95.5

The results presented in Table 4 indicated that there was a maximum efficiency in extraction using 60% ethanol. This could be important, since it meant necessity of less organic solvent than traditionally used to perform extractions; also, the use of an aqueous phase may prevent dissolution of other components, lipids for example, in the solvent. Finally, Soxhlet extraction of the pigments was more efficient than static extraction by *ca*. 20%.

Conclusions

Results of the present study proved that rice was the best substrate among the eleven tested for SSF of *Monascus*, regarding pigment production. Other substrates could also be used, with a fair pigment production, especially corn, wheat and cassava. Cassava bagasse gave a low pigment yield, although efficient pigment production with this substrate could be achieved with the supplementation of nutrients to the culture medium. Optimum fermentation time for SSF in rice was 7 d and in cassava bagasse 10-11 d. The production of yellow pigments was superior to that of red pigments, but the ratio red/yellow (ABS 500/ABS 400) grew during the course of the process. Regarding pigment extraction, it was found that the best solvent is methanol, closely followed by DMSO and ethanol. Since methanol is toxic and DMSO is more expensive than the alcohols, the most suitable solvent to be used for extraction of *Monascus* pigments produced in SSF is ethanol, because it is cheap, non-toxic and volatile, and only 8% less efficient than methanol. A mixture of ethanol-water with *ca*. 60% ethanol (w/w) was more efficient than other concentrations. Extraction using Soxhlet extractor was more efficient than static ones. Finally, it could be mentioned that there was no direct correlation between efficiency in extraction and polarity of solvents, but polar solvents seemed to perform better than too-polar (water) or non-polar (hexane).

Acknowledgement

The authors wish to thank CNPq, Brazilian National Council for Research and Technological Development, and Fundação Araucária, for their financial support.

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