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PIGMENT PRODUCTION IN *PENICILLIUM*: DIFFERENT METHODS OF OPTIMIZATION IN SUBMERGED FERMENTATION

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Abstract

There is great interest worldwide in the use of natural colorants due to the serious health issues associated with synthetic pigments. To that end, microbes are the most reliable sources of natural pigments available. In particular, filamentous fungi are the most active and efficient sources of pigment production due to the low cost of fermentation and easy downstream processing. However, Monascus, though a well-known pigment-producing filamentous fungus, co-produces mycotoxin—citrinin, which limits its industrial applications, whereas Penicillium is reported to produce industrially significant, Monascus-like pigments without the co-production of citrinin. Due to which, recent studies are focused on pigment optimization in Penicillium in order to get higher yields. Hence, this review focuses on summarizing the optimum fermentation conditions involving environmental (pH, temperature, and agitation), microbial (inoculum type, age, and size), and nutritional factors (carbon and nitrogen sources) as well as incubation periods for the maximum pigment production in Penicillium in submerged fermentation. It also explains different methods of optimization that include direct experimentation to evaluate the target parameters, pre-experimentation, or statistical tools such as factorial or fractional factorial design (FFD), and response surface methodology(RSM) based on second-order central composite and Box-Behnken design to predict the significance and impact of different factors on the product. It further discusses the significance of self-immobilization biomembrane-surface liquid culture (SIBSLC), a new method of cultivation to increase pigment yield. Beside optimization, pigment upscaling is also required for their industrial application which is a future challenge that needs to be emphasized.

Keywords: *FFD, Optimization, Penicillium, RSM, Submerged Fermentation, SIBSLC*

INTRODUCTION

Pigments are significant in industrial and biotechnological applications. There is extensive utilization of various natural and synthetic pigments in the cosmetic, food, textile, pharmaceutical, and nutraceutical industries (9). However, synthetic pigments are produced from petrochemical sources via chemical processes that release harmful substances that negatively affect human, plant, and animal health and have damaging effects on the environment and water bodies. Furthermore, synthetic dyes are non-renewable and non-biodegradable, with their disposal causing serious issues (10-12). Hence, as a consequence of the toxic side-effects of synthetic pigments, the interest in the exploration of natural pigments has increased (4).

Natural pigments, on the other hand, are derived from renewable sources; hence, they are ecofriendly and biodegradable (13). However, natural pigments obtained from plants and animals/insects are not reliable due to their seasonal availability, low water solubility, and instability against extreme pH, heat, and light (Gunasekaran & Poorniammal, 2008). Hence, nowadays, microbial fermentation is the major source of producing more soluble and stable natural pigments (14). Additionally, microorganisms grow rapidly which allows high productivity, in addition to their availability throughout the year (15). However, microbial pigments from yeast, bacteria, and microalgae (16-19) are not significant due to their high cost and

low productivity (20). Hence, the attention is focused on ascomycetes and basidiomycetes thanks to the low cost of fermentation and easy downstream processing, which makes them industrially applicable (21, 22). Among the potent producers of microbial pigments, filamentous fungi are the most metabolically active in the production of stable and economically valuable substances. They secrete extracellular enzymes and secondary metabolites like pigments, organic acids, and food additives in addition to antibiotics (23). Filamentous fungi are also known to produce a number of pigments, such as red, orange, yellow, chestnut, bronze, and brown (24, 25). Pigments are categorized into four major classes, namely polyketide, amino acid derivatives, terpenes, and non-ribosomal peptides, each of which is synthesized by different metabolic pathways (26). Pigments produced by filamentous fungi are melanins, quinones, naphthoquinone, anthraquinone, flavin, ankaflavins, carotenoids, violacein, azaphilones, and indigo, belonging to the aromatic polyketide group of pigments (4, 27, 28).

The most dominant pigment-producing fungi belong to genera *Aspergillus, Penicillium, Fusarium, Paecilomyces, Monascus, and Trichoderma* (29). *Monascus* is well-known to produce three types of pigments: red (Monascorubin, Monascorubramine, and Rubropunctamine), yellow (Ankaflavin and Xanthomonascin), and orange (Rubropunctatin) (30-32). However, it co-produces citrinin; a mycotoxin that affects kidneys and liver (33-35). Hence, the interest of scientists is driven towards exploring new fungal species with the potential to produce safe natural pigments (9, 36, 37). As some *Penicillium* species are known to produce *Monascus* like pigments using similar metabolic pathways without the co-production of citrinin (31, 37).

Besides the application of fungal pigments in different industries, such as textile, food, and pharmaceutical, they also possess biological properties such as anticancer, antioxidant, antimicrobial, antineoplastic, and antirejection; as well as blocking enzyme activity, lowering cholesterol, and acting as probiotics (38-40). However, fungal pigments become commercially significant only when their productivity is high and are able to withstand production and processing conditions, in addition to their approval as non-toxic to human health by food and drug authorities. And as pigment approval and industrial production is an expensive and time-consuming process, only the processes that promise higher productivity and are economically feasible are of practical interest. Therefore, it is essential to understand the potential of the relative pigment-producing process before its application. To that end, the optimization of fermentation conditions is the only way to get an improved yield of pigments as well as check the efficiency of the process on a lab scale.

Most of the studies on the pigment production in *Penicillium* were carried out in submerged fermentation. As the growth and pigmentation are mainly influenced by physiochemical factors such as temperature, pH, humidity, agitation, carbon sources, nitrogen sources, and C/N ratios, as well as microbial parameters such as inoculum age, type, and size, recent studies are focused on improving culture conditions in order to increase pigment production through the optimization of environmental, nutritional, and microbial factors. In submerged fermentation, the maximum pigment production is expressed as OD at maximum absorbance wavelength, as mg or grams of pigment/L, AU (unit of absorbance), $P_{P/X}$ (pigment yield on biomass), and $P_{P/S}$ (pigment yield on the substrate).

This review paper provides a comprehensive and detailed analysis of the optimization processes in submerged fermentation for higher yields of pigments. It also includes different methods such as experimentation, fractional factorial design (FFD), response surface methodology (RSM), and self-immobilization biomembrane-surface liquid culture (SIBSLC) in order to optimize fermentation conditions.

METHODS OF OPTIMIZATION

EXPERIMENTATION

A method in which experiments are performed on a lab-scale to evaluate the effects of various factors, such as the type of media, pH, temperature, agitation, carbon sources, nitrogen sources, incubation period, and microbial factors, on the pigment yield (41, 42).

EFFECTS OF DIFFERENT TYPES OF MEDIA

Different types of culture media, such as natural and synthetic, were tested for the growth and pigment production in different *Penicillium* species. Natural growth media is mainly made up of potatoes and is the preferred choice in *Penicillium* sp, *Mangrove Penicillium* DLR-7, and *Penicillium purpurogenum* Li-3 (42-45). On the other hand, synthetic media, such as PDB (potato dextrose broth), CDB (czapek dox broth), SDB (Sabouraud dextrose broth), MEB (malt extract broth), YE (yeast extract), YMB (yeast extract malt extract broth), PGY (peptone glucose yeast), YPD (yeast extract peptone glucose), and czapek yeast extract, were tested for different *Penicillium* species. Among the tested synthetic culture media, PDB (14, 46, 47), CDB (48, 49), and SDB (25, 50-52) were the most favorable culture media for the production of pigments. Table I below summarizes the details of the pigments produced by different *Penicillium* species in selected media.

Table I. Optimum culture media for pigmentation in different *Penicillium* species

***Penicillium* species**	**Culture media**	**Pigment**	**Maximum absorbance wavelength**	**References**
Penicillium sp.	PDB	Red	530nm	(14)
Penicillium purpurogenum GH2	Czapek dox broth supplemented with xylose	Red	500nm	(48)
Penicillium purpurogenum DPUA 1275	Czapek yeast extract media	Yellow Orange Red	400nm 470nm 490nm	(49)
Mangrove Penicillium DLR-7	Natural potato media	Red Yellow	510nm 420nm	(45)
Penicillium purpurogenum	Sabouraud dextrose broth	Red	510nm	(52)
Penicillium purpurogenum BKS9	Sabouraud dextrose broth	Red	500nm	(50)
Penicillium minioluteum	Sabouraud dextrose broth	Red	490nm	(51)
Penicillium purpurogenum Li-3	PDB (Natural potato based)	Red	500nm 494nm	(44)
Penicillium sp. GBPI P155	PDB	Orange	530nm (shoulder peak)	(46)
Penicillium sp. HSD07B	PDB	Red	505nm	(47)
Penicillium mallochii TACB-16	Sabouraud dextrose broth	Orange-red	450nm	(25)
Penicillium sp.	PDB (Natural potato-based)	Yellow	650nm	(43)
Penicillium sp.	PDB (Natural potato based)	Red	490nm	(42)
Penicillium sclerotiorum strain AK-1	PDB	Dark yellow	250-500nm	(41)

EFFECTS OF pH

pH is the major environmental factor that affects the structure, morphology, development of conidia, physiology, permeability of the cell wall and cell membrane, and the synthesis of metabolites in fungi (53, 54). pH has a regulatory effect on the enzyme activities involved in metabolic pathways leading to the formation of pigments as secondary metabolites(55). The fungal growth and metabolite formation is limited at a certain pH range (56).

Research conducted to determine the effects of pH on pigment production has used a wide range of values, from pH 2-13. Though different optimum initial pH values were reported in different *Penicillium*

species, most of the optimum pH values fall in the acidic range, between 4 to 6.5, in both static and shaking flasks (25, 41, 42, 48, 50-53, 57-59). However, pH 3 is the lowest optimum initial pH reported for the growth and pigment production in *Mangrove Penicillium* DLR-7 and *Penicillium* sp GBPI P155 (45, 46). Whereas *Mangrove Penicillium* DLR-7 also demonstrated the production of yellow pigment at pH 2 with maximum absorption at 420nm. Though at this pH, *Penicillium* showed little growth with the absence of spores (45). As low pH inhibits conidia development and increases pigment production, indicating that low pH might influence the transport of nutrients and ions between the media and the microbial cell by affecting the solubility of salts and the ionic state of nutrients (53, 54).

On the other hand, a few research findings have reported neutral to alkaline pH to promote pigment production in a few *Penicillium* species. For instance, *Penicillium* sp and *Penicillium murcianum* gave maximal pigment production at pH 9 (14, 60). Additionally, the growth of *Penicillium* also followed the same trend as the pigment production as the maximal growth of almost all *Penicillium* species occurred in the acidic range of pH 4 to 6.5(41, 42, 50, 52, 57, 58), except for *Penicillium aculeatum* ATCC 10409, *Penicillium* sp, and *Penicillium purpurogenum* GH2 as their maximum growth were at pH 8, 9 and, 7 (14, 48, 53).

EFFECTS OF TEMPERATURE

Temperature is an important environmental factor as it affects the metabolism of the fungi, hence, affecting growth and pigmentation. And as fungi require a longer period for their growth in liquid fermentation, they can be exposed to contamination risks. Therefore, a temperature optimum for their growth is preferred. Additionally, temperature also regulates the activity of multi-enzyme complex polyketide synthetase catalyzing the synthesis of polyketide pigments (53).

According to many research findings, the temperature between 24-30^{0}C is optimum for growth and pigment production in most *Penicillium* species (14, 25, 41-43, 45, 48, 50, 52, 53, 57-60) when grown in a wide range of temperature between 10^{0}C-35^{0}C for temperature optimization. However, some *Penicillium* species, such as *Penicillium minioluteum* and *Penicillium* sp GBPI P155 (46, 51), have the potential to produce pigments at low temperatures, such as 15^{0}C. For instance, *Penicillium* sp GBPI P155, being psychrotolerant, gave maximum pigment yield at 15^{0}C, which is a survival response to environmental stress in order to compensate low metabolic activity at this temperature (61). The optimum temperature specific to the specie also corresponds to the environmental conditions of the area from which it was isolated. Furthermore, for some *Penicillium* species an optimum temperature for growth is different from the optimum temperature required for pigmentation. For instance, *Penicillium purpurogenum* Li-3 produced pigment at 20^{0}C when a two-stage temperature-controlled culture was used in which the temperature of the 1st stage was 32^{0}C, which was sufficient for the growth, then the temperature was shifted from 32^{0}C to 20^{0}C, which was optimum for pigment production. Hence, the separation of the growth phase from the pigment synthesis phase led to a significant increase in pigment yield (44). Table II below describes the optimum pH and temperature values for growth and pigmentation in different *Penicillium* species.

EFFECTS OF AGITATION

Agitation plays an essential role in aerobic fermentation as it facilitates oxygen uptake and the transfer of nutrients between media and microbial cells (62). Research findings have reported an optimum agitation speed between 150-200 rotations per minute for maximum biomass formation and pigment production in *Penicillium aculeatum* ATCC 10409 [11.12g/L biomass, 1.38g/L pigment], *Penicillium* sp [3.2g/L biomass, 920mg/L pigment], and *Penicillium purpurogenum* Li-3[1.5 OD_{500nm}](14, 44, 53) when more than one agitation speeds were tested to determine the optimum agitation speed for growth and pigmentation. Agitation speed above this optimum range might cause mechanical damage to mycelium by shearing.

In many other *Penicillium* species, fixed agitation speed, either 150rpm or 200rpm (25, 42, 47-49, 58, 59), is used, making it difficult to distinguish whether it is optimum for growth or pigment production, or both. However, the study is focused on pigmentation and, hence, stirring speed might be optimum for

pigment production. Mostly, the stirring speed that is favorable for both growth and pigmentation and which is not damaging to mycelium is always preferred.

Table II. Optimum pH and temperature for growth and pigmentation in different *Penicillium* species

Penicillium species	Optimum pH		Optimum temperature		References
	Growth	Pigment	Growth	Pigment	
Penicillium sp. AZ		5:		30^{0}C:	(59)
Penicillium sp.	9: 4.75g/L	9: 90 mg/L	30^{0}C: 2.65g/L	30^{0}C: 950mg/L	(14)
Penicillium reticulosum Blr1	5.5: 4.4 g/L	5.5: 0.91g/L	28-29^{0}C:	28-29^{0}C:	(57)
Mangrove Penicillium DLR-7	3: 1.8g/L	3: 500mg/L	25^{0}C:	25^{0}C:	(45)
Penicillium purpurogenum	5:	5: 2.02 AU/mg	27:	27: 110 AU/mg	(52)
Penicillium aculeatum ATCC 10409	8: 11.12g/L $Y_{X/C}$: 0.69	6.5: 1.38g/L 1.479 OD_{445nm} $Y_{P/X}$: 0.155	30^{0}C: 11.12g/L $Y_{X/C}$: 0.69	30^{0}C: 1.38g/L 1.479 OD_{445nm} $Y_{P/X}$: 0.155	(53)
Penicillium purpurogenum BKS9	6: 0.877g/50ml	6: 0.79 OD_{500nm}	30^{0}C:	30^{0}C:	(50)
Penicillium UCP 1371	4:	4:	28^{0}C:	28^{0}C:	(58)
Penicillium SIS $A_9P_110^{-3}D$					
Penicillium minioluteum		5.6: 1.015 OD_{490nm}		15^{0}C: 1.012 OD_{490nm}	(51)
Penicillium purpurogenum Li-3			32^{0}C:	20^{0}C 2.98 OD_{500nm}	(44)
Penicillium sp. GBPI P155	3: 9g/L	3: 3.24 OD_{495nm} 2.74 OD_{530nm}	25^{0}C: 7.31g/L	15^{0}C: 3.37 OD_{494} 3.12 OD_{530nm} 35^{0}C: 3.39 OD_{494nm} 2.93 OD_{530nm}	(46)
Penicillium purpurogenum GH2	7: 6.045g/L	5: 2.460g/L Pigment yield $Y_{P/X}$: 9: 0.8041	34^{0}C: 6.04 g/L	24^{0}C: 2.460g/L Pigment yield $Y_{P/X}$: 34^{0}C: 0.8041	(48)
Penicillium murcianum		9:	28^{0}C:	28^{0}C:	(60)
Penicillium mallochii TACB-16		5:		30^{0}C:	(25)
Penicillium sp.			30^{0}C: 1.319g/L	30^{0}C: 1 OD_{650nm}	(43)
Penicillium sp.	6.5: 4.5g/L	6.5: 2.965 OD_{490nm}	24, 27, 30^{0}C Insignificant growth change	24-30^{0}C 24^{0}C: 20.02OD_{490nm}	(42)
Penicillium sclerotiorum strain AK-1	6: 7.983g/L	5: 0.563g/L	30: 9.743g/L	30: 0.364g/L	(41)

$Y_{P/X}$ indicates pigment yield, $Y_{X/C}$ indicates biomass yield, and empty blocks show that their values are not experimentally determined.

EFFECTS OF CARBON SOURCES

Fungi are heterotrophic microorganisms and require an exogenous nutrient source. Hence, the growth and metabolite production is influenced by the composition of the media. However, the utilization of just any nutrient is not sufficient as metabolism is also required for the synthesis of metabolites. Therefore, it is essential to select suitable nutrients in order to get the desired products by knowing the nutrient requirement of the microorganism, as well as the metabolic pathway that will favor its metabolism. This way, the culture medium could be supplemented with the required substrates to be converted into the products of interest efficiently with increased productivity in short time (49).

Tested carbon sources include monosaccharides (glucose, fructose, xylose, galactose, mannose, rhamnose, dextrose, and arabinose), disaccharides (sucrose, lactose, and maltose), polysaccharide (soluble starch), glycerol, and carboxymethyl cellulose. Among the tested carbon sources, starch (14, 49, 59), sucrose (49, 63, 64), xylose (45, 52, 57), maltose (46), mannitol (42) dextrose (41, 51), and glucose (47, 65) are the most preferred carbon sources metabolized by *Penicillium* to produce pigments.

Carbon sources that promoted the growth are glucose (45, 47, 59, 65, 66), fructose (14, 46, 49), xylose (52, 57, 66), and dextrose (51). Disaccharides and polysaccharides favor pigment production while monosaccharides promote growth. Mostly, growth and pigmentation are not directly related when carbon sources are considered, except for a few *Penicillium* species that have one optimum carbon source for maximum growth as well as for maximum pigment yield (41, 42, 47, 51, 52, 57).

EFFECTS OF NITROGEN SOURCES

Tested nitrogen sources include both organic and inorganic sources. Organic nitrogen sources include yeast extract, beef extract, peptone, urea, tryptone, malt extract, and amino acids (alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, histidine, lysine, phenylalanine, tyrosine, and tryptophan). Whereas, inorganic nitrogen sources include sodium nitrate, potassium nitrate, ammonium nitrate, ammonium chloride, ammonium sulfate, and monosodium glutamate. Among the organic nitrogen sources, yeast extract (41, 49, 57) and peptone (14, 46, 51, 52, 57, 60) are the best regarding pigment production. Whereas among the inorganic nitrogen sources, sodium nitrate (52, 57) and ammonium nitrate (57, 67) are significant in yielding more pigment. On the other hand, regarding growth, malt extract (49), yeast extract (46, 57), and peptone (14, 57) are mostly favored. Additionally, sodium nitrate (57) and ammonium nitrate (57, 59) also promote growth. However, in most of *Penicillium* species, there is no direct relationship between growth and pigment production as they use different nitrogen sources for each phenomenon.

Mostly, organic nitrogen sources are preferred over inorganic ones. However, *Penicillium* sp. AZ is known to produce violet (PP-V) and red (PP-R) pigments in culture media supplemented with starch, yeast extract, ammonium nitrate in citrate buffer, but as the ammonium nitrate is removed from the media, it is switched from producing violet and red pigments to yellow (PP-Y) and orange (PP-O), indicating the impact of inorganic nitrogen sources on pigment production. It is hypothesized that the presence of ammonium nitrate in the culture media provides nitrogen atom, or NH, for the substitution of pyranoid oxygen at C-7 in the pigments PP-O and PP-Y to form PP-V and PP-R involving Schiff base formation. However, it is also assumed that the low pH of the media does not favor this chemical reaction to occur. Therefore, it would be a biological one. (67). Hence, the type of pigment produced not only varies from specie to specie in a genus but also within the strain under different culture conditions that might change the physiology of the microorganism.

C:N RATIO

Carbon to nitrogen ratio is an essential factor that contributes to the productivity of the *Penicillium* species. An optimum C/N ratio is specific for each *Penicillium* specie/strain. According to some publications, the C/N ratio 1:1 is optimum for *Penicillium* spp [4.2g/L biomass, 1550mg/L pigment] (14), 21:1 for *Penicillium reticulosum* [3.55g/L biomass, 0.50g/L pigment] (57), and 15:1 for *Penicillium purpurogenum* DPUA 1275(49).

The combination of carbon and nitrogen sources in a culture medium affects the growth and production of pigments. Hence, their efficient selection is very crucial for the optimization process. Chintapenta et al., 2014 reported maximal biomass in glucose+alanine/xylose+alanine and the highest red pigment yield in xylose-glycine media in *Mangrove Penicillium* DLR-7. Furthermore, mannose-alanine, starch-glycine, glucose-glutamic acid, and xylose-aspartic acid also gave good pigment yield. However, although mannose+glycine promotes pigment production, if glycine is replaced by histidine, then pigment production decreases. Similarly, if glycine in the xylose-glycine pair is replaced by lysine or alanine, then pigment yield also decreases. Whereas, the combination of arginine and lysine did not show any sign of pigment production. Hence, the combination of carbon and nitrogen sources not only affect the rate of pigment production, but also the types of pigment produced, as the presence of tryptophan, histidine, and lysine showed the production of yellow pigment instead of red (45).

de Araújo Alencar et al., (2016) determined the effects of carbon and nitrogen combinations on yellow and red pigment production in *Penicillium* UCP 1371 and *Penicillium* SIS $A_9P_110^{-3}D$. Wherein, in the case of *Penicillium* UCP 1371, the highest production of yellow and red pigment was reported in starch-tryptone media. Additionally, while all tested carbon sources promoted pigment production, among the nitrogen sources, only tryptone was the most favorable. However, in the case of *Penicillium* SIS $A_9P_110^{-3}D$, the highest production of both red and yellow pigments was favored by the media containing glucose-tryptone. On the other hand, glucose-tryptone-containing culture media gave optimum growth of *Penicillium* UCP 1371 while starch-tryptone promoted the growth of *Penicillium* SIS $A_9P_110^{-3}D$(58). Hence, neither carbon nor nitrogen sources can exhibit a positive effect on biomass or pigment yield if they are inaccurately combined (de Araújo Alencar et al., 2016). Table No III describes optimum carbon and nitrogen sources for maximum growth and pigmentation in different *Penicillium* species.

Table III. Optimum carbon sources and nitrogen sources for growth and pigmentation in different *Penicillium* species

***Penicillium* species**	**Optimum carbon sources**		**Optimum nitrogen sources**		**References**
	Growth	**Pigment**	**Growth**	**Pigment**	
Penicillium sp. AZ	Glucose: 55g/L	Soluble starch: 19.3 A_{500nm}		Ammonium nitrate: 19.3 A_{500nm}	(59)
Penicillium pinophilum	Glc/Xyl: u_{max}: 0.08-0.09/h Biomass yield: Y_{SX}: (Glc) 0.33g/g				(66)
Penicillium persicinum	Glc/Xyl: u_{max}: 0.08-0.09/h Biomass yield: Y_{SX}: (Glc, Xyl) 0.56 and 0.51g/g				(66)
Penicillium brasilianum	Glc: u_{max}: 0.16/h Xyl: u_{max}: 0.14/h				

	Biomass yield: Y_{SX}: (Glc, Xyl) 0.47 and 0.48g/g				(66)
Penicillium sp.	Fructose: 5.50g/L	Soluble starch: 670mg/L	Peptone: 4.25g/L	Peptone: 880mg/L	(14)
Penicillium purpurogenum DPUA 1275	Fructose: 11.98g/L	Starch: Yellow: $1.77UA_{400nm}$ Orange: $0.84UA_{470nm}$ Red: $1.01UA_{490nm}$	Malt extract: 14.61g/L	Yeast extract: Yellow: $2.01\ UA_{400nm}$ Orange: $0.94\ UA_{470nm}$ Red: $0.71\ UA_{490nm}$	(49)
Penicillium reticulosum Blr1	Xylose: 3.32g/L	Xylose: 0.55g/L	$NaNO_3$: 2.76g/L NH_4NO_3: 3.36g/L Yeast Extract: 3.78g/L Peptone: 4.04g/L	$NaNO_3$: 0.34g/L NH_4NO_3: 0.35g/L Yeast Extract: 0.35g/L Peptone: 0.36g/L	(57)
Mangrove Penicillium DLR-7	Glucose: 2.8g/L	Xylose: 850mg/L	Xylose+alanine: 3.12g/L	Xylose+glycine: 1030mg/L	(45)
Penicillium purpurogenum	Xylose:	Xylose (2%): 8.41 AU/mg		Peptone (2%): $5\ AU_{510nm}$/mg Sodium nitrate: $8\ AU_{510nm}$/mg	(52)
Penicillium UCP 1371	Glc-tryptone: 10.6g/L	Yellow: Starch and tryptone : $2.855\ UA_{400nm}$ Red: Starch and tryptone : 0.853	Glc-tryptone : 10.6g/L	Yellow: Starch and tryptone : $2.855\ UA_{400nm}$ Red: Starch and tryptone : $0.853\ UA_{530nm}$	(58)
Penicillium SIS $A_9P_110^{-3}D$	Starch and tryptone: 15.7g/L	Yellow: Glc-tryptone : $3.385\ UA_{400nm}$	Starch-tryptone : 15.7g/L	Yellow: Glc-tryptone : $3.385\ UA_{400nm}$	(58)

Penicillium minioluteum	Dextrose:	Red: Glc-tryptone : 0.978 UA_{530nm} Dextrose and Maltose: 1.015 OD_{490nm}		Red: Glc-tryptone : 0.978 UA_{530nm} Peptone: 1.013 OD_{490nm}	(51)
Penicillium sp. GBPI P155	Fructose: 9.5g/L	Maltose: 3.25 OD_{495nm} 2.73 OD_{530nm}	YE: 11.11g/L	Peptone: 0.86 OD_{495nm} to 0.91OD_{495nm} (10^{th} to 30^{th} day)	(46)
Penicillium sp. HSD078	Glucose:	Glc (15%): 2.75g/L			(47)
Penicillium murcianum				Peptone (20%):	(60)
Penicillium sp.	Starch, mannitol, sucrose gave the same growth as PDB.	Mannitol: 15.12OD_{490nm} 1% Mannitol: 21.08 OD_{490nm}	Tested nitrogen sources increased growth.	Tested nitrogen sources decreased pigment yield.	(42)
Penicillium sclerotiorum strain AK-1		Dextrose: 11.989g/L		Yeast extract: 0.364g/L	(41)

u_{max} shows maximum growth rate, OD is optical density, AU is unit of absorbance and empty blocks show that their values are not experimentally determined.

EFFECTS OF INCUBATION PERIOD

Incubation period is an important factor regarding the optimization process as every microorganism requires an optimum time to grow and produce pigments at the maximal level. The incubation period is specific for every microbe and varies among the species of the *Penicillium* genus. The optimum incubation days for pigmentation in different *Penicillium* species are 7 (68), 8 (42), 12 (45, 49, 57), 14 (49), 15 (65), 18 (50), and 28 (25, 51), while the optimum incubation days for the maximum growth are 3 (68), 5 (49), 6 (42), 12 (45, 57), 15 (50), and 18 (25). Additionally, there is no direct relationship between growth and pigmentation with respect to incubation period as pigments are secondary metabolites and are secreted in the decline or decelerated phase of growth.

Pandey et al., (2018) reported a specific optimum incubation period for each of the other parameters/factors tested. In the case of pigmentation, an incubation period of 15 days is sufficient at the optimum temperature between 15^0C and 35^0C in the optimum carbon source maltose, while 10 to 30 days of incubation is required in the optimum nitrogen source peptone, whereas an incubation period of 20 days is optimum at optimum pH 3 (46). In the case of growth, on the other hand, maximum biomass formation at 25^0C, pH 3 and in maltose occurred on the 15^{th} day of fermentation, while in yeast extract, maximum growth was achieved on the 20^{th} day of incubation (46).

In some *Penicillium* species, the fixed incubation periods of 5 (14, 52), 7 (44, 58), 10 (48, 53, 60), 15 (41), and 32 (43) days are selected, making it difficult to distinguish whether it is optimum for growth or pigmentation. Whereas in the case of other *Penicillium* species, pigmentation is checked on different time intervals and even days to get the optimum period. Table No IV below shows the different optimum incubation periods for growth and pigmentation in different *Penicillium* species.

EFFECTS OF INOCULUM AGE

Inoculum age affects pigmentation indirectly by affecting growth. Mostly, 5-7 days old inoculum was used for inoculating culture media. Gunasekaran & Poorniammal, 2008 reported that 4 days old inoculum is the best for the maximum growth (3.2g/L) and pigment production (850mg/L) in *Penicillium* sp (14).

Table IV. Optimum incubation period for growth and pigmentation in different *Penicillium* species

Penicillium species	Optimum incubation period		References
	Growth	Pigment	
Penicillium novae-zelandiae HSD07B		15th day: 2.39g/L	(65)
Penicillium purpurogenum DPUA 1275	5th day (120hrs): Spore suspension: 10^7: 14.31g/L 10^8: 14.17g/L 5th day (120hrs) Mycelial disc: 15: 17.54g/L	Spore suspension: 10^8: 5th day (120 hours): Yellow: 1.15UA_{400nm} 12th day (288 hours): Orange: 0.42UA_{470nm} Red: 0.34UA_{490nm} Mycelial disc: 5: 14th day (336 hours): Yellow: 3.08UA_{400nm} Red: 2.27UA_{490nm} Orange: 1.44UA_{470nm}	(49)
Penicillium reticulosum Blr1	12th day: 5.17g/L	12th day: 0.58g/L	(57)
Mangrove Penicillium DLR-7	12th day: 3.1g/L	12th day: 1050mg/L	(45)
Penicillium purpurogenum GH2	Mycelium: 10% v/v 3rd day (72 h): 7.06g/L	Mycelium: 10% v/v 7th day (172 h): 10.6 OD_{500nm} Productivity/h: 0.050 ODL/h $Y_{Y/B}$: 4.51 ODL/h $Y_{Y/S}$: 0.770 ODL/h	(68)
Penicillium purpurogenum BKS9	15th day: 0.847g/50ml	18th day: 0.761 OD_{500nm}	(50)
Penicillium minioluteum		28th day: 1.012 OD_{490nm}	(51)
Penicillium sp.	15th day: 25^0C 7.31g/L 15th day: pH 3	15th day: 15^0C 3.37 OD_{495nm} 3.12 OD_{530nm} 15th day: 35^0C 3.39 OD_{495nm} 2.93 OD_{530nm} 20th day: pH 3	(46)

GBPI P155	9g/L 20th day: Fructose 9.5g/L 15th day: YE 11.11g/L	3.24 OD_{495nm} 2.74 OD_{530nm} 15th day: Maltose 3.25 OD_{495nm} 2.73 OD_{530nm} 10th day: Peptone 0.86 OD_{495nm} 30th day: Peptone 0.91 OD_{495nm}	
Penicillium mallochii TACB-16	18th day: 46.33mm colony diameter	28th day: 0.9 OD_{450nm}	(25)
Penicillium sp.	6th day: 10^8: 7g/L 5th day: 10^6: 4.52g/L	8th day: 2.866 OD_{490nm}	(42)

$Y_{Y/B}$ indicates pigment yield on biomass, $Y_{Y/S}$ indicates pigment yield on substrate and empty blocks show that their values are not experimentally determined.

EFFECTS OF INOCULUM TYPE AND SIZE

Although there are many methods in literature to inoculate filamentous fungi, spore suspension and mycelial discs are the most used ones. Many research studies use spore suspension for inoculation (25, 47, 48, 50, 52, 53, 57, 60). According to a research report by Elattaapy & Selim, 2020, when different concentrations of spore suspension were used to determine the effects of inoculum size on growth and pigmentation, it was concluded that 10^8 spore suspension is optimum for growth while 10^6 is optimum for pigment production in *Penicillium* sp. (42).

Santos-Ebinuma et al., (2013) reported that 10^8 spores/ml and 15 mycelial discs are optimum for growth while 10^8 spores/ml and 5 mycelial discs are optimum for pigment production when determining the effects of spore suspension and agar mycelial disc as well as their concentration on the growth and pigmentation in *Penicillium purpurogenum* DPUA 1275. Furthermore, five mycelial discs produce more pigment than the spore suspension (10^8 spores/ml)(49). A similar finding was reported by Morales-Oyervides et al., 2015 using mycelium as the inoculum. Morales-Oyervides et al., 2015 observed that mycelium not only reduces the length of the lag phase but also reduces the time required to reach maximum pigment yield (68).

Big difference in growth and pigmentation was observed between the spore suspension and the mycelium inoculation methods. A major reason is the presence of a long lag phase in the spore suspension and a short one in the mycelium inoculation. When inoculation is done with spore suspension, the initial 24h represent the lag phase in *Penicillium purpurogenum* DPUA 1275(49), while in *Penicillium purpurogenum* GH2, the lag phase is even longer, consisting of 5 days (120h)(68). This presence of the lag phase in the case of spore suspension is due to the time spores require to adapt and maintain themselves in a new culture medium, as they are under physiological stress due to the scrapping from the mycelial surface during the preparation of spore suspension (49, 68). Another possible reason for less growth in spore suspension is that mycelium has 20% more initial biomass in comparison (49). Additionally, inoculation with mycelium results in the shortening of the lag phase due to the presence of spores, enzymes, and metabolites that are already produced during the inoculum preparatory phase (49, 68).

Morales-Oyervides et al., 2015b observed that mycelium produces 55% more pigment than spore suspension per hour. Pigment yield on the substrate was 59% higher using mycelium in contrast to spore suspension. Similarly, pigment yield on biomass was also higher than in the case of spore suspension. The initial concentration of mycelium 10% v/v is optimum for growth and pigmentation. However, further increase in the initial concentration of mycelium led to too much biomass, which caused a reduction in pigment production due to nutrient starvation (68).

RELATIONSHIP BETWEEN INCUBATION PERIOD AND INOCULUM TYPE

Santos-Ebinuma et al., (2013) reported that *Penicillium purpurogenum* DPUA 1275 produced three different types of pigments (yellow, orange, and red) in different time periods. The maximum production of yellow pigment was on the 5th day (120h), while the red and orange pigments were maximally obtained on the 12th day (288h) using 10^8 spores/ml. However, using 5 agar mycelial discs, the highest production of yellow and red pigments occurred after the 9th day (216h), while the orange pigment was maximally produced after the 12th day (288h), with the optimum fermentation day for all three pigments being the 14th (336h) (49). Whereas the optimum growth was on the 5th day (120h) using either of the inoculation methods (49).

Furthermore, the yellow pigment was produced earlier than the red and orange ones, which indicated that either the structure of the yellow pigment was modified into red and orange or the yellow pigment was produced and disappeared during the process due to its degradation by light, heat, or chemicals (49). Additionally, the production of the red and orange pigments was initiated after 168h, when the carbon source was almost completely utilized and the microorganism had entered into the decline phase of growth, suggesting that they are secondary metabolites (49).

Morales-Oyervides et al., (2015b) described growth, pigmentation, and substrate consumption during the incubation period in *Penicillium purpurogenum* GH2. Herein, the initial 120h represents the long lag phase followed by rapid growth from 120 to 188h, before becoming constant until the end using spores as the inoculum. Additionally, the substrate is rapidly consumed from 172 to 196h, with no substrate left by the end. Whereas the highest pigment production is obtained at 196h of fermentation, which depicts the decline phase of growth.

Furthermore, Morales-Oyervides et al., (2015b) reported that in the case of mycelium, rapid growth is started at 24h, reaching a maximum at 72h, with further incubation reducing the growth until it becomes constant at the end of the fermentation (220h). Additionally, the substrate is rapidly consumed between 48h and 96h, corresponding to the exponential phase of growth. Whereas maximum pigment yield is obtained at 172h of fermentation. However, the inoculum preparatory phase for mycelium consists of 3 additional incubation days (72h). Hence, the time required to initiate pigment synthesis in both inoculation methods is almost the same, though the time required to reach maximum pigment yield is reduced by 40% using mycelium in contrast to spore suspension (68). Table V shows the optimum inoculum size and type for maximum growth and pigmentation in different *Penicillium* species.

Table V. Optimum inoculum size and type for growth and pigmentation in different *Penicillium* species

***Penicillium* species**	**Optimum inoculum type/size**		**References**
	Growth	**Pigment**	
Penicillium purpurogenum GH2	Mycelium: 10% v/v 3rd day (72 h): 7.06g/L	Mycelium: 10% v/v: 10.52 OD_{500nm} Productivity/h: 0.050 ODL/h $Y_{Y/B}$: 4.51 ODL/h $Y_{Y/S}$: 0.770 ODL/h	(68)
Penicillium purpurogenum DPUA 1275	Mycelial discs: 15: 17.54g/L	Mycelial disc: 5 14th day (336 hours): Yellow: 3.08UA_{400nm} Red: 2.27UA_{490nm} Orange: 1.44UA_{470nm}	(49)
Penicillium sp. HSD078	Spore suspension:	Spore suspension: 1(inoculum proportion): 2.75g/L pigment	(47)
Penicillium sp.	Spore suspension: 10^8 spores/ml: 7g/L	Spore suspension: 10^6 : 20.53 OD_{490nm}	(42)

FACTORIAL OR FRACTIONAL FACTORIAL DESIGN (FFD) AND RESPONSE SURFACE METHODOLOGY (RSM)

Fractional factorial and response surface methodology based on second-order central composite design and Box-Behnken design are pre-experimentation or statistical tools that are effective in predicting the results of an experimental design by telling the significance and influence/effect of independent variables over the dependent or response variables, which not only saves time but also reduces the number of experiments. Response surface methodology also shows the interaction effect among the factors by a quadratic polynomial equation.

Hailei et al., (2011) reported glucose and inoculum proportions as two independent variables to be statistically significant among the six independent variables (pH, temperature, glucose, inoculum quantity, inoculum proportion, and rotation speed) regarding the production of red pigment in Penicillium sp. HSD07B when co-cultured with *Candida tropicalis* as suggested by fractional factorial design. Whereas, response surface methodology using central composite design predicted the maximum pigment yield of 2.76g/L with 15g glucose and 1 inoculum proportion, which agrees to the experimental value of pigment yield of 2.75g/L. This value was 3.4 times higher than the pigment obtained under non-optimized culture conditions (47).

Hailei et al., (2012) described the use of fractional factorial design to screen the influence of six factors (glucose, pH, temperature, membrane diameter, depth of fermentation broth, and rotary speed) on the production of red pigment as a response variable in *Penicillium novae-zelandiae* HSD07B (65). Hailei et al., 2012 reported that glucose and membrane diameter were selected as two significant factors for parameter optimization, and their effects were studied via response surface methodology based on central composite design (65). The statistical analysis predicted a maximum pigment yield of 4.26g/L when glucose concentration was 9.32g/L and membrane diameter was 9.74cm, which agrees with the experimental pigment yield of 4.25g/L. Hence, just by optimizing the culture parameters, there is a 78% increase in the pigment yield compared to the initial non-optimized culture conditions (65).

Santos-Ebinuma et al., (2014) analyzed the effects of six variables (pH, temperature, yeast extract, sucrose, stirring speed, and incubation time) statistically by two fractional factorials, one full fraction, and response surface methodology based on the central composite design in *Penicillium purpurogenum* DPUA 1275. The first fractional factorial design predicted that pH, stirring speed, yeast extract, and sucrose were significant factors regarding pigment production (63). Whereas, according to the second fractional factor design, agitation, yeast extract, and sucrose were significant factors; while the full factorial design estimated that sucrose and yeast extract were the most important variables that significantly increased pigment yield (63). Table VI below shows the significance of factors and their interactions on the production of yellow, orange, and red pigments predicted by FFD (1st fractional factorial, 2nd fractional factorial, and full factorial design).

Not only yeast extract and sucrose are significant regarding the production of red pigment in *Penicillium purpurogenum* DPUA 1275, but their concentrations are equally important. According to a report by Santos-Ebinuma et al., (2013), the production of red pigment (CYA media containing 30g of sucrose and 5g of yeast extract at pH 6.5, temperature 30^{0}C, and 336h of incubation) was indicated by 1.052UA (49), which was 2.8 folds lower than the absorbance (2.955UA) obtained in the study in which the concentration of sucrose and yeast extract was 50g and 12g respectively, as suggested by fractional factorial design and response surface methodology. This absorbance value represented a 78% increase in the production of red pigment (64). Whereas in another study prior to this one on the same fungus, the effects of six independent variables on the production of red pigment were determined statistically and found that at pH 4.5, 50g of sucrose, and 10g of yeast extract, the production of red pigment reached 1.66UA, which shows 23% increase in the production of red pigment compared to the previous work in which the concentration of sucrose and yeast extract was 30g and 5g (63).

Hernández et al., (2019) evaluated the effects of pH and NaCl optimization in malt extract media and pH, NaCl, and peptone optimization in peptone glucose yeast extract media on yellow pigment production in *Penicillium murcianum* via full factorial and response surface methodology based on the second-order central composite and Box-Behnken design. Hernández et al., 2019 reported that the model

predicted pH 9 and 7.5g/L of NaCl in malt extract and pH 9, 20g/L of peptone, and 6g/L of NaCl in peptone glucose yeast extract media to be optimum for maximum yellow pigment production, as indicated by 1.57 UA and 1.42 UA at maximum absorbance wavelength. There was a 107.4% increase in yellow pigment production through the optimization of the initial pH, NaCl, and peptone concentration in contrast to non-optimized conditions (60).

Table VI. Significance of factors and their interactions regarding the production of yellow, orange, and red pigments predicted by FFD

Factors/ Interaction effect	First Fractional Factorial Design			Second Fractional Factorial Design			Full Factorial Design			References
	Yellow	Orange	Red	Yellow	Orange	Red	Yellow	Orange	Red	
pH	*	*	*							(63)
Temperature	*									(63)
Agitation	*	*		*		*			*	(63)
Incubation Period	*	*	*							(63)
Yeast Extract	*	*	*	*	*	*	*	*	*	(63)
Sucrose	*	*	*	*	*	*	*	*	*	(63)
pH-Incubation Period	*	*	*							(63)
Agitation-Yeast Extract	*	*	*				*	*	*	(63)
Agitation-pH	*	*	*	*	*	*				(63)
Agitation-Temperature	*									(63)
Agitation-Sucrose	*	*	*				*	*	*	(63)
Agitation-Incubation Period		*								(63)
pH-Sucrose			*	*		*				(63)
pH-Yeast Extract				*						(63)
Yeast Extract-Sucrose							*			(63)

* Indicates the significance of factors and their interactions

SELF-IMMOBILIZATION BIOMEMBRANE-SURFACE LIQUID CULTURE (SIBSLC)

Self-immobilization biomembrane-surface liquid culture (SIBSLC) is a novel cultivation procedure used to enhance the potential of metabolite production in microorganisms, specifically in fungi. SIBSLC is the cultivation procedure based on the biomembrane-liquid surface culture in which the inoculum is prepared using sterile peanut oil. And as peanut oil is lighter than water, the fungal spores float and germinate on the surface of the liquid broth and form a self-immobilized biomembrane over the surface (65).

This special method of cultivation was developed to increase the production of red pigment in submerged fermentation using monoculture of *Penicillium novae-zelandiae* HSD07B in a shake flask. Previously, this strain was able to secrete red pigment in liquid culture only when co-cultured with *Candida tropicalis,* which requires a complex process of co-culture (47). Hence, this cultivation procedure was devised

to overcome the co-culture issues, thanks to SIBSLC being a simple, cost-effective, efficient, and energy-saving cultivation method in the optimization of fungal growth cultures for fungal cultures (65).

Hailei et al., (2012) reported that red pigment is secreted in the decline phase of growth when the upper cells in the mycelia are unable to get enough nutrients from the media due to the thickening of the biomembrane with time. Hailei et al., 2012 further explained that when *Penicillium novae-zelandiae* HSD07B and *Candida tropicalis* were cultured individually in submerged fermentation with 9.32g/L glucose, glucose limitation occurred only in the culture of *Candida tropicalis* while *Penicillium novae-zelandiae* HSD07B was unable to cause glucose exhaustion, which is the main reason for red pigment production during co-culture. Hence, the pigment was absent when *Penicillium novae-zelandiae* HSD07B was cultured individually as only *Candida tropicalis* causes glucose exhaustion and the subsequent secretion of red pigment during co-culture (65). Furthermore, in SIBLSC, the vertical growth of hyphae caused the thickness of mycelium, due to which the upper cells were unable to get enough glucose that led to glucose exhaustion and, consequently, pigment production as a stress response to nutrient starvation (65).

CONCLUSION

This review paper showed that *Penicillium* can serve as an alternative source to produce safe and non-toxic pigments whose production can be improved through the optimization of their fermentation conditions via different methods including experimentation, fractional factorial design, response surface methodology and self-immobilization biomembrane-surface liquid culture. Furthermore, their upscaling will enable them to be significant commercially in various industries such as food, cosmetics, textile, and pharmaceutical.

Future Recommendations:

This review is focused on optimization in liquid culture due to the large number of publications available on submerged fermentation. However, there is limited information about optimization in solid-state fermentation (SSF), the potential of which could be tested in the future. SSF is more economically valuable thanks to the use of cheap agro-industrial wastes as a substrate for the growth of microorganisms. Further studies should also be conducted on agro-industrial wastes both in liquid state fermentation (LSF) and solid state fermentation (SSF) to develop an efficient, low-cost, and sustainable bioprocess required for the upscaling and the commercial application of pigments.

Of course, optimization alone is not sufficient for industrial application as pigments extraction, characterization, stability under production and processing conditions, safety tests regarding toxicity and mutagenicity, and upscaling are also required. These are the major subjects of today's research on the pigments from *Penicillium.* However, more detailed, and comprehensive research is still required to make them more suitable and reliable food, textile, and cosmetic colorants.

Moreover, knowing the genetic makeup of microorganisms and the metabolic pathways leading to the synthesis of the pigments will enable researchers to manipulate them via metabolic and genetic engineering technology (recombinant DNA technology) to produce pigments on a larger scale.

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