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Monascus spp.: A source of Natural Microbial Color through Fungal Biofermentation

Abstract

The search for naturally produced substitutes for chemical food colorants has led to a resurgence of interest in pigments synthesized by fungi such as *Monascus spp.* This fungus has been used in Asia for many centuries as a natural color and flavor ingredient in food and beverages. The red pigments are of particular interest, because red is the most popular food color and true natural pigments suitable for applications in food industries are difficult to obtain. As a result of recent efforts to replace synthetic food dyes with natural colorants, pigments produced by *Monascus spp.* have attracted worldwide attention. *Monascus* color, categorized as a natural color, has also been widely used as a food supplement and in traditional medicine. The major objective of this review deals with production of natural microbial color by *Monascus spp.* and addresses the parameters involved in fungal biofermentation. The fungal strains of *Monascus spp.* can be either fermented in solid state fermentation (SSF) or in submerged fermentation (SmF). SSF and SmF are two commonly used techniques during various fermentation processes. One important aspect in the development of a biofermentation process is the ability and suitability of the *Monascus* strain to be employed with a suitable medium.

Keywords: *Monascus spp*; Fungi; Biofermentation; Proliferation; Pigments

Review Article

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Abbreviations: SSF: Solid State Fermentation; SmF: Submerged fermentation

Introduction

Many synthetic food colorants, particularly red ones, have been banned for use in food products due to their toxicity. This has promoted research into food colorants from natural sources. The less stringent tests necessary for the use of natural colorants and the increase in demand for natural ingredients by the increasingly health conscious have led food manufacturers to relook into the use of natural food colorants [1]. This has also resulted in a proliferation of interest in the development of natural food colorants in recent years. The health concern issues of consumers increase the trend towards replacement of synthetic colorants with natural pigments [2]. It has become clear that no single natural colorant can be used for the whole range of food products, due to sensitivity to physical parameters. It is also true that a traditional food colorant is more likely to obtain approval from regulatory authorities and the acceptance of consumers [3]. The task of food biotechnology is to incorporate modern biotechnology methods to improve the productivity and scale-up in the production of these traditional food colorants.

Color is an important food characteristic. It is also a means for the ultimate acceptance of food. In order to enrich or compensate for the loss of color during processing, food colorants have become important food additives. Color additives are also indispensable in manufacturing industries. Dyes and pigments are used to create new food products and to modify the color of established food products, which show color shifts as a result of manufacture and storage. Dyes and pigments are classified as certified and uncertified food colors [4]. Certified colors are manufactured synthetically to meet strict government specifications. Uncertified colors are usually naturally derived products. Most precursors of chemically based colorants are generally derived from petrochemicals [5]. There is considerable interest worldwide in the development of food colorants from natural sources. One indication of this is the number and distribution of food colorant patents issued in the years 1969 through 1984; the interest in natural colorants is clear, since there were 356 patents on natural sources compared to 71 on synthetics [6]. As synthetic food colorants have been prohibited due to their toxicity, the development of comparatively safe natural food colors is now attracting the great interest of researchers. One alternative method for food colorant production is through microbial fermentation.

Colorants from microbial species offer considerable advantages since they can be produced in any quantity and are not subject to the vagaries of nature [7]. The best known microbial colorants are those produced by the *Monascus* group, especially *M. purpureus*, which is traditionally grown on rice in the Orient. *Monascus* species produce a red color, which may be incorporated into foods. Production of natural pigments from animals, plants and microorganisms is gaining more importance in recent years [8]. These include anthocyanins, betacyanins, cochineal and carmine (extract of the female cochineal insect *Dactylopius coccus*), lycopene, carotenoids, annatto and saffron, turmeric, riboflavin, grapes, beets, paprika (extract of plant), etc [9]. Microorganisms could be a promising source for natural

food colorants but, except for the fungus *Monascus* in the Orient, few attempts have been made to use them. The presence of carotenoids, anthroquinone pigments and chlorophylls has been demonstrated in bacteria, yeasts, fungi and algae [10]. Due to their sensitivity to environmental parameters, no single natural colorant can be used for the whole range of food products, e.g. Safflower Yellow is sensitive to heat treatment while ß-carotene from carrot is sensitive to light; Berry color appears red at pH 3 but turns to purple at pH 4 to 6, and eventually becomes blue at pH 8. The food industry would need many more natural colorants than the available synthetic colors in order to color different food products [8,11].

It is also true that traditional food colorants are more likely to obtain approval from regulatory authorities and the acceptance of consumers. Thus, the main task of the food biotechnologist is to apply modern biotechnological methods to improving the productivity and application of these traditional food colorants. Many microorganisms contain pigments and may serve as sources of food colors. *Monascus sp.*, a fungus that produces red pigments, shows promise for application in food systems. *Monascus* pigments have been used to color food for centuries and the traditional method of pigment production involves the growth of the fungus on a solid medium such as steamed rice. This method does not permit easy control of environmental parameters and requires intensive space. There have been reports that *Monascus* can be cultured in submerged culture systems. The submerged fermentation approach could overcome the drawbacks of the former process. *Monascus* is usually produced according to empirically found rules and, so far, this fermentation has never been described completely using scientific parameters [12]. Despite this, little work has been performed on synthetic media to produce pigments, especially in submerged fermentation [13,14].

The Microorganism

Monascus belongs to the phylum Eumycota, subphylum Ascomycotina, class Ascomycetes, order Eurotiales and the family Monascaceae. The genus *Monascus* includes nine species: *M. pilosus*, *M. purpureus*, *M. ruber*, *M. floridanus*, *M. argentinensis*, *M. eremophilus*, *M. lunisporas*, *M. pallens* and *M. sanguineus* [15-18]. This fungus is a source of various secondary metabolites of polyketide structure [19,20] and famous for their fermented products [17].

M. purpureus is a strain of *M. ruber* van Tieghem, a destructive mold that grows on starch and silage and naturally exists in dairy products [21]. The yeast causes fermentation of cellobiose, maltose, fructose and glucose to occur, but does not ferment cane sugar [22]. *Monascus sp.* is distinguished as a fungus by its ascospores [23]. These are usually spherical, being 5µm in diameter, or ovoid (6×5µm). In the early stages, the young part of the mycelium is white. However, it rapidly changes to a rich orange and later to a distinctly rich red color, reflecting the increasing acidity of the medium and the production of red-orange hyphae. A deep crimson color is found in the substratum as the culture ages [24]. Figure 1 shows some *Monascus spp.* available in culture collection at laboratory in Malaysian Agricultural Research and Development Institute (MARDI). The colony of *Monascus spp*. was thin and spreading with reddish in color, indicating that a substantial amount of red pigment had been produced during growth.

Figure 1: Several species of *Monascus spp.* from culture collection at laboratory in MARDI.

Pigments

It has been reported that *Monascus sp.* produces a mixture of six major pigments of polyketide origin [21] (Figure 2). In recent years, two novel yellow pigments have been recovered [25]. The orange pigments, monascorubrin (M.W.: 382.46) and rubropunctatin (M.W.: 354.40), are synthesized in the cytosol from acetyl coenzyme A by the multienzyme complex of polyketide synthase I [19,20]. These compounds possess a unique structure responsible for their high affinity to compounds with primary amino groups (so called aminophiles). Reactions with amino acids yield the major compounds which are water-soluble red pigments, monascorubramine (M.W.: 367.44) and rubropunctamine (M.W.: 339.39) [22,26]. The mechanism of formation of the yellow pigments, ankaflavin (M.W.: 386.49) and monascin (M.W.: 358.43), has not yet been elucidated [20]. These compounds were suggested to originate from chemical oxidation of monascorubrin and rubropunctatin [27,28]. However, their structures strongly suggest that the yellow pigments are reduced derivatives of the orange ones. Thus, the suggestion by Yongsmith et al. [25] that ankaflavin and monascin have their own biosynthetic pathway seems to be more probable.

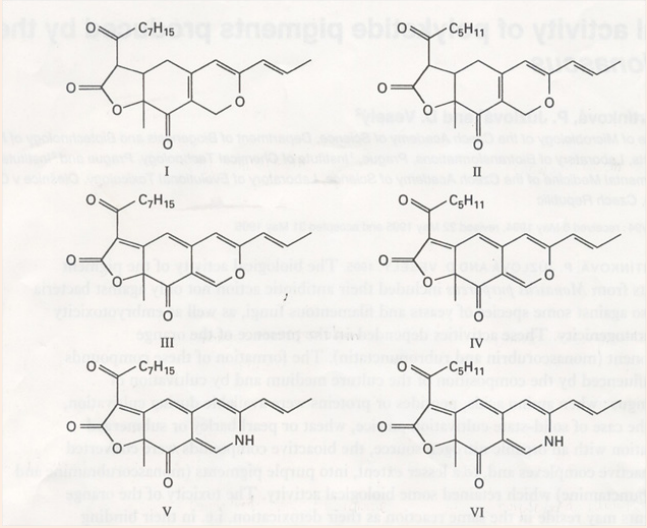

Figure 2: Structures of the yellow [ankaflavin (I), monascin (II)], orange [monascorubrin (III), rubropunctatin (IV)] and red [monascorubramine (V), rubropunctamine (VI)] pigments of *Monascus* (Sources [21]).

Red Fermented Rice

Red fermented rice is the fermented product of rice on which the red fungus *Monascus* has been grown. This fungus is also commonly known as red yeast rice, red rice, red leaven, beni-koji (Japanese), hung-chu, hong qu, angkak and zhitai (Chinese) [29]. In traditional practice, red fermented rice is produced by combination of two microorganisms, *M. purpureus* and yeast. *M. purpureus* is capable of breaking down rice starch into simple sugar, while yeast later continues further conversion into alcohol. In practice, the fungus is fermented on rice and the mycelium develops rapidly until the rich crimson pigment has permeated the grains completely, giving the product its characteristic red color [30]. The whole mass is then ground to a powder and used as a coloring material in food, etc.

Today, red fermented rice is still used in traditional Chinese medicine and in powdered forms as a food coloring in Asia and in Chinese communities in North America, most commonly for coloring fish, alcoholic beverages and cheeses [12]. Considerable interest has been shown in using *M. purpureus* as a nitrite/nitrate substitute for the preservation of meats and as a potential replacement for synthetic dyes [21]. European manufacturers of meat products have recently popularized its use in coloring sausages and salami [12]. It is interesting to note that a century ago in Asia, red fermented rice was used as a "food disinfectant" [10,14,31,32]. The nutritional and medicinal properties of red fermented rice are not fully appreciated by the Western world. *Monascus* pigments have not been approved by the Food and Drug Administration (FDA) of the USA for use as food colors, but they enjoy a Generally Recognized as Safe (GRAS) status in Asia [11]. This might be due to controversy over their safety which has been provoked because a mycotoxin is also produced along with the *Monascus* secondary metabolites by certain strains and under certain cultivation conditions [33,34]. However, spurred by renewed interest in natural remedies, recent biochemical and pharmacological studies have identified red fermented rice as a beneficial dietary supplement for maintaining a healthy balance of cholesterol and related lipids in the body [35]. The genus *Monascus* produces the most famous secondary metabolites which have been purified and identified, including monacolins that are responsible for reducing cholesterol levels in blood [33,36,37]. γ-Aminobutyric acid (GABA), dimerumic acid with medical effects and citrinin as an antibacterial agent have also been identified as secondary metabolites [34,36,38-40]. At the same time, purified pigments from *M. purpureus* show high potential for application in antibacterial therapy and also possess anti-oxidant and antibiotic properties [41]. The annual consumption of *Monascus* pigments in Japan alone amounted to 600 tons and was valued at 1440 million yen according to a survey done in 1992 [11].

Production

Although the traditional production of red pigment for use as a coloring agent is conducted on solid support (rice), most studies performed in the laboratory have been done using submerged fermentation (SmF), which presents easily controllable conditions. Solid state fermentation (SSF), however, generally gives a higher yield and productivity of pigment than SmF, but usually needs complex media, which are difficult to use for the elucidation of metabolic pathways and other factors during growth. A comparison was made between submerged and solid media of similar composition, the solid media obtained from the submerged medium by addition of a gelling agent, followed by extrusion in rice-sized particles. The solid media thus prepared supported the production of up to three times more pigment than the corresponding submerged media, but fermentation over rice was still superior [42].

Solid State Fermentation

The traditional manufacture of red fermented rice is known

only in certain localities in China. However, Lotong et al. [43] have described procedures for preparing red fermented rice at laboratory scale. The traditional manufacture of red fermented rice includes the following steps: rice is washed, soaked in water for about 1 day and drained thoroughly. The moist rice is then transferred to a glass beaker or suitable container to allow plenty of air space above it and autoclaved for 30min at 121°C. Upon cooling, the rice is inoculated with a sterile water suspension of ascospores prepared from a 25-day- old culture of *M. purpureus* grown on Sabouraud's agar. At the time of inoculation, the rice should appear rather dry. A wet mushy substrate is undesirable. The inoculated rice is thoroughly mixed and then incubated at 25 to 32°C for about 3 days. By this time the rice will have taken on a red color and should be stirred and shaken to redistribute the moisture and kernels with respect to depth from the surface of the fermenting mass. It may be necessary to add some sterile water to replenish moisture lost during incubation. Within about 3 weeks, the rice should take on a deep purplish-red color and kernels should not stick together. After drying at 40°C, the kernels are easily crumbled by slight force and may be reduced to a powder before using in foods or beverages.

The performance of SSF is influenced by several factors such as the strain of the microorganism employed, the variety of rice, substrate moisture content, nitrogen source, trace elements and aeration [43-45]. Corn can be used as a substrate for producing red pigment. However, non-glutinous varieties of rice are most suitable for preparing red rice, since kernels of glutinous varieties tend to stick together and thus reduce the surface to volume ratio of solid material which is critical for pigment production [43].

In the traditional process of red rice fermentation, the inoculated rice is cultured in bamboo or wooden trays. The treatment of rice and methods used to prepare the inoculum differ slightly between factories. In the culturing steps, however, methods for the successful production of red rice are the know-how of each producing company. One of the most important parameters is the low initial moisture content. If the fermentation starts at low moisture content, the culture dries out to such a level that the fungus grows poorly and pigmentation does not occur before fermentation is complete [46]. To overcome this problem, an appropriate amount of water is occasionally added throughout the fermentation period [43]. One of the conditions for successful fermentation of fungus for pigment production is employing a low initial substrate humidity (25-30% w/w) which prevents the risk of bacterial contamination and rice grains sticking together [42], and keeps the glucoamylase activity of the fungus low in favor of pigment production [47]. The production process consists of maintaining non-agglutinating rice immersed in water for up to 24 h, and then steaming (autoclaving), addition of an inoculum (a portion of previously fermented rice) and addition of inoculum at time intervals to maintain humidity [31,32]. 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 on solid substrates is around 56% at pH 6 [48].

Another important factor is the oxygen supply. Han et al. [49] recommended oxygen and carbon dioxide partial pressures of 0.5 and 0.02 atm, respectively. Red pigment production is more sensitive than growth to oxygen and carbon dioxide concentrations in the atmosphere. In order to achieve sufficient aeration of the mycelium, it is advisable to separate grains from agglomerates formed during sterilization or cultivation. This separation is quite easy when fermentation is carried out in plastic bags or in a fermenter with a moving bed ("swing" fermenter) [43].

With this microorganism, fermentative production of pigments can be obtained in both SSF and SmF [24]. However, SSF leads to higher yields of pigment that are released into the solid substrate (usually rice grains), while in SmF, the pigments are mainly retained intracellularly, causing inhibition of further production [11]. SSF results in a higher pigment yield than fermentation in shake flasks and it was concluded that this phenomenon could be due to minimal inhibition by the product [50]. In SSF, pigments are released into grains while during SmF pigment accumulates in the mycelium. However, Evans et al. [5] showed that the better pigment yields by SSF are probably not caused by the extractive effect of rice grains because addition of sorbent particles into SmF does not result in an increase of pigment production. These authors suggested that the reason might be rather the attachment of the mycelium to the grains. Johns et al. [48] suggested that the microscopic porous structure of rice favorably influences cultivation because the substitution of this substrate for carrageenan particles containing all nutrients is not successful. Lin et al. [51] compared various kinds of substrates and found that the use of steamed bread led to the best pigment yield. In addition to rice and bread, oat, corn or wheat grains can serve as substrates for the SSF of *Monascus* species [44,51]. In order to recreate the physiological conditions of SSF, a strain of *M. purpureus* was immobilized in/on different inert carriers such as Ca-alginate, polyurethane sponge, active carbon and pearlite, and cultivated in SmF and repeated-batch processes [52]. Examples of pigment fermentation by *Monascus* species are summarized in Table 1 [53-64].

Table 1: Production of pigments from different media by solid state fermentation.

Microbial source	Medium	Parameters	Reference
M. purpureus KACC 42430	Corn cob	60% initial moisture content; incubation at 30°C; incubation period 7 days; fermentation in 250mL Erlenmeyer flask	[53]
Monascus sp. LPB 31	Rice	53% initial moisture content; incubation at 32°C; incubation period 16 days; fermentation in columns and drum bioreactor	[54]
M. purpureus ATCC 16365, BCC 6131, DMKU and FTCMU *M. ruber* TISTR 3006	Polished adlay	Incubation at 32-35°C; incubation period 28 days; fermentation in autoclavable polypropylene plastic bag	[55]

M. purpureus LPB 97	Jackfruit seed (powder)	50% initial moisture content; incubation at 30°C; incubation period 7 days; fermentation in 250mL Erlenmeyer flask	[56]
M. purpureus M9	Rice, corn, whole sorghum grain, dehulled sorghum grain, sorghum bran	Incubation at 30°C; incubation period 14 days; fermentation in jar	[57]
M. purpureus KJR2	Durian seed	Incubation at 30°C; incubation period 14 days; fermentation in 300mL Erlenmeyer flask	[58]
M. purpureus MTCC 369	Corn, sugarcane bagasse	Incubation at 30°C; incubation period 7 days; fermentation in 250mL Erlenmeyer flask	[59]
M. ruber MTCC 2326	Broken rice	Incubation at room temperature; incubation period 15 days; fermentation in 250mL Erlenmeyer flask	[60]
M. ruber ICMP 15220	Rice	70% initial moisture content; incubation at 30°C; incubation period 7 days; fermentation in packed bed solid state bioreactor	[61]
M. purpureus MTCC 369	Long grain, non-glutinous rice	Incubation at 30°C; incubation period 14 days; fermentation in 250mL Erlenmeyer flask	[62]
M purpureus ATCC 16392	Rice	55% initial moisture content; incubation at 30°C; incubation period 10 days; fermentation in Raimbault column solid state bioreactor	[63]
M. purpureus FTCC 5391	Broken rice, rice husk, pineapple waste, pressed coconut flesh	65% initial moisture content; incubation at 32°C; incubation period 7 days; fermentation in covered 500mL beaker	[64]

Submerged Fermentation

The composition ratios and productivity of pigments can be influenced by the *Monascus* strain, medium composition and the fermentation conditions in which they are produced. Therefore, many studies have attempted to enhance the production of *Monascus* pigments through strain improvement, carbon source change and improvement of culture conditions [65].

Effect of carbon source

Numerous substances of variable molecular sizes have been utilized effectively as substrates in media for growth of *Monascus* species and pigment production. Carbon sources supplied in the medium are of great importance to fungi since they provide the carbon needed for the biosynthesis of cellular constituents such as carbohydrates, proteins, lipids and nucleic acids, and their oxidation provides energy for the cell [66]. Glucose is reported by most researchers [13,22,67-71] to be a superior substrate for pigment production by *Monascus* species. Lin et al. [47,71] indicated that the utilization of carbon sources for growth appears to be strain-specific; glucose and its oligo- and polysaccharide are better than other carbon sources both for growth and pigment production. However, Tseng et al. [72] found glucose to be less suitable for this purpose. This may be caused by strain differences or by other differences in medium composition (glucose concentration, type of nitrogen source). Different carbon sources at varying concentrations produce different types of pigments. However, according to Lin et al. [13], glucose is better than other carbon sources both for growth and pigment production. The quantity of pigment produced is commonly affected by different glucose concentrations. When the glucose concentration is too high it will inhibit the formation of secondary metabolites. Furthermore, it can make the growth slightly acidic but not favorable for pigment production. An initial glucose concentration of 40g/L was found to be enough to give maximum growth and pigment production [6]. A high glucose concentration (50g/L) led to growth rates, pigment synthesis and considerable ethanol production, perhaps due to induction of respirofermentative metabolism (Crabtree effect) in the aerobic SmF of *M. purpureus* by high glucose levels [67]. It is recommended that glucose be maintained below 20g/L in the culture medium.

On the other hand, after comparing 24 carbon substrates, Lin [50] found soluble starch, galactose and maltose to be most suitable for the production of red pigments in SmF. Lin et al. [13] claimed that maltose is the best carbon source for red pigment production among maltose, glucose and fructose, and gave the maximum yield at 5% (w/v) concentration. Between potato starch, corn starch, wheat flour, rice powder and glucose, rice powder was found to favor red pigment production in SmF, and 5-7% (w/v) rice powder produced the highest pigment [36,50]. Nevertheless, the fermentation medium with fructose as carbon source proved to be most suitable for mycelium growth and pigment production, with maltose and glucose being the second most productive [72]. When sucrose and lactose were used as carbon sources, mycelium growth and pigment production were inhibited. This result might be due to the strain being unable to utilize sucrose and lactose for fermentation and producing some alkaline substances in the SmF. According to Lin et al. [13,47,71], *M. purpureus* utilizes sucrose and lactose for reproduction, but cannot utilize sucrose for fermentation because *M. purpureus* lacks the transfer enzyme. Some strains of *M. ruber* can be also

grown on cellulose but pigment production is negligible [73].

Stimulation of pigment production by ethanol as a carbon source in some *Monascus* strains could originate from a higher cellular pool of acetyl CoA formed during fermentation on ethanol in comparison with that on sugars [69,74,75]. Ethanol at a concentration of 2% (v/v) was used as the sole carbon source for pigment production by *M. purpureus*, and the specific pigment production was higher than that obtained with fermentation on maltose [74]. However, until now, ethanol has never been intensively studied as a sole carbon source for pigment production by *M. purpureus*. It was only mentioned that ethanol supports pigment production at a greater rate than sugars [69]. Maltitol and glycerol were tested as substrates for pigment production; the former was a superior substrate but the latter resulted in poor pigment production [50]. Fatty acids can be transformed by *Monascus* into methyl ketones, but cannot be utilized as the sole carbon source [76-78]. Different types of carbon source are used depending upon the *Monascus* species, as shown in Table 2 [79-84].

Table 2: Carbon sources for pigment production by various microorganisms.

Carbon Source	%(w/v)	Microorganism	Reference
Glucose	1	*Monascus sp.* KB20M10.2	[79]
	2	*M. ruber* ATCC 96218	[80,81]
		M. anka (M-9)	[93]
	2.6	*M. ruber* van Tieghem	[82]
	5	*M. purpureus* UQM 192F (FRR 2190)	[68]
		Monascus sp. B683	[65]
	10	*M. purpureus* N11S	[31]
Maltose	3	*M. purpureus* CCM 8152	[75]
	5	*Monascus sp.* TTWMB6093	[13]
Sucrose	1	*M. kaoliang* KB9	[25]
Ethanol	2	*M. purpureus* CCM 8152	[75]
Rice starch	5	*Monascus sp.* B683-M171	[11]
Co-product of biodiesel	Pure	*M. ruber*	[83]
Corn steep liquor	5-10	*M. ruber*	[84]

Effect of nitrogen source

Most fungal strains are capable of utilizing inorganic or organic nitrogen sources. Inorganic nitrogen may be supplied as ammonia gas, ammonium salts or nitrates while organic nitrogen sources may be supplied as amino acids, protein, peptone, yeast extract or urea [85]. Ammonia is a good nitrogen source and can be used by most fungi. However, attention must be paid to the specific salt to be used due to pH variations during ammonium absorption that may cause inconvenience to the culture [86]. Most fungi grow well on a mixture of amino acids but the effectiveness of these compounds as the sole nitrogen source varies considerably [85].

Various requirements for nitrogen source, either inorganic or organic, are provided to the medium in pigment production. Inorganic nitrogen does not influence pigment formation but organic nitrogen such as malt extract, yeast extract, peptone and soybean flour or their combination promotes growth as well as pigmentation [86-88]. The combination of three organic nitrogen sources, i.e. malt extract, yeast extract and peptone, was used for yellow pigment production by *Monascus* species [70,79,89]. The effect of the nitrogen source on SmF of glucose-utilizing *Monascus* species was described in detail by Carels et al. [28,90]. The effect of different nitrogen sources on pigment production by a number of *Monascus* species concluded that when the source of nitrogen is yeast extract or nitrate, red pigments are formed, whereas with ammonium or ammonium nitrate, orange pigments are formed [28,90]. Yeast extract stimulates conidiation, represses the sexual cycle and increases biomass production [21]. Due to the formation of copious amounts of conidia, pigment production remains at a relatively low level. It was proposed that the orange pigments enter reactions with amino acids because the pH (above 5) in cultures assimilating an organic nitrogen source is favorable for this interaction [91]. Sodium nitrate supports sporulation, limited growth and gives intermediate pigment yields; the use of ammonium chloride results in repression of conidiation and the sexual cycle and leads to the best pigment yields [21,91]. In this medium, the dramatic pH decrease impairs pigment–amine interactions giving origin to red pigments according to Carels et

al. [92] and they also reported that nitrogen source determines pH evolution. In addition to ammonium chloride, peptone also yields superior growth and pigment production when compared with sodium nitrate [27,28,92]. The study also reported that monascorubramine (red pigment) is the major product in all media in spite of the low pH of some fungal cultures.

For the formation of red pigments in MOPS-buffered (3-(N-morpholine) propane sulfonic acid) culture, monosodium glutamate (MSG) is the most favorable nitrogen source [13]. In an unbuffered culture of another *Monascus* strain, ammonium glutamate gave superior pigment yields [75]. Hajjaj et al. [80,93-96] reported that a well-defined chemical medium with MSG as the nitrogen source used in SmF of *Monascus* shows better control of pigment production. This promotes production and excretion of extracellular pigments [10,22] and also leads to the formation of a glutamic acid–pigment complex [26]. Lee et al. [94] observed that increased MSG concentrations increase biomass but concentrations above 1.5 g/L of MSG give increased biomass but decreased pigment production. However, Yoshimura et al. [69] observed that utilization of nitrogen sources for pigment production appears to be strain-specific since other strains produce much more pigment with MSG and sodium nitrate. Tseng et al. [72] reported that basal broth medium containing organic nitrogen (yeast extract) at 8g/L with the addition of 0.3g/L sodium nitrite might be over the maximum requirement for growth and pigment formation, thus resulting in a decreased weight of dry mycelia and pigment production. Some examples of nitrogen used during SmF for pigment production are shown in Table 3.

Table 3: Nitrogen sources for pigment production by various microorganisms.

Microorganism	Nitrogen Source	%(w/v)	Reference
Monascus sp. B683-M171	MSG	0.3	[11]
M. ruber van Tieghem		0.5	[26,82]
M. ruber ATCC 96218		0.5	[95,80]
M. purpureus CBS 10907		0.5	[26]
Monascus sp. TTWMB 6042	MSG	1.26	[22]
	NH_4NO_3	0.3	[13]
M. purpureus N11S	NH_4NO_3	0.3	[31]
M. purpureus UQM 192F (FRR 2190)	NH_4Cl	0.2	[68]
	Bacto Peptone	0.5	
Monascus sp. B683	NH_4Cl	3	[65]
M. anka (M-9)	$NaNO_3$	0.3	[77]
	Peptone	1	[96]
	Yeast extract	0.5	
M. kaoliang KB9	$NaNO_3$	0.02	[25]
	Yeast extract	0.03	
	Casamino acid	0.03	
Monascus sp. KB20M10.2	Peptone	2	[70,89]
	Yeast extract	2	
	Malt extract	2	

Effect of pH

The pH change during cultivation of a microorganism in SmF depends on the nitrogen source [27,75] and, to a lesser extent, on the carbon source [12,75]. The optimal initial pH value must also be selected with respect to the carbon and nitrogen sources used [50]. Studies by Chen et al. [68] revealed that culture pH clearly affects fungal growth and pigment synthesis. However, the final mycelium dry weight was often similar between pH 4.0 and 6.5 and they observed red pigment production was always higher at pH 6.5. Without controlling the pH and with an initial pH of 6.5 in batch fermentation, the value of pH dropped during the early

stages of fermentation and then increased to around 5.8 when carbon sources become exhausted. This phenomenon was almost the same as the shake flask culture in which pH decreased during the early stage of fermentation but began to increase to around 8.0 towards the end. This trend was positively correlated with pigment production [69]. The changes of pH of the medium during the fermentation period may be one of the possible explanations for the transformation of orange pigments to red pigments. Red and yellow *Monascus* pigments produced by fermentation are changed by adjusting the medium composition and pH of the fermentation broth [27,28,90,97]. Red pigments are formed in yeast extract or nitrate medium at pH 6.5 and orange pigments are formed in ammonium or ammonium nitrate medium at pH 2.5. More red pigments are produced in fermentations with a final culture pH of above 6. pH evolves differently depending on the nitrogen source and different pHs determine the color of the pigment formed [90]. On the other hand, when pH is kept constant at pH 6.0, 7.0 and 8.0 throughout the fermentation, the pigment productivity, measured as optical density, is less than in fermentation without pH control [70]. Buhler et al. [83] reported that *M. ruber* favors release of red pigment into the medium when the pH range is maintained between 5.45 and 6.23.

Regardless of the initial pH, the final pH of cultures utilizing the same carbon and nitrogen sources is approximately the same [24,27,75]. Yoshimura et al. [69] reported that maintenance of pH at a constant value during the entire cultivation period is not profitable. On the contrary, Lin et al. [13] carried out successful cultivation at a constant pH by using MOPS buffer. 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 [21], usually in the range of pH 7 to 8 [25]. Fermentation media for various *Monascus sp.* are summarized in Table 4 [98-100].

Table 4: Optimum culture pH for pigment production by various microorganisms.

Microorganism	pH	Reference
M. purpureus CCM 8152	4.6	[75]
Monascus sp. B683-M171	5.0–5.7	[65]
M. purpureus C322	5.0–5.6	[52]
M. purpureus N11S	5.5	[10]
Monascus sp. TTWMB 6093	5.5	[22]
		[47,71]
M. purpureus	5.5	[72]
Monascus sp. NRRL 1993	6	[5]
Monascus sp. TTWMB 6042	6.3	[13]
M. ruber van Tieghem	6.5	[26]
		[82]
M. purpureus UQM 192F (FRR 2190)	6.5	[67]
M. ruber ATCC 96218	6.5	[95]
Monascus sp. KB20M10.2	6.5	[79]
Monascus sp. J101	6.6	[81]
		[98,99]
		[100]

Effect of other medium components

The only trace element reported to support growth and pigment production by *Monascus* species is zinc [78,101,102]. This effect could be due to the participation of zinc in the uptake and utilization of the carbon source. According to Lin et al. [13], high concentrations of phosphate and magnesium sulfate ($MgSO_4$) inhibit pigment production and the growth is a crescent linear function of $MgSO_4$ concentration, in the range from 0.5 to 16 mM. The addition of corn oil stimulates pigment production twofold compared to the control, while the addition of 0.4% (v/v) Tween 80 neither affects glucose uptake, nor retards the growth rate, but enhances the pigment productivity (six to eight times, reaching 8535 units of absorbancy/g dry matter) [103].

The addition of individual amino acids (such as lysine, histidine, arginine, aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine and phenylalanine) influences neither growth nor pigment production [90]. On the other hand, McHan et al. [102] reported that almost all protein amino acids except lysine stimulate growth. Pigment production is also increased by the addition of nonprotein amino acids, especially methanproline and azetidinecarboxylic acid. Leucine, valine, lysine and methionine have strong negative effects on the formation of hydrophilic red pigments, i.e. pigments containing an amino acid side chain. Leucine is one of a few amino acids that have a strong negative effect on pigment formation. In addition, leucine supports poor pigment formation when used as the sole nitrogen source, yet it neither represses pigment synthase(s) nor inhibits its action. The negative effect of leucine is caused by enhanced decay of pigment synthase(s) [45]. Addition of a crystallization inducer, poly(oxyethylene) sorbitan esters of palmitic acid (Tween), to the fermentation medium results in the production of extracellular pigments [104]. Addition of an adsorbent resin (Amberlite XAD-7) to the immobilized *Monascus* culture greatly increases both the maximum pigment yield and the production rate above those of free-cell fermentations [5]. Peters et al. [77] reported that *M. purpureus* is able to oxidize fatty acids and methyl ketones growing on a synthetic medium.

Effect of inoculum size

The age of the inoculum, the medium used for its cultivation, and therefore their physiological states are of the utmost importance in many fermentation processes. If the inoculum used for the production of secondary metabolites is not in the correct physiological state, a considerable decrease in production will occur because the early hours of fermentation determine the future direction the culture will take [97]. Sekiguchi et al. [105] observed in detail that with *Penicillium urticae* the type of inoculum used greatly influences the level of enzymes present at the beginning of the fermentation and hence the final level of secondary metabolites produced. Although different levels of pigment-synthesizing enzymes were not measured in *Monascus*, it was reported that in order to obtain well-phased cultures in the production medium it was necessary to have an inoculum that was actively growing and that had no nutritional limitations [97]. They found that only vegetative biomass, very few conidia and no secondary metabolites are produced. The age of the inoculum is always critical, maximum efficiency only being achieved within very fine limits of 1-2 h.

Calam [106] demonstrated the importance of inoculum for maximum production of secondary metabolites (penicillin and griseofulvin) by different microorganisms, especially the type of morphology exhibited by fungi. According to Smith et al. [107] different yields are obtained in penicillin and griseofulvin fermentations using different types of inoculum. From their study, it was shown that biochemical factors, such as the levels of enzyme activity and efficiency, are at least as important as morphology in determining yield, being carried forward from the inoculum to the production stage. Calam et al. [108] also investigated the time at which inoculum quality is determined and the events associated with the process. Their results are again in agreement with the observations with *Monascus*, in that the initial stage of growth is very important in fixing the future metabolic and morphological pattern of the behavior of the culture [97].

Effect of temperature

Although growth is not affected, pigment production is greatly influenced by temperature. The optimal cultivation temperature for individual *Monascus* strains varies from 25°C to 37°C [47]. Palo et al. [109] studied various conditions of fermentation and found that the optimum temperature for pigment formation is 27°C. Growth occurs at temperatures as low as 20°C and as high as 37°C but poor pigmentation results are obtained at these extremes. Panagou et al. [23] reported that *M. ruber* ascospores isolated from thermally processed green olives are capable of surviving heat treatments and subsequently grow under reduced oxygen levels, resulting in food spoilage. For pigment production, a higher temperature might be more appropriate. A summary of optimum temperatures for the production of *Monascus* pigments is shown in Table 5[110-111].

Table 5: Optimum temperature for pigment fermentation by various microorganisms.

Microorganism	Temperature (°C)	Reference
Monascus sp. NRRL 1993	28	[5]
M. ruber van Tieghem	28	[26]
		[82]
Monascus sp. KB20M10.2	28	[79]
	30	[88]
M. kaoliang KB9	28-32	[25]
M. anka (M-9)	29	[96]
Monascus sp. TTWMB 6093	30	[22]
		[47]
M. purpureus CCM 8152	30	[75]

M. bakeri KB10	30	[87]
M. kaoliang KB9		
M. ruber ATCC 96218	30	[80,95]
Monascus sp. UQM 192F (FRR 2190)	30	[67]
Monascus sp. B683	30	[48]
		[11]
M. purpureus Went CRC3150	30	[110]
M. purpureus C322	30	[52]
M. purpureus	30	[72]
Monascus sp. J101	30	[81]
		[98,99]
		[111]
		[100]
M. purpureus N11S	35	[14]
Monascus sp. TTWMB 6042	37	[13]

Effect of agitation and aeration

Microorganisms normally vary in their oxygen requirement. The largest utilization of oxygen during the growth of aerobic microorganisms is for respiration. Oxygen acts as a terminal electron acceptor for oxidative reactions to provide energy for cellular activities [112]. Productivity of several fermentations is limited by oxygen availability and it is therefore important to consider the factors, which affect the efficiency in supplying microbial cells with oxygen [113].

Agitation provided in the fermentation process affects mycelium formation. Pigment production has been shown to be proportional to mycelial growth. It was reported that the pigment production in a stirred tank fermenter is high at high agitation rates [69]. These researchers observed that in fermentation with an agitation speed of 300 rpm the mycelial growth is in the form of pulp and pigment production is low. At agitation speeds of 600 and 800 rpm, mycelial growth is in the form of intermediate pulp and pellets, and pigment production is substantially higher. However, when the agitation speed is increased to 1200 rpm, growth of the fungus is mainly in pellet form and pigment production drops to about half that of fermentation at 600 to 800 rpm. From the study, it can be concluded that high agitation speed provides high shear rates that cause the mycelium of the fungus to split, which, in turn, reduces pigment production. Krairak et al. [114] reported that at low agitation speed sedimentation of cells is observed; therefore, the agitation speed must be sufficiently high to obtain cell culture in the form of cell suspension. Musaalbakri et al. [115] observed that fermentation in a 2 L stirred tank bioreactor is successful at a constant agitation speed of 600 rpm and dissolved oxygen tension (DOT) levels higher than 90%, which are the best conditions for pigment production and cell morphology. Another finding from this study is that a high DOT level (>80%) during growth phase was found to be one of the main factors in the production of cell mycelia which have high ability in synthesizing red pigment; it seems that the use of air for the oxygen supply, even at a very high flow rate, is not capable of maintaining DOT at high levels. Moreover, an increase in cell concentration during fermentation causes high broth viscosity. Therefore, a higher agitation speed is necessary during fermentation to avoid cell sedimentation, especially of growth in pellet form in the case of high cell concentrations. Another aspect that is interesting to investigate is the effect of agitation speed, during growth phase and production phase, on red pigment production. Krairak et al. [114] suggested an agitation speed of 100 rpm (during the early stages of fermentation with low cell concentration) and an increase to 250 rpm (during maximum cell growth with high cell concentration) during *Monascus* fermentation for improvement of red pigment fermentation. As mentioned by Mohamed et al. [116], mixing filamentous fungi entails a delicate balance between promoting a high volumetric oxygen transfer coefficient while keeping low hydrodynamic stresses in the culture microenvironment.

Pigment production is greatly influenced by medium composition and oxygen supply [98,99,117]. *Monascus* species require sufficient aeration and therefore SmF can proceed only in shaken, preferably baffled flasks [75] or in a well-stirred and aerated fermenter such as a fermenter equipped with an airlift system [118]. Researchers have observed that mass transfer of oxygen to the aqueous phase of the fermentation volume is improved significantly through the use of an airlift fermenter. Recently, Yang et al. [119] observed the influence of oxygen on *Monascus* pigment and citrine production by *M. ruber* by shifting the oxygen supply at different stages of production, the initial growth phase, mid-stage phase and later stage. The availability of oxygen appears to affect biomass levels less than pigment production; red pigment production in particular needs vey high oxygen levels [120]. This might occur due to shear forces that may destroy the mycelium and can be overcome by using roller bottles [96].

Effect of other physical factors

Pigment formation is independent of visible light. Broder et al. [73] recommended the cultivation of *Monascus* species in total darkness for improvement of red pigment production. Wong et al. [121] reported that different morphologies, photoresponses and antibacterial activity of *M. purpureus* can be obtained by induction or mutation using fast-neuron and X-ray irradiation. However, the different mutants of *M. purpureus* obtained by irradiation using light sources of various wavelengths [such as white light, blue (430nm), red (660nm), infrared (730nm) and ultraviolet (245)] do not show a significant effect on conidiation for red pigment production.

Fermentation Media for Pigment Production

Different medium formulations have been proposed and used for pigment fermentation by *Monascus* species and examples are summarized in Table 6. The carbon sources used for pigment production range from simple C3 molecules such as glycerol up to a complex carbon source such as starch. Glucose is mainly used in all cultivation media with a concentration ranging from 20 to 200g/L. However, at industrial scale, rice starch can be used in cases of bulk production of red pigment through SSF in a non-purified form that is utilized in pigment production, food preservation and other medical purposes. Also, different organic nitrogen and inorganic nitrogen sources are added to the cultivation medium to enhance pigment production. MSG, with a concentration ranging from 0.5 to 15g/L, or yeast extract, malt extract, peptone and combinations are the main common organic nitrogens. On the other hand, ammonium nitrate (NH_4NO_3) is mainly used as the inorganic nitrogen source for pigment production, followed by sodium nitrate ($NaNO_3$). The phosphate source is in the form of either potassium dihydrogen phosphate (KH_2PO_4) or dipotassium hydrogen phosphate (K_2HPO_4) with a concentration ranging from 0.1 to 6g/L. Other trace inorganic salts for supplementation with Mg^{++}, Fe^{++}, Zn^{++} and K^{++} cations are also added in smaller amounts.

Table 6: Media for *Monascus* fermentation and pigment production.

Strain	Medium Composition (g/L)	Reference
Monascus sp. J101	Sucrose, 100; casamino acid, 5; yeast extract, 3; $NaNO_3$, 2; KH_2PO_4, 1; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $FeSO_4.7H_2O$, 0.01; agar, 20; stock cultures	[98-100]
	Glucose, 50; Bacto Peptone, 20; KH_2PO_4, 8; CH_3COOH, 2; NaCl, 1; $MgSO_4.7H_2O$, 0.5; seed cultures	[98,99]
	Glucose, 70; MSG, 5; yeast extract, 3; $NaNO_3$, 2; KH_2PO_4, 1; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $FeSO_4.7H_2O$, 0.01; fermentation medium; pH 6.6	[111]
M. purpureus CCRC 31499, 32966, 31530 *M. ruber* CCRC 31535 *M. pilosus* CCRC 31527	Shrimp and crab shell powder (SCSP)/sucrose/chitin; yeast extract, 1; polypeptone, 1; KH_2PO_4, 1; $MgSO_4.7H_2O$, 0.5; $FeSO_4.7H_2O$, 0.1; $NaNO_3$, 3; KCl, 0.5; pH 7.0	[35]
M. purpureus	i. Glucose, 20; malt extract, 20; peptone, 1.0; agar, 20; dH_2O; stock cultures ii. Glucose, 100; yeast extract, 8; basal broth medium; pH 5.5	[72]
M. ruber	Glucose, 20; MSG, 5; K_2HPO_4, 5; KH_2PO_4, 5; $CaCl_2$, 0.1; $MgSO_4.7H_2O$, 0.5; $FeSO_4.7H_2O$, 0.01; $ZnSO_4.7H_2O$, 0.01; $MnSO_4.H_2O$, 0.03; pH 6.0	[95,80]
Monascus sp. KB20M102.2	i. Cassava starch, 20; malt extract, 3; yeast extract, 3; peptone, 5; agar, 15; stock cultures ii. Glucose, 30; malt extract, 20; yeast extract, 20; peptone, 20; inoculum iii. Glucose 10; malt extract, 8.8; yeast extract, 8.8; peptone, 8.8; fermentation medium; pH 6.5	[70,79,89,114]
M. purpureus FTC 5391	i. Potato dextrose agar; stock cultures ii. Yeast extract, 3; malt extract, 3; peptone, 5; glucose, 20; inoculum iii. Glucose, 50; MSG, 12; K_2HPO_4, 2.5; KH_2PO_4, 2.5; $MgSO_4.7H_2O$, 1.0; KCl, 0.5; $ZnSO_4.7H_2O$, 0.01; $FeSO_4.7H_2O$, 0.01; $MnSO_4.7H_2O$, 0.03	[64,115]

M. ruber CCRC 31535 *M. purpureus* CCRC 31497, 31498, 31499, 31501, 31504, 31530, 31540, 31542, 31615, 32966 *Monascus sp.* CCRC 32087, 32808, 32809 *M. anka* M-13 *Monascus sp.* S2	i. Rice powder, 30; peptone, 9; glycerin, 30; glucose, 110; $MgSO_4.7H_2O$, 1; KNO_3, 2; potato dextrose agar; stock culture ii. Dextrose, 100; peptone, 10, KNO_3, 2; $NH_4H_2PO_4$, 2; $MgSO_4.7H_2O$, 0.5; $CaCl_2$, 0.1; pH 6.0; inoculum iii. Rice powder, 20; glucose, 40; MSG, 10; peptone, 10; pH 5.0; fermentation medium	[30]
M. purpureus	Rice powder/glucose/maltose/sucrose, 50; MSG/peptone/NH4NO3/NH4Cl, 15; potato dextrose agar; stock cultures	[110]
M. purpureus C322	i. MPI: glucose, 40; K_2HPO_4, 3; yeast extract, 10; agar; pH 5.6; stock cultures ii. MPII: glucose, 40; K_2HPO_4, 1; $MgSO_4.7H_2O$, 0.5; NaCl, 0.5; $FeSO_4.7H_2O$, 0.1, $NaNO_3$, 3; pH 5.0 iii. MPIII: glucose, 40; K_2HPO_4, 1; $MgSO_4.7H_2O$, 0.5; NaCl, 0.5; $FeSO_4.7H_2O$, 0.1, NH_4Cl, 0.5; pH 5.5 iv. MSG: maltose, 50; MSG, 12.6; K_2HPO_4, 2.4; KH_2PO_4, 2.4; $MgSO_4.7H_2O$, 8; KCl, 0.5; $FeSO_4.7H_2O$, 0.01; $ZnSO_4.7H_2O$, 0.01; $MnSO_4.7H_2O$, 0.03; pH 5.5	[52]
M. kaoliang KB9	Cassava starch, 20; yeast extract, 3; peptone, 5; malt extract, 3; agar; parent strain; stock cultures	[25,87,88]
M. bakeri KB10 20M10.2 *M. purpureus* CBS 10907	i. Sucrose, 10; KH_2PO_4, 0.1; $MgSO_4.7H_2O$, 0.05; $NaNO_3$, 0.2; KCl, 0.05; $FeSO_4.7H_2O$, 0.001; yeast extract, 0.3; casamino acid, 0.5; stock cultures ii. Cassava starch, 20; yeast extract, 3; peptone, 5; malt extract, 3; inoculum iii. Cassava starch, 30; soybean flour, 40; fermentation medium i. Prickly pear juice ii. Ethanol, 25; MSG, 4; K_2HPO_4, 2.5; KH_2PO_4, 5; KCl, 0.5; $MgSO_4.7H_2O$, 1; $FeSO_4.7H_2O$, 0.01; $ZnSO_4.7H_2O$, 0.01; $MnSO_4.H_2O$, 0.03; pH 5.5	[117]
Monascus sp. B683-M171	i. Rice, 50; MSG, 3; K_2HPO_4, 2.5; KH_2PO_4, 2.5; $MgSO_4.7H_2O$, 1; pH 5-5.7 ii. Tapioca starch, 50; MSG, 4.5; K_2HPO_4, 1.8; KH_2PO_4, 1.8; $MgSO_4.7H_2O$, 0.5; pH 5.5	[11]
M. purpureus CCM 8152, 8152/10, ATCC 16362 *M. ruber* CCM 8111, 8112	i. Glucose, 9.13; NH_4NO_3, 0.045; KH_2PO_4, 0.007; $MgSO_4$, 0.006; malt extract, 0.53; agar; stock cultures ii. Glucose/fructose/xylose/maltose/starch, 30; ethanol, 20; NH4Cl/yeast extract/peptone, 0.25	[19,20]
M. ruber	i. Glucose, 26; MSG, 5; K_2HPO_4, 5; KH_2PO_4, 5; $MgSO_4.7H_2O$, 0.5; $CaCl_2$, 0.5; $FeSO_4.7H_2O$, 0.5; $ZnSO_4.7H_2O$, 0.01; $MnSO_4.H_2O$, 0.03; pH 6.5	[82]
Monascus sp. TTWMB 6093	i. Glucose, 20; yeast extract, 3; malt extract, 3; peptone, 5; agar, 15; stock culture ii. Glucose, 20; yeast extract, 3; malt extract, 3; peptone, 5; inoculum iii. Maltose, 50; MSG, 12.6; K_2HPO_4, 2.4; KH_2PO_4, 2.4; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $FeSO_4.7H_2O$, 0.01; $ZnSO_4.7H_2O$, 0.01; $MnSO_4.H_2O$, 0.03; pH 5.5	[13,22,47,71]
M. purpureus CCM 8152	Ethanol, 20; NH_4Cl, 0.95; KH_2PO_4, 2.4; $MgSO_4.7H_2O$, 0.5; pH 4.6	[75]

M. purpureus UQM 192F (FRR 2190)	i. Potato dextrose agar; stock cultures ii. Glucose/maltose, 50; NaNO3, 3/NH4Cl, 2/Bacto Peptone, 5; KH-2PO4, 1; MgSO4.7H2O, 0.5; trace metals	[67,68]
M. purpureus DSM 1379, CBS 109.7 *M. ruber* ATCC 96218 *M. pilosus* CBS 286.34	Glucose/ethanol, 20; MSG, 5; KH_2PO_4, 5; K_2HPO_4, 5; $MgSO_4.7H_2O$, 0.5; $CaCl_2$, 0.1; $FeSO_4.7H_2O$, 0.01; $ZnSO_4.7H_2O$, 0.01; $MnSO_4.H_2O$, 0.03; pH 6.5	[26,397408]
M. purpureus DSM 1379	Glucose, 40/10/0.26; $NaNO_3$, 3; KH_2PO_4, 1; $MgSO_4$, 0.5; $ZnSO_4.7H_2O$, 0.0081; $MnSO_4.H_2O$, 0.03	[77]
M. purpureus N11S	i. Medium YEA: yeast extract, 8; glucose, 100; agar, 15; stock cultures ii. Medium YEB: yeast extract, 8; glucose, 100; fermentation medium	[10,31,32]
M. purpureus N11S	i. Medium YEA: yeast extract, 8; glucose, 100; agar, 15; stock cultures ii. Glucose, 40/80/150/200; NH_4NO_3, 0.1-50; K_2HPO_4, 6; KH_2PO_4, 5; $MgSO_4$, 0.5; KCl, 0.5; $FeSO_4.7H_2O$, 0.01; $ZnSO_4.7H_2O$, 0.01; $MnSO_4.H_2O$, 0.03	[14]
M. purpureus	i. Malt extract agar; stock cultures ii. Malt extract liquid; fermentation medium	[121]
Monascus sp. (39 strains)	i. Glucose, 40; KH_2PO_4, 3; yeast extract, 1; agar, 15; stock culture ii. Glucose, 40; KH_2PO_4, 1; MgSO4.7H_2O, 0.5; NaCl, 0.5; $FeSO_4.7H_2O$, 0.1; fermentation medium with different nitrogen sources, pH 2.5	[27,92]

The production of pigment has also been carried out successfully using immobilized cells. Fenice et al. [52] studied extracellular pigment production by immobilized *M. purpureus* in repeated-batch processes using different immobilizing carriers such as Ca-alginate, polyurethane sponge, active carbon and pearlite and cultivated in an air lift bioreactor. They found that, with Ca-alginate, pigment production was at a maximum while the cell leakage was negligible and the bead mechanical stability was good; with this carrier, extended repeated-batch fermentation (nine batches, 55 days) was carried out and gave clear indications as to the possibility of maintaining high pigment productivity.

Pigment Analysis Methods and Amounts Produced

The analysis of pigment produced by *Monascus* is usually done by measuring absorbance of pigment solutions in the range near 400, 470 and 500 nm, for yellow, orange and red pigments, respectively [22,48]. The ratio of absorbance at 500 nm to the absorbance at 400 nm gives the ratio between red and yellow pigments [10]. Some researchers analyzed total extracellular and intracellular pigments, summing up the absorbance of extracellular pigments (the absorbance of the liquid culture medium, filtered) and intracellular pigments (the absorbance of an alcoholic extract of the mycelium, filtered) [51,75]. When the fermentation has a solid substrate, the method used must be extraction by water or solvent, with posterior absorbance reading. According to Mapari et al. [122], the pigment characteristics of fungal extracts are comparable to water-soluble natural pigments derived from sources currently in use. The amount of solvent used is usually 5mL for each gram of fermented material, and the extraction time varies from 1 to 12 h. Since the samples must, in many cases, be diluted in order to enable reading on a spectrophotometer, absorbance values should not be directly compared, but rather extrapolated to specific absorbance, taking into consideration the dilution factor of the sample [103]. Red pigment solubility is highest in an aqueous solution containing 60–70% ethanol [75]. Other solvents may be used (acetonitrile, dimethyl sulfoxide, isopropanol), but the only common solvent that extracts pigments more efficiently than ethanol is methanol; it is, however, not recommended for food use because it is toxic.

The fact that the yellow, orange and red pigments of *Monascus* are produced as a mixture probably affects analysis by simple measurement 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 SmF, for example, 220 OD_{510}/mL in optimized conditions by Kim et al. [100,123], to thousands of absorbance units/g dry substrate in SSF, for example, 5430 OD_{500}/g dry matter [22]. The best procedure for pigment analysis is probably liquid chromatography, which allows separation and quantification of individual pigments; using this method, Hajjaj et al. [80] considered one 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, which may contain several substances with different absorbances.

Batch culture

Batch culture is widely used as the method of production

for many secondary metabolites using an aerated stirred tank fermenter. Although the traditional production of red fermented rice for use as a coloring agent is conducted on a solid support (rice), most studies performed in the laboratory have been done using batch fermentation, which presents easily controllable conditions [124]. When only the coloring fraction is necessary, without substrate residues, for instance for use in beverages, the pigment must be extracted with an organic solvent, then the solvent must be evaporated, for example using a spray drier. In that case, it may be interesting to work with batch fermentation in which, although the pigment is diluted in a high liquid volume, the biomass and other eventual insoluble components of the fermentation broth may be readily separated by filtration, and then the solvent evaporated. According to Lee et al. [44], *Monascus* pigment production, although traditionally done by SSF, is industrially performed by batch fermentation. This is due to solid substrate difficulties in aeration, humidity, temperature and pH control, while these parameters are easily controlled in batch fermentation; besides that, a flexible batch fermentation plant may use the same fermenter for several processes, which is industrially interesting [125]. pH control, for example, may permit direction of the fermentation in order to favor red pigment production [126].

The biosynthesis of pigments by fungi is poorly understood. Studies by many researchers have revealed that pigment production in SmF by *Monascus sp.* is affected by numerous environmental factors, particularly the type and concentration of nitrogen source in the medium [27,28,68]. Unfortunately, the results are often difficult to interpret, due to the use of shake flask cultures, in which the pH changes during cultivation and is dependent on the nitrogen source used [68]. Furthermore, individual pigment concentrations have not been determined. Wong et al. [14] reported that a *Monascus* species isolated from the koji of Kaoliang liquor had a maximum yield of pigment in a medium containing 50g/L of rice powder and 5g/L of sodium or potassium nitrate. In addition, they also reported that nitrogen sources such as MSG, peptone and yeast extract or casamino acids are also good nutrients for pigmentation. Carels et al. [27] observed the effect of different nitrogen sources on pigment production of a number of *Monascus sp.*, and concluded that when the source of nitrogen is yeast extract or nitrate, red pigment is formed, whereas with ammonium or ammonium nitrate, orange pigment is formed. For better control of pigment production, a well-defined chemical medium with glutamate as nitrogen source was used in SmF of *M. purpureus* [95]. This medium formulation promoted production and excretion of extracellular pigments [10] and also led to the formation of a glutamic acid–pigment complex [26].

Batch fermentation using media based on gelatinized cassava starch [45,87], soybean flour [87] and sweet potato [127] has been carried out in an attempt to increase the concentration of carbohydrates using these particular substrates, which are cheap and colorless. Using the starch as a block, there are plenty of carbon sources for the microorganism, but it does not increase the medium viscosity with free starch and thus does not compromise oxygen transfer known to be necessary [11,45,65]. Improvement of pigment production and optimization of medium composition and some of the culture conditions such as aeration rate, agitation, pH, dissolved oxygen and addition of substrates during batch culture have been studied in the literature [66-68,87-89,119]. Buhler et al. [62] showed the feasibility of the production of pigment by *M. ruber* in a batch bioreactor using a co-product of biodiesel without previous treatment as a substrate. According to Lee et al. [67] the production of both yellow and red pigments appears to be parallel to the growth curve, and both pigment production and growth end at around 140 h of culture time. The production of pigments in *Monascus* cultures appears to be growth-associated. Other researchers also observed the growth-associated pigment production kinetics of *Monascus* [49,69].

Current Trends and Future Perspectives

The open literature clearly demonstrates the great importance of studying *Monascus* in controlled, well-phased cultures and, above all, the importance of studying the early developments of the culture, because it is this phase together with the physiological state of the inoculum which determines the later stages of development. Apart from being a potential producer of industrially interesting pigments, *Monascus* provides a very elegant model of how fungi can use their metabolism to survive in different environments and how quickly they can adapt. *Monascus* seems to have evolved in a way whereby it can quickly change its metabolism, switching from one glycolytic pathway to another, thereby changing the form from sporulation to secondary metabolism and vice versa in a very short period of time. *Monascus* pigments are classified as versatile natural microbial colors that have a wide range of applications in food besides their use in nutraceutical/pharmaceutical industries. *Monascus* produces pigments that contain compounds responsible for inhibiting cholesterol synthesis and can decrease the level of total cholesterol and serum triglycerides [126]. A recent invention showed how *Monascus* fungus can be used as a bio-agent to treat agricultural by-products through biofermentation and conversion to *Monascus* fermented products. *Monascus* fermented products have high potential as an alternative protein-rich or functional feed ingredient in the diet of growing chickens (specific for layers). It will carry some benefits for cholesterol properties, for example targeting low cholesterol eggs due to the bioactive components of functional *Monascus* fermented products such as monacolin K and polysaccharides [127]. Therefore, the production of *Monascus* fermented products by utilizing agro-industrial by-products may not only reduce process costs but also help in environmental management.

The ability of *Monascus spp*, in producing red pigment may be improved further by the application of genetic engineering techniques or genomics levels due to the increasing demands of the industry for the development of natural pigments [128,129]. On top of that, with the current situation, the use of nutraceutical supplements such as *Monascus* fermented products by the communities is growing tremendously due to health concerns. Many studies reported in open literature review that different *Monascus* fermented products preparations contain dissimilar amounts of monacolin levels as well as different production of

bioactive compounds by *Monascus* spp. In other words, different strains of *Monascus* cultivated in different growth conditions may produce *Monascus* fermented products with different types and quantities of pigment, monacolin and monacolin derivatives. Therefore, the methods for improving of products quality and safety as well as proper information of *Monascus* fermented products that are available for consumers need to be developed.

Concluding Remarks

In order to design a reasonable and feasible biotechnological process for production of natural microbial color, optimal process control, medium formulation and an accurate experimental design should be investigated. Detailed investigations are required in order to establish the best medium for *Monascus* biofermentation. Kinetic studies are also important to give a better understanding of the process and also to give an idea regarding the productivity of *Monascus* culture, through the determination of specific growth rate, specific product formation rate and substrate consumption rate. Information on kinetics is important for the future scaling-up and development of process control for improvement of pigment production.

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