15 Production of Pigments

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15.1 INTRODUCTION

There is a marked trend towards the use of natural additives in the food and feed industry. In the case of natural pigments, there is a renewed interest in traditional technologies for the production of pigments such as carmine (from insects), annatto and curcuma (from plants) or *Monascus* (from a fungus). There has also been a lot of research on new alternatives, in the last few decades. Because of the intensive metabolite production that is possible in bioprocesses, there is a high interest in pigment production by fermentation. This chapter describes in brief the importance of natural pigments, presents some of the commercially successful, discusses the potential of other microorganisms for pigment production by SSF, and finally describes aspects of the production of *Monascus* by solid-state fermentation.

15.2 NATURAL PIGMENTS

Natural pigments are generally regarded as a preferable alternative to synthetic additives, because of their vegetal or animal *origin*. The preference towards pigments of natural origin makes sense, because in terms of metabolic pathways, "natural" substances might be more easily transformed in our body, since we have many matabolic pathways in common with other organism. At the other side, several natural pigments present anti-oxidant properties, a useful property consistent with the trend towards the development of nutraceutical foods.

This very antioxidant property (which means that the pigment itself is easily oxidized) makes some pigments unstable under extreme conditions of light, pH or temperature, when compared to its synthetic counterparts. However, the artificial pigments present two problems: first, there are several artificial pigments which were banned from the list of permitted color additives in the last few years, because of suspected toxicity or allergenicity; second, even synthetic additives proved harmless in adequate levels, it still appears as "artificial color" on the food package – which is currently, bad advertising.

Natural organic pigments have been known for long. The most important pigments were plant extracts, such as curcuma and indigo, or animal extracts such as carmine. Until the 19th century, natural organic pigments were the most important either for food and cosmetic use or for the textiles industry (the paint industry, on the other side, had always counted on several inorganic pigments which are, however, toxic for humans). After 1850, with the synthesis of mauve by W. Perkins, there has been a flourishing of organic pigment industry and, as a complement, the weakening of traditional pigment agro industry such as indigo, in India. The great variety in synthetic organic pigments stimulated its use without any concern about its toxicity. In 1900, about 80 artificial pigments were used in foodstuffs. At that time, there was no regulation regarding the use or the purity of such substances; however, there was reasonable toxicity in some cases. Since 1971, the carcinogenic effect of several substances in the human body is being studied (Nazaré, 2001). Today, most synthetic pigments have its use forbidden for food and cosmetic use (FDA, 2002).

Increasing doubts regarding the safety of several artificial colors has stimulated its substitution by natural color additives (Kim et al., 1995). Some of the artificial colors, such as azorubin or tartrazin, may cause allergies (Fabre et al., 1993); even the list of permitted artificial colors is constantly reduced due to new information about adverse effects of known pigments. There are 9 artificial color additives currently permitted in drugs, foods and cosmetics ("FD&C colors") in USA, 2 of which with restricted use. At the other side, there are 21 natural color additives (5 of which with restricted use), permitted for the same uses. These natural colors are from diverse origin, from fruit and vegetable juices to caramel (food-grade carbohydrates thermally processed) (FDA, 2002). The same situation prevails in other countries: the list of permitted color additives in the European Union is presented in Table 1 (Arlt, 2004).

Analysis of Table 1 shows that while several natural pigments are permitted in as much as needed amounts in foods, none of the artificial pigments have derestricted use: they must be used at controlled levels. This indicates that the natural pigments market is rather promising, especially because the pigments now permitted do not span over the whole visible spectrum (Vargas et al., 2000). Finally, refrigeration, canning, dehydration, fuming, bottling and exposition to light, air and extremes of humidity and temperature are factors which tend to alter the natural food color, making color additives essential for the recovery of product quality (Griffiths, 2005).

At the moment there is an increasing demand for natural pigments in food industry, both from natural and vigetal sources. New sources of natural pigments such as betalains from beet and anthocyanins from grapes, and optimizing

Table 1 – Food colors permitted in the European Union

Use at quantum satis in any food[a]	*Use limited to a maximum level, in some specific foods*	*Restricted use (permitted for some foods only)*
Riboflavin (E101)	Curcumin (E100)	Amaranth (Bordeaux Red) (E123)
Clorophylls and clorophyllins (E140)	Tartrazin yellow (E102)	Eritrosin (E127)
Copper complexes of clorophylls and clorophyllins (E141)	Quinoline yellow (E104)	Red 2G (E128)
Caramel (E150a)	Sunset yellow FCF, sunset orange S (E110)	Brown FK (E154)
Caramel sulphite (E150b)	Cochonile, carminic acids, carmines (E120)	Canthaxantin (E161g)
Caramel ammonia (E150c)	Azorubin, carmoisin (E122)	Aluminum (E173)
Caramel sulphite-ammonia (E150d)	Ponceau Red 4R, Carmine Red A (E124)	Silver (E174)
Carbo medicinalis (E153)	Allura red AC (E129)	Gold (E 175)
Carotenoids (E160a)	Sea blue V (E131)	Litorubin BK (E180)
Paprika extracts, capsanthin, capsorubin (E160c)	Indigotin, indigo carmine (E132)	Anatto, bixin, norbixin (E160b)
Beetroot red, betanins (E162)	Brilliant blue FCF (E133)	
Anthocyanins (E163)	Green S (E142)	
Calcium carbonate (E170)	Brilliant black BN, black PN (E151)	
Titanium dioxide (E171)	Brown HT (E155)	
Iron oxides and hydroxides (E172)	Licopene (E160d)	
	Beta 8'-carotenal (C30) (E160c)	
	Ethyl Ester of beta-apo-8' carotenoic acid (C30) (E160f)	
	Lutein (E161b)	

a - use "*at quantum satis*": as much as needed, but following good manufacturing practices

their extraction processes open new evenues for its application in food industry. Furthermore, it is estimated that up to 70% of the world plants have not yet been exploited towards their potential for production of natural substances, and that only 0.5% were totally investigated for. At the other side, the growing demand in the food industry supersedes the offer of traditionally produced pigments, and requires new alternatives – such as production of pigments by fermentation (RIA, 1999). The only pigments presented in Table 1, which are currently produced economically by fermentation are riboflavin and beta-carotene.

15.3. MICROBIAL PIGMENTS – PRODUCTION AND MARKET

Currently, the pigments produced by microorganisms and used commercially are riboflavin (vitamin B2, a yellow pigment permitted in most countries) by *Eremothecium ashbyii* and *Ashbya gossypi*; the pigments from *Monascus* (discussed later on) produced by *M. purpureus* and *M. ruber*; carotenoids (yellow pigments produced by several microorganisms, but currently produced commercially only from micro algae) such as β-carotene (by *Dunaliella salina* and *D. bardawil*) and astaxanthin (by *Haematococcus pluvialis*), and ficobiliproteins such as phycocianin (a blue pigment used in food and cosmetics), produced by *Spirulina* sp. Indigoids, anthraquinones and naphtoquinones are pigments which hold potential use in the near future (Jacobson & Wasileski, 1994). In recent years, active search for microorganisms producing non-toxic metabolites has been performed by several researchers (Babitha et al., 2004; Pandey and Babitha, 2005; Downham & Collins, 2000; Mapari et al., 2005).

Fungi are the most adequate microorganisms for pigment production in SSF. The use of cyanobacteral cultures such as *Spirulina* in SSF is limited because light has a poor penetration on the substrates, and even though some of these microorganisms may be cultivated in heterotrophic cultures, light still is necessary for the stimulation of photo-pigments (chlorophyll and phycobilins) production. Bacteria, at the other side, may grow in solid substrates but usually show a better development in liquid media. There is an enormous amount of pigmented fungal metabolites. They frequently also present pharmacological activity, which reduces the possibilities for food use. Table 2 shows some fungal strains with potential for application in SSF production of pigments.

Table 2. Selected fungal strains with potential for pigment production in SSF

Substance	*Microorganism*	*Strains*
ß-Carotene	*Blakeslea trispora*	DSM 2387, DSM 2388, ATCC 14271
Other Carotenoids	*Sphingomonas echinoides*	DSM 1805
	Acremonium diospyri	DSM 2939, ATCC 9066
	Dacrymices deliquescens	ATCC 13293, ATCC 13295, ATCC 13292
	Mucor azygosporus	ATCC 15087
	Neurospora crassa	ATCC 10816, ATCC 26187
Red Pigments	*Monascus sp.*	DSM 1604, ATCC 16360, ATCC 16362, ATCC 16365, ATCC 16367, ATCC 16427
Riboflavin	*Acremonium diospyri*	DSM 2939, ATCC 9066
	Candida sp	DSM 70109, ATCC 10539, ATCC 9058
	Eremothecium ashbyii	ATCC 26614, ATCC 36179
	Pichia guilliermondii	DSM 70051

The market for natural pigments produced by bioprocesses is hard to estimate, either due to the lack of statistics of regional, low-technology products or to the fact that the production is pulverized over many small companies worldwide. In the specific case of *Monascus* pigments, the consumption of these pigments in Japan raised from 100 ton in 1981 to 600 ton in 1992, and was estimated as U$12 million (Lee et al., 1995; Hajjaj et al., 1997).

Natural pigments frequently cost 5 to 10 times more than its synthetic equal (Spears, 1998). The best case is that of β-carotene produced by microalgae, which costs approximately U$1000/kg against U$ 500/kg produced by synthesis; despite the higher price, β-carotene produced by fermentation may compete in markets where it is important that all the ingredients be "natural"; besides, the microbial pigment is a mixture of cis- and trans- isomers, with anti-cancer effects that synthetic β-carotene, mostly cis-, does not show.

The world market for pigments of natural sources (excluding nature-identical and caramels) was estimated in 1987, as U$ 35 million; in 2000, this market was around U$ 250 million (Downham & Collins, 2000). Based on this growth tendency (600% in thirteen years, against 200% for the whole color market in the same period), today the market for natural pigments (which excludes nature-identical and caramels) is probably on the order of U$ 350 to 600 million. The biggest markets for food pigments are Europe and United States. The utilization distribution is not proportional to the food consumption (or to the population), because pigments are used in *processed* foods: there is a potential demand for

developing countries, in which an improvement on the economic profile possibly will cause an improvement on the consumption of processed foods.

15.4. MONASCUS PRODUCTION BY SSF

15.4.1. Monascus products

Easily encountered in diverse ecosystems, fungi from the genus *Monascus* are traditionally used in oriental countries, originally in China and Thailand, to prepare a fermented rice with strong red color, which finds several applications ranging from conferring color to products such as wine, cheese and meat, to medicinal uses and as a meat preservative (Wong & Koehler, 1981). While some *Monascus* metabolites are important due to their strong color, others show hypocholesteremic and antimicrobial properties. Because of their high potential as food color additives, this microorganism was studied with more intensity in the last decade (Martínkova et al., 1995; Zhang et al., 2000, Babitha et al., 2006a, 2006b, 2007a, 2007b). Currently, several companies sell the dry, pulverized, fermented rice product as a nutritional supplement with ability to reduce cholesterol levels, and others sell the dried product or purified extracts as food colors.

15.4.2. Metabolites structure and biosynthesis

Fungi of the genus *Monascus* produce a series of secondary metabolites, which include several azaphilone pigments with a similar polyketide structure. These pigments are produced as a mixture of red, orange and yellow compounds which are commonly used without further separation, although the main commercial interest lies on the red compounds. According to some authors, there are more than 10 pigments produced by the culture, but only some of them were elucidated structurally (Shin et al., 1998). The main *Monascus* pigments are illustrated in Figure 1.

The orange pigments, monascorubrin and rubropunctatin, are synthesized in the cytosol from acetyl coenzyme A through a multi-enzymatic polyketide synthase complex. These pigments are not hydro-soluble and are unstable in extreme pH (Hajjaj et al., 2000), but present structures with high affinity to compounds containing primary amino groups (thus called aminophiles). Reactions with amino acids lead to formation of *water-soluble* red pigments, monascorubramine and rubropunctamine. The mechanism of yellow pigment formation is not yet clear; some authors consider that these are products of the alteration of orange pigments, as others believe it to be pigments with their own metabolic pathway (Lin & Demain, 1991; Jůzlová et al., 1996). The polyketide pathway is fairly well known, but details of the biosynthesis of *Monascus* pigments are important in order to allow better manipulation of conditions and medium composition regarding

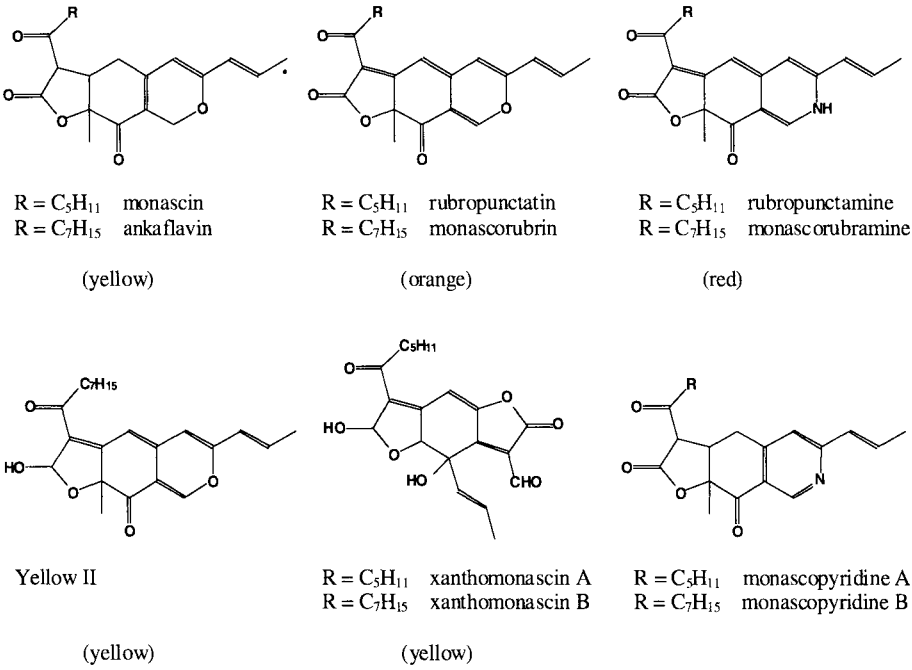

Figure 1. Structures of *Monascus* sp. pigments

fungal metabolism. There are reports of analogues to *Monascus* pigments produced by a non-*Monascus* fungus, *Penicillium* sp. AZ, using the similar metabolic pathways (Ogihara et al., 2000).

Because of their affinity to amino groups, *Monascus* pigments are frequently associated with proteins (Wong & Koehler, 1981) or with the cell wall, forming a complex that may be of difficult extraction. Other authors consider that there may be a fixation of the pigments to lipids of the fungal biomass, so that the extraction would involve cell breakage and dissolution in an organic solvent (St. Martin, 1990). Also due to this affinity for amino groups, it is possible to convert orange lipid soluble pigments to red water soluble ones by reaction with amino acids and analog compounds *in vitro* (St. Martin et al., 1991). In that case, nitrogen from the amino group (from the amino acid or analog) takes the place of the oxygen of the ring on rubropunctatin or monascorubrin, yielding analogs of rubropunctamine or monascorubramine, but presenting a radical linked to the N in substitution to the H of the natural red pigments.

15.4.3. *Monascus*: Strains and Morphology

The genus *Monascus* contains five main species pertaining to the family *Monascaceae* and to the class *Ascomyceta* (*M. pilosus*, *M. sanguineous*, *M.*

eremophilus, *M. pallens* and *M. lunisporas*) (Pitt & Hocking, 1997, Park et al., 2004), whose most important characteristic is the ability to produce secondary metabolites of polyketide structure (Jůzlová et al., 1996), some of them with strong yellow, orange or red color. Most strains used for pigment production are ultimately species of *M. pilosus*, eventually referred to as *M. purpureus* or *M. ruber*.

The colonies in potato-dextrose-agar (PDA) after 7 days are 20 to 30 mm in diameter, plane, eventually with small aerial development, sparse, with flocculent superficial texture, mycelium initially white (1 to 2 days), turning to orange and then to brick red as the culture develops, with formation of cleistotechia and aleurioconidia (Figure 2). There usually is the formation of soluble pigments that diffuse through the agar. The cleistotechia are spherical, from 30 to 60um in diameter, formed as a hyphal knot from a well defined stalk, with cell walls turning to brown with maturation; ascospores are ellipsoid, hyaline, 5-7×4-4,5um in size, with a smooth cell wall. There may be the formation of aleurioconidia in pedicels, laterally to hyphae, but most commonly terminal, sometimes growing isolated but more commonly in chains up to 10 cells, spherical to piryform, frequently rounding on maturation, with 10-14mm in diameter or 10-18×8-14mm in size, with thick walls, smooth and brown. Most species produce also chlamidoconidia and arthrospores. The colonies of *M. ruber* grow more quickly than other species of Monascus (Pitt & Hocking, 1997).

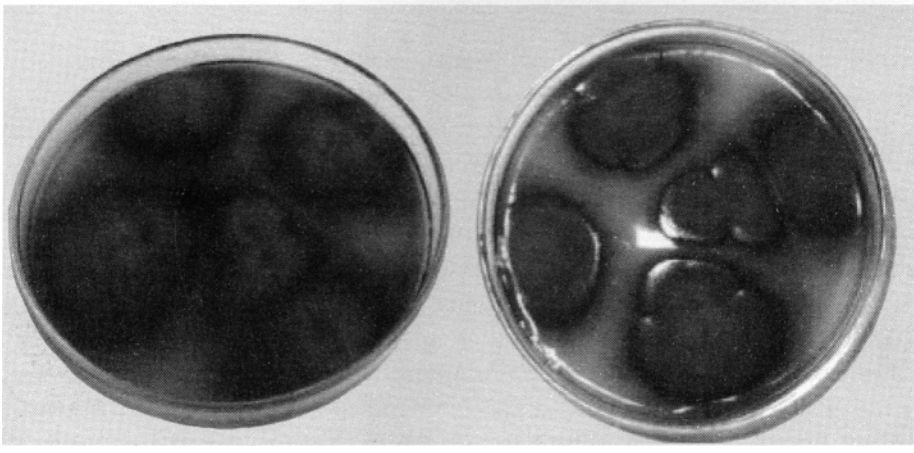

Figure 2. *Monascus* cultures in PDA after 7 days incubation at 32°C. Note the diffusion halo of water soluble pigments and the characteristic color of mycelium.

Source: Carvalho et al., 2004

15.4.5. *Monascus* cultivation conditions

15.4.5.1. Temperature

The optimal temperature range for most *Monascus* species is 28-32°C, with some strains requiring temperatures as low as 25°C and others as high as 37°C (Lin & Demain, 1991).

15.4.5.2. Presence of oxygen

The strains of the genus *Monascus* are incapable of anaerobic growth using glucose as a substrate, but may grow in oxygen-limiting conditions. Under these conditions, there is a higher production of ethanol and CO_2, but a lower production of pigments. In conditions of higher aeration, ethanol production decreases and pigment production increases. It was observed that increase in the partial pressure of CO_2 increased the pigment production (Pastrana et al., 1995). In high glucose concentrations (above 20 g/L in liquid culture), a Crabtree-like effect occurrs, that is, the shift to a predominantly anaerobic metabolism, with production of ethanol, even in good aeration conditions (Chen & Johns, 1994; Rosenblitt et al., 2000, Carvalho 2004). With lower glucose concentrations or with other sugars, it is possible to divide production in two phases: initially glucose is converted to ethanol and biomass, and then ethanol is converted to biomass and pigment (Hamdi et al., 1996). In a defined liquid medium with oxygen-limiting conditions, red pigment production is growth-associated, whereas in oxygen excess pigment production may be inhibited by the effect of an unknown product (Hajjaj et al., 2000). The influence of oxygen concentration in solid substrate fermentation was also investigated; at 0.02atm CO_2, an increase of the oxygen partial pressure up to 0.5 atm gave high pigment yields, whereas low CO_2 pressures with 0.21atm O_2 gave higher pigment yields (Han & Mudget, 1992).

15.4.5.3. pH

Growth has been observed in a wide range of pH, from 2.5 to 8.0, with the ideal range being from 4.0 to 7.0 (Yongsmith et al., 1993). Within this range, the variation in growth is small, with a little better development at pH 4.0 (Lin & Demain, 1991; Chen & Johns, 1993). Although the growth was better at pH 4, the biomass yield was higher at pH 6.5 (Chen & Johns, 1993). The production of pigments is affected in a different way: at lower pH, there is predominance of yellow pigments, and at a higher pH, there is a predominance of red pigments. At pH 2.5, there was production mostly of a bright red pigment with a skeleton similar to the traditional pigments (Yongsmith et al., 1993); at pH 4 there was a stimulation of synthesis of ankaflavin (yellow pigment), and the total production of pigments increased with a increment in

pH until pH 5.5. Above this value, there was a decrease in pigment production (Lin & Demain, 1991), although the production of red pigments was higher than that of yellow ones. At pH 7, there was no more production of yellow pigments (Yongsmith et al., 1993). The change of pH during growth depends on nitrogen sources, in the first place, and also the carbon sources. Regardless of initial pH, the final pH tends to be the same (Jůslová et al., 1996), usually in the range of 7 to 8 (Yongsmith et al., 1993).

15.4.5.4. Liquid vs. solid fermentation

Although the traditional production of ang-kak for use as a coloring agent is conducted on a solid support (rice), most of the studies performed in laboratory have been done using liquid culture media, which present easily controllable conditions. Solid-state fermentation (SSF), however, gives a higher yield and productivity of pigment than liquid fermentation. A comparison was made between liquid and solid media of similar composition, the solid media obtained from the liquid by addition of a gelling agent, followed by the extrusion in rice-sized particles. The solid media thus prepared supported the production of up to three times more pigment than the corresponding liquid media, but the cultivations over rice were still superior (Johns & Stuart, 1991).

15.4.5.5. Humidity in SSF

The traditional process of ang-kak production is the same used for other koji varieties (koji is the general name given to rice fermented by several fungal species, usually *Aspergillus*), hence the alternative name "red koji". The production process consists of maintaining non-agglutinating rice immersed in water up to 24h, and then steaming (autoclaving), addition of an inoculum (a portion of rice previously fermented) and addition of water at intervals to maintain humidity (Palo et al., 1960; Wong et al., 1981). The humidity needed in the rice fermentation varies according to the traditional description, but it is usually recommended that the humidity be enough to permit growth of mycelium through the grain, without *disintegrating* the grain. The ideal humidity for pigment production in solid substrates is around 56%, with pH 6 (Johns & Stuart, 1991).

15.4.5.6. Carbon sources

The most common way to produce *Monascus* in SSF is the cultivation of steamed rice. This is also the best natural substrate in terms of color production; however, several other carbon sources have been used as substrates for *Monascus* growth in liquid media, and these are presented here because they might be used as the medium of choice in SSF over suitable supports.

The most common carbon sources in formulated media are glucose, sucrose and starch. The highest growth is usually observed with glucose (St. Martin, 1990). The pigment production depends on several factors such as nature and concentration of the substrate, pH and nitrogen source. The volumetric production of pigments in submerged media is higher with starch and dextrin, while specific production is higher with maltose and almost as good with glucose (Lin & Demain, 1991), but that comparison between sugars must be done carefully, taking into account the concentrations. In glucose concentrations lower than 20g/L, growth and red pigment production are excellent; glucose concentrations higher than 20g/L lead to a Crabtree-like behavior, with ethanol production and reduced cell growth and pigment formation, even in the presence of oxygen. This indicates that there could be a repressive effect from glucose, and that the use of another sugar might avoid this effect. As a matter of fact, in high concentrations (50g/L), maltose is better than glucose, especially in the presence of peptones. These different concentrations for sugars show stronger effect on production of yellow rather than red pigments, although with maltose, red pigment formation is stimulated (Chen & Johns, 1994). A good alternative carbon source is ethanol (Jůslová et al., 1994), which is naturally produced by the fungus in oxygen-limiting or excess glucose conditions (Pastrana et al., 1995; Hamdi et al., 1997). Since biomass formation is favored by the use of carbohydrates, the process could be performed in two steps (for example, maltose-ethanol) in order to enhance the efficiency of ethanol use for pigment production (Jůslová, 1996). There are some contradictions on the effect of carbon sources: for instance, Lin (1991) did not encounter repressive effects for any carbon source, using concentrations of up to 10% carbohydrate. These contradictions may be possibly attributed to different organisms and aeration rates used.

15.4.5.7. Nitrogen sources

Different N-sources used for *Monascus* growth range from inorganic nitrogen (ammonium and nitrates) to peptones. In the traditional ang-kak production, there is no need of addition of nitrogen sources, since rice has 5 to 8% proteins, (dry basis) (Franco, 1992). When using other substrates, the addition of a nitrogen source (especially organic nitrogen) stimulates pigment production. According to Lin (1991), the use of monosodium glutamate as a nitrogen source stimulates pigment production, which has been confirmed by other authors. The kind of pigment and its excretion by the cell are also related to the nitrogen source: organic nitrogen, as in amino acids, favors the formation of red pigments (Yongsmith et al., 1993; Jůslová et al., 1996), and the use of peptones is better for pigment production and cell growth, as well as for pigment secretion at pH 6.5 (Chen & Johns, 1993), although the use of polypeptones favor the formation of yellow pigments (Jůslová et al., 1996). It

is possible that the stimulating effect of amino acids on the production or liberation of pigments is caused by an enhancement in solubility, since the derivatives of *Monascus* pigments linked to amino acids, are more soluble than the original pigments. Studies demonstrate that the use of ammonia as a nitrogen source may favor the orange pigment production (Jůslová et al., 1996). A comparison of the relative amounts and color quality of pigments produced using several amino acids show that yellow and orange pigments are unaffected by the nitrogen source, whereas red pigments differ in tone and solubility (Jung et al., 2003)

15.4.5.8. Other factors

According to Lin (1991), high concentrations of phosphate and of magnesium sulphate inhibited pigment production and the growth was a crescent linear function of $MgSO_4$ concentration, in the range from 0.5 to 16mM. The addition of corn oil stimulates (doubled) the pigment production, while the addition of 0.4% Tween 80 neither affect glucose uptake, nor retard the growth rate, but enhanced the pigment productivity (6 to 8 times, reaching 8535 units of absorbancy/g dry matter) (Chiu & Poon, 1993).

15.4.5.9. Complex culture media

Culture media for *Monascus* pigments production are very diverse, ranging from defined compositions to natural ones. Being a common contaminant in grains, and having been isolated from several substrates with a high solids concentration, *Monascus* grows in a wide variety of natural substrates. Some natural substrates already tested, besides rice and other cereals, are cassava starch (Yongsmith et al., 1993; Lee et al., 1995; Carvalho et al., 2001), prickly pear juice (Hamdi et al., 1996), and dairy milk (Kujumdzieva et al., 1997). In some cases, as in cassava starch, it is necessary to supplement these substrates with yeast extract and peptones, as vitamins and organic nitrogen supplements. The components of the complex culture media used were already discussed in the text, and include several sugars (most commonly glucose), oligoelements and organic nitrogen sources (amino acids, peptones) or inorganic nitrogen (ammonium, nitrates). A medium used in some works is composed of: glucose 40g/L; NH_4NO_3 3 g/L; K_2HPO_4 6g/L; KH_2PO_4 6g/L; $MgSO_4.7H_2O$ 0.5g/L; KCl 0.5g/L; $FeSO_4.7H_2O$ 10mg/L; $ZnSO_4.7H_2O$ 10mg/L; $MnSO_4.H_2O$ 3mg/L; final pH 6.3 (Wong 1981, Lin and Demain, 1991).

15.4.5.10. Culturing Monascus over rice

For the production of *Monascus* pigments in SSF, best conditions are forced aeration (with water-saturated air) of 1NmL/min.g of wet substrate (56% humidity)

in an 8cm height bed, at 32°C, for 7-8 days, after inoculation with 10g/kg of a spore suspension with at least 10^6CFU/mL. Under these conditions, a maximum specific growth velocity of 0.039^{h-1} and a specific pigment production velocity of 27.5 AU/g biomass.h^{-1} were obtained. Aeration and humidity play a critical influence in pigment formation, and careful design of the fermenter must be done prior to scale-up; in tray fermentation, although forced aeration is not necessary, the bed height must be reduced to 2-3cm (Carvalho et al., 2006).

15.4.6. Production and extraction in SSF

The production of *Monascus* in SSF is very straightforward, except for the fact that, depending on the fermentation conditions, the substrate may lose its rigidity, so that a suitable solid layer must be used (generally 2 to 15 cm). Figure 3 illustrates *Monascus* pigments production over rice, in trays.

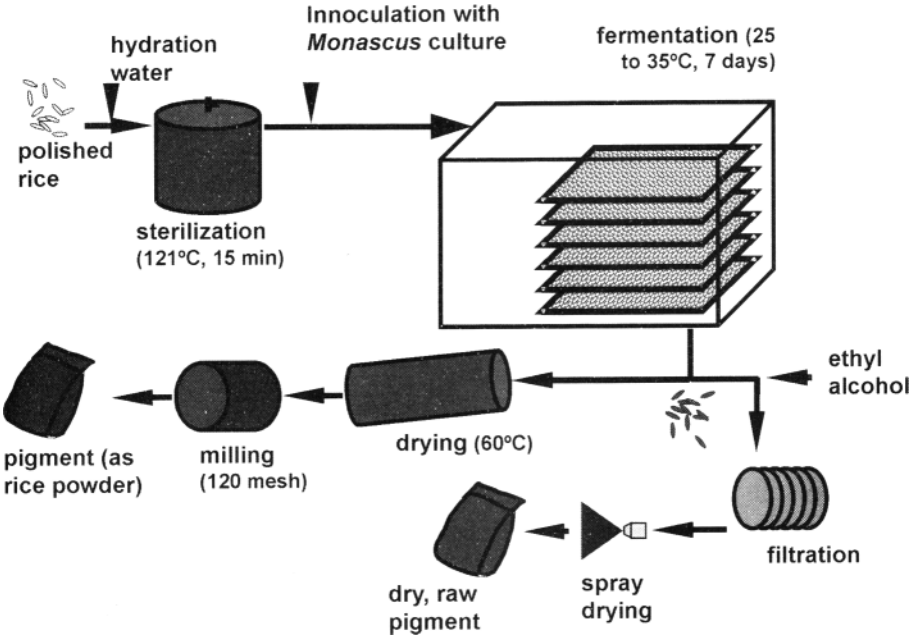

Figure 3. *Monascus* production in trays

When only the coloring fraction is necessary, without substrate residues – for use in beverages, for an instance – the pigment must be extracted with an organic solvent, such as 60-70% ethyl alcohol (pure ethanol is less efficient; methanol is very efficient, but toxic). After the extraction, the solvent is evaporated and the pigment eventually processed. (Carvalho et al., 2006). Depending on extraction conditions, an adequate filler must be used to enhance processing.

15.4.7. Pigment analysis, toxicity and stability

There are several studies on the toxicity of *Monascus* pigments, which are apparently safe in the quantities tested. The many years for which these pigments have been used suggested low or non-existent toxicity (Lin & Demain, 1991). Since the strains from the genus *Monascus* started to be studied systematically, it has been believed that the pigments produced also presented antibiotic properties; later, it was verified that this activity is due mainly to other substance, named monascidin A (Wong & Koehler, 1981). Further studies showed that this substance was, in fact, citrinin, a mycotoxin with nephrotoxic action produced by several fungi (Blanc et al., 1995a), but that not all *Monascus* strains produced citrinin. Unfortunately, it seems that all good pigment producers also produce citrinin. Finally, studies over the toxicity of purified fractions of pigments showed that there was indeed an antibiotic activity for *Monascus* pigments, especially the orange ones and, in lower degree, the red ones (Martínková et al., 1995).

Although there is not yet a definitive conclusion about pigment toxicity and citrinin production in industrial processes, several actions may be taken to prevent or reduce this problem: the use of strains which produce less citrinin; the control of the nitrogen source (organic nitrogen sources favor red pigment production and decrease citrinin production); the control of cultivation conditions (aeration, pH, solid-substrate vs. liquid fermentation); the transformation of orange pigments into red, non toxic complexes, using amino acids (Blanc et al., 1995, 1995a; Jùzlová et al., 1996) and the extraction in low citrinin-solubility conditions, which may be achieved controlling pH, since citrinin is strongly acidic. There is much space for strain development, as may be noted by the examination of Table 3 (Carvalho, 2004):

Table 3 - Pigment production and productivity, and citrinin production in rice for several *Monascus* strains.

Strain	*Relative absorbance (500nm)*	*Productivity day^{-1}*	*Citrinin μg/g product*
CCT 3802	0.15[a]	0.021	20
ATCC 6405	0.47[a]	0.047	23
ATCC 16365	0.47[a]	0.067	83
UFPE 3196	0.14[a]	0.01	29
Commercial product[b]	1.00[a]	0.07	31
NRRL 1991	0.16	0.023	25
NRRL 2897	0.14	0.020	22
CCT 3802	0.15	0.021	20
LPB 31	0.87	0.124	18

[a]values from Miyashira, 2003, [b]strain unknown

Table 3 shows that there is a high diversity among different *Monascus* strains, with a large span in production, productivity and citrinin production. It is very likely that future production will be made with pigment overproducers which do not produce citrinin.

15.4.7.1. Analysis

Pigment production by *Monascus* is usually evaluated by measuring absorbances for pigment solutions on the ranges near 400, 470 and 500nm for yellow, orange and red pigments, respectively (Johns & Stuart, 1991; Lin & Demain 1992). The ratio of absorbance at 500nm/absorbance at 400nm gives the ratio between red and yellow pigments (Wong & Koehler, 1981). In SSF, it is necessary to make a solvent extraction step, followed by absorbance reading. The amount of solvent used is, usually, 5mL solvent for each gram of fermented material, and the extraction time 1h under agitation. Red pigment solubility is highest in an aqueous solution containing 60-70% ethanol (Carvalho, 2006).

The fact that the yellow, orange and red pigments of Monascus are produced as a mixture probably affects the analysis by simple measure of absorbance. Nonetheless, the vast majority of authors estimate pigment production by this method, with pigment production ranging from hundreds of absorbance units/ml culture media in submerged fermentations (e.g. 220 OD_{510}/mL, in optimized conditions by Kim et al., [2002]), to thousands of absorbance units/g dry substrate, in SSF (e.g, 5430 OD_{500}/g dry matter, by Lin & Demain, [1992]). The best procedure for pigment analysis is liquid chromatography, wich allows to separate and quantify individual pigments; with this method, Hajjaj *et al.* (2000) considered 1 unit OD_{480} correspondent to 15mg/L of red pigment with M = 498 g/mol. Naturally, this equivalence should not be applied to crude pigment extracts, wich may contain several substances with different absorbances.

Biomass analysis may be adequately done via ergosterol analysis, rather than glucosamine (which may have interferences from pigments) (Carvalho et al., 2006)

15.4.7.2. Pigment use and stability

Surprisingly, only few works deal on the stability of *Monascus* preparations, considering that several industries produce this pigment. Some documentation can be found at the producers such as Allok (www.allok.com). According to Lin & Demain (1992), these pigments are fairly stable to autoclaving and in a wide range of pH. According to Fabre et al., (1993), sausages or pates colored with red pigments of *Monascus* presented a residual color of 92 to 98% after three months at 4°C, with good sensorial acceptance. However, the pigments

were unstable to light (only 20% residual color after 50 days) and heat (45% residual color after 2h at 100°C. They are more stable at neutral or basic pH (Fabre et al., 1993, Lee & Chen, 2000). Table 4 shows the residual color of *Monascus* pigments incubated in aqueous solutions at several pH and temperatures.

Table 4. Residual color (in %) of aqueous pigment solutions, after 25h incubation at several pH and temperatures.

	Temperature, °C				
pH	**0**	**32**	**40**	**60**	**100**
4.1	80	86	79	49	13
4.7	87	89	82	46	15
6.0	83	87	81	57	16
7.3	83	84	86	72	27
7.9	84	92	88	79	53

Source: Carvalho et al., 2005

As can be seen at Table 4, *Monascus* pigments should be used in food applications with neutral pH and moderate temperatures. However, the pigment stability is very good in lower water activity, such as in alcoholic beverages, pates and sauces.

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