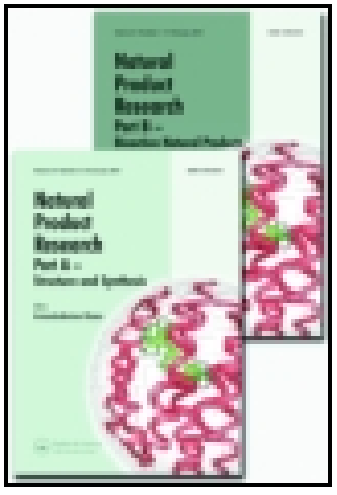

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Investigating the influence of pH, temperature and agitation speed on yellow pigment production by *Penicillium aculeatum* ATCC 10409

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In this study, the combined effect of pH, temperature and agitation speed on yellow pigment production and mycelial growth of *Penicillium aculeatum* ATCC 10409 was investigated in whey media. Different pH levels (5, 6.5 and 8), temperatures (25, 30 and 35°C) and agitation speed levels (100 and 150 rpm) were tested to determine the best conditions to produce a fungal yellow pigment under submerged fermentation. The best production of yellow pigment (1.38 g/L) was obtained with a pH value of 6.5, a temperature of 30°C and an agitation speed of 150 rpm. In contrast, the maximal biomass concentration (11.12 g/L) was obtained at pH value of 8, a temperature of 30°C and an agitation speed of 100 rpm. These results demonstrated that biomass and yellow pigment production were not directly associated. The identification of the structure of unknown *P. aculeatum* yellow pigment was detected using UV absorption spectrum and FT-IR spectroscopy.

Keywords: agitation speed; fungal pigment; *Penicillium aculeatum*; pH; temperature; whey

1. Introduction

Filamentous fungi are readily available raw materials that can be tailored to make microbial cell factories for the production of food-grade pigments because of their chemical and colour versatility in their pigment profile, easier large-scale controlled cultivation, and a long-term history of well-known production strains for the production of a variety of other biochemicals including colorants (Dufosse et al. 2014). Microorganisms belonging to the genus *Aspergillus* and *Penicillium* have also been studied as potential producers of natural pigments. The production of Monascus-like pigments from *Penicillium* strains has recently been reported with a potential use in the food industry, as citrinin production does not occur. These pigments are

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homologues of Monascus pigments with similar chromophore polyketide. Monascorubrin, xanthomonasin A and threonine derivatives of rubropunctatin were identified in the extract of *Penicillium aculeatum* IBT 14263 on YES medium (Mapari et al. 2008).

In pigment production, temperature and pH as a whole activate mechanisms probably related to genetic and metabolic control or of the defence mechanism (Dikshit & Tallapragada 2011). The high concentration and high productivity of pigments might be regulated by the effect of the pH and temperature of the culture medium, as well as the regulation of molecules, such as ATP (adenosine triphosphate), which have an important function in the regulation of metabolic pathways, couple reaction and functional yields at the level of the membrane and cellular wall (Zhou et al. 2009). Therefore, this study represented that pigment production and cellular growth of *P. aculeatum* ATCC 10409 in a submerged culture can be controlled by the temperature, pH and agitation speed of the medium.

2. Results and discussion

2.1. *Combined effect of pH, temperature and agitation speed on yellow pigment production*

P. aculeatum ATCC 10409 produced a yellow pigment under different treatments of initial pH, temperature and agitation in a submerged culture. From day six of incubation, signs of pigment production appeared and continued to accumulate throughout the fermentation period, however, the yield reached its highest level at the 10th day. The highest level of yellow pigment production was obtained with a treatment of pH 6.5, 30°C and 150 rpm. Yellow pigment ranged determined between 0.1 and 1.38 g/L (Figure 1(b)). Our results indicated that pigment production was slightly affected by the initial pH of the medium. For example, Figure 1(b) indicates the results at pH 5, 6.5 and 8 at 30°C and 150 rpm; pH had a significant effect on pigment production. This study divulges the influence of pH on pigment production. The pigment yield varied with changes in pH. At lower pH, consolidation of yellow pigment production was observed.

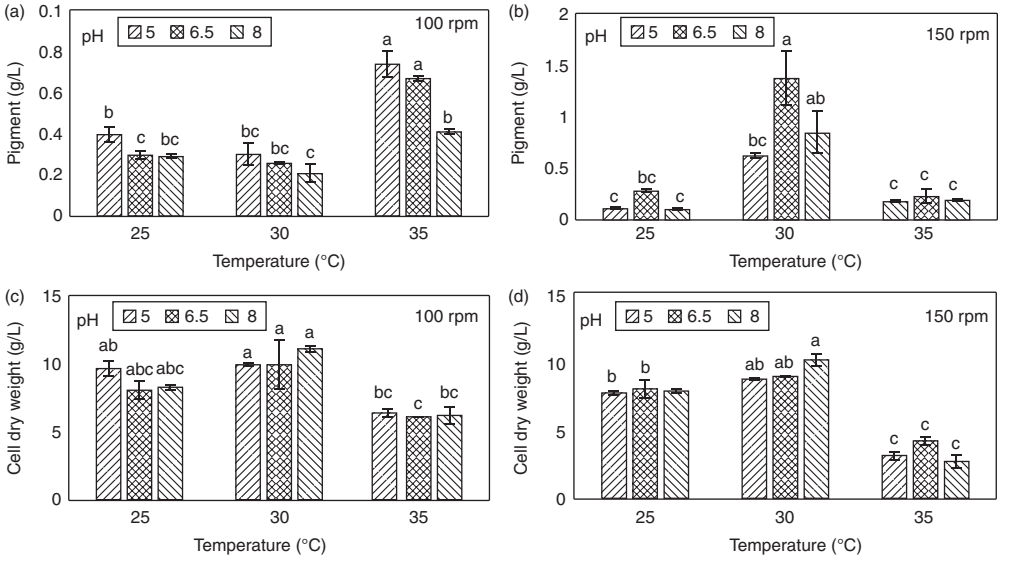

Figure 1. Effect of pH and temperature on pigment production at the agitation speed of 100 rpm (a) and 150 rpm (b) and effect of pH and temperature on cell dry weight at an agitation speed of 100 rpm (c) and 150 rpm (d). Different letters are significantly different according to Tukey's test ($P < 0.05$).

In order to increase the amount of pigment production, it is important to obtain the kinetic parameters. Studying cell behaviour and maintaining the parameters affect the final yield of pigment production and growth. The pH of the culture medium has been reported to play a key role in pigment synthesis. The pH of the culture medium is one of the determining factors for the metabolism and, hence, for the biosynthesis of secondary metabolites. The pH is related to the permeability characteristics of the cell wall and membrane and thus has an effect on either ion uptake or loss to the nutrient medium. The medium pH may affect cell membrane function, cell morphology and structure, the solubility of salts, the ionic state of substrates, the uptake of various nutrients and product biosynthesis. In general, cells can only grow within a certain pH range, and metabolite formation is also often affected by the pH level (Nomila Merlin et al. 2013).

Different pH levels influenced the physiology of fungi, conidial development and pigment synthesis. Reducing the pH inhibits the formation of conidia and increases pigment production, suggesting that the pH of the medium might affect the transport of certain media constituents, such as glucose and nitrogen sources (Lee et al. 2001). The pH can affect the activity of enzymes involved in the biosynthesis of pigments (Pisareva et al. 2005).

Temperature is another important factor as it influences the metabolic activity of fungi and, subsequently, their growth. *P. aculeatum* ATCC 10409 was cultivated under various temperatures for pigment production. Consequently, the optimal temperature for pigment production was found to be 30°C. Similar information had been reported by Gunasekaran and Poornimal (2008).

The fungi usually require long periods for submerged culture, exposing them to contamination risks; this optimal temperature is regarded as favourable for *Penicillium* sp. A temperature of 30°C in combination with acidic pH and high agitation speed favours good production of the pigment. This temperature might be involved in the regulation of enzymatic processes inside the fungal cell. The enzymatic activity (multi-enzyme complex – polyketide synthetase) seems to be optimum for pigment production at 30°C (Figure 1(b)); hence the maximum production of yellow pigment was observed at this temperature. At 25 and 35°C (Figure 1(b)), there was a reduction in pigment yield. Thus, it is observed that temperature plays a pivotal role in cell metabolism and therefore influences pigment yield. Thus, it is observed that temperature plays a pivotal role in cell metabolism and thus influences pigment yield.

The yellow pigment production was studied in various agitation speeds. The level of yellow pigment production varied at different agitation speeds. From the results, it can be concluded that agitation had a significant effect on yellow pigment production (Figure 1(a), (b)).

The obtained experimental data showed a higher yield of pigments ($Y_{P/X}$) for the treatment at pH 6.5, 30°C and 150 rpm. They ranged between 0.013 and 0.155 for T7 and T5, respectively (Table 1(b)).

2.2. *Combined effect of pH, temperature and agitation speeds on growth*

The cultivation was performed as described in the materials and methods section; the samples for analysis were collected after 10 days. The biomass detected through the gravimetric method showed a maximal growth of this microorganism with the treatment of pH 8, 30°C and 100 rpm (Figure 1(c)). The determined dry weight ranged between 2.69 and 11.12 g/L (Figure 1(c), (d)). Figure 1(d) indicates that an inhibition in cellular growth occurs as a result of temperature but not of pH. At conditions of pH 8, 30°C and 100 rpm, biomass production was good and maximal (11.12 g/L); however, yellow pigment production was very low (0.21 g/L). The results showed no relationship between biomass concentration and pigment production. Similar information has been reported by Mendez et al. (2011).

The consumption of lactose as carbon source is shown in Table 1(a), (b). Lactose consumption increased by increasing the temperature from 25 to 30°C and reducing from 30 to 35°C; this was paralleled with changes in growth.

Table 1. Effect of pH and temperature on absorbance $Y_{P/X}$ and $Y_{X/C}$ at an agitation speed of 100 rpm (a) and 150 rpm (b).

			Response (± standard error)[q]				
Trials[r]	T[s] (°C)	pH	Final pH	Absorbance 445 nm	$Y_{P/X}$	$Y_{X/C}$	Consumption of lactose (g/100 mL)[t]
100 rpm (a)							
T1	25	5	8.05 ± 0.012^{a}	$0.446^{b} \pm 0.015$	0.042 ± 0.006^{c}	0.72 ± 0.038^{a}	1.341 ± 0.018^{c}
T2	30	5	8.01 ± 0.032^{a}	0.255 ± 0.005^{c}	0.03 ± 0.006^{b}	0.63 ± 0.017^{ab}	1.576 ± 0.02^{b}
T3	35	5	5.84 ± 0.069^{c}	0.674 ± 0.01^{a}	0.117 ± 0.004^{a}	0.49 ± 0.028^{bc}	1.303 ± 0.007^{c}
T4	25	6.5	8.01 ± 0.078^{a}	0.241 ± 0.083^{c}	0.038 ± 0.006^{b}	0.51 ± 0.052^{abc}	1.59 ± 0.031^{b}
T5	30	6.5	7.77 ± 0.196^{a}	0.221 ± 0.001^{c}	0.028 ± 0.005^{c}	0.56 ± 0.092^{abc}	1.766 ± 0.031^{a}
T6	35	6.5	6.35 ± 0.28^{bc}	0.545 ± 0.024^{ab}	0.11 ± 0.0001^{a}	0.4 ± 0.003^{c}	1.512 ± 0.003^{b}
T7	25	8	8.07 ± 0.118^{a}	0.221 ± 0.001^{c}	0.035 ± 0.0006^{c}	0.55 ± 0.026^{abc}	1.506 ± 0.036^{b}
T8	30	8	8.02 ± 0.035^{a}	0.191 ± 0.013^{c}	0.019 ± 0.004^{c}	0.69 ± 0.038^{ab}	1.624 ± 0.059^{ab}
T9	35	8	6.72 ± 0.169^{b}	0.475 ± 0.041^{b}	0.067 ± 0.008^{b}	0.51 ± 0.043^{abc}	1.215 ± 0.026^{c}
150 rpm (b)							
T1	25	5	8.23 ± 0.092^{ab}	0.059 ± 0.005^{c}	0.014 ± 0.001^{b}	0.51 ± 0.012^{ab}	1.521 ± 0.011^{ab}
T2	30	5	8.02 ± 0.043^{ab}	0.811 ± 0.054^{ab}	0.071 ± 0.004^{b}	0.52 ± 0.007^{ab}	1.668 ± 0.011^{a}
T3	35	5	6.2 ± 0.22^{d}	0.115 ± 0.005^{bc}	0.058 ± 0.008^{b}	0.27 ± 0.035^{c}	1.179 ± 0.055^{c}
T4	25	6.5	8.23 ± 0.191^{ab}	0.333 ± 0.005^{bc}	0.035 ± 0.001^{b}	0.53 ± 0.043^{ab}	1.518 ± 0.007^{ab}
T5	30	6.5	8.15 ± 0.017^{ab}	1.479 ± 0.301^{a}	0.155 ± 0.032^{a}	0.53 ± 0.003^{ab}	1.692 ± 0.026^{a}
T6	35	6.5	7.7 ± 0.104^{bc}	0.374 ± 0.151^{a}	0.057 ± 0.021^{b}	0.38 ± 0.023^{bc}	1.093 ± 0.015^{c}
T7	25	8	8.42 ± 0.015^{a}	0.054 ± 0.004^{c}	0.013 ± 0.001^{b}	0.56 ± 0.029^{a}	1.412 ± 0.04^{b}
T8	30	8	8.17 ± 0.065^{ab}	1.451 ± 0.286^{a}	0.082 ± 0.017^{b}	0.64 ± 0.064^{a}	1.597 ± 0.097^{ab}
T9	35	8	7.23 ± 0.069c	0.093 ± 0.003^{bc}	0.073 ± 0.008^{b}	0.24 ± 0.035^{c}	1.098 ± 0.038^{c}

Note: Values with different letters in the same column are significantly different according to Tukey's test ($P < 0.05$).
[q] SE, $n = 3$ samples.
[r] Data are obtained at the time of maximum pigment production (10 day).
[s] Temperature.
[t] The amount of total lactose in whey was 3.238 g/100 mL.

The efficiency of growth and carbon substrate utilisation by the tested strains was measured by calculating the growth yield coefficients $Y_{X/C}$. They ranged between 0.24 and 0.72 for T9 (Table 1(b)) and T1 (Table 1(a)), respectively. An interesting fact is that there is no correlation between growth yield coefficients ($Y_{X/C}$) and the amount of the pigment synthesised. The trials with equal values of $Y_{X/C}$ indicated different amounts of total pigment. For instance, T5 with $Y_{X/C} = 0.53$ had a total pigment production of 1.38 g/L, while T4 possessed the same value of $Y_{X/C}$ and a pigment production of 0.28 g/L (Table 1(b) and Figure 1(b)). This observation indicates that the pigmentation is not connected with the growth efficiency. Similar information has been reported by Pisareva et al. (2005).

The production of biomass can be related to an oxidative metabolism caused by a significant production of ATP that would accelerate the processes of oxidation leading to the formation of biomass (Mendez et al. 2011).

Experiments were conducted at agitation speeds of 100 and 150 rpm. It is known that the intensive flow of liquid, caused by agitation, forces the air bubbles to disintegrate into a large number of small bubbles. An additional beneficial effect of agitation is to diminish the size of mycelia aggregates; this makes oxygen more easily accessible to the cells (Jafari et al. 2007). Agitation speed increased the amount of dissolved oxygen and dispersion of macromolecules in the medium. It might, therefore, have contributed to the greater growth. However, the shearing effect induced by the higher agitation speed on the cells may contribute negatively towards cell growth. Similar observations were also reported by Lee et al. (2001).

2.3. *FT-IR spectral analysis*

The main absorbance peaks included 3398.3, 2931.6, 1593.1, 1380.9, 1130.2 and 1018.3. The peaks at 3398.3, 1130.2 and 1018.3 suggested a hydroxide bond in molecules. The peak at 2931.6 and 1593.1 indicated that there might be NH groups present. The peak at 2931.6 was moderately sharp, indicating that there were multiple CH_2 groups present. The peaks at 1593.1 and 1380.9 indicate the presence of benzol structure and CH_3 group. The IR spectrum of the yellow pigment was also characterised as fungal ankaflavin (Figure S1).

3. Experimental

3.1. *Strain and culture condition*

P. aculeatum ATCC 10409 (isolated from textile) was procured from the American type culture collection (ATCC). The strain was conserved in a spore's solution at −20°C, and it was inoculated into a 100 mL Erlenmeyer flask containing 20 mL of potato dextrose agar prepared and sterilised by an autoclave at a temperature of 121°C for 15 min, then cooled to room temperature and incubated at 25°C for 7 days.

A concentration of 4×10^7 spores/mL (estimated based on the 0.5 McFarland methods) was inoculated into 500 mL Erlenmeyer flasks with 100 mL of whey media. The whey media was sterilised in an autoclave at a temperature of 121°C for 15 min, then cooled to room temperature and was incubated in the dark at 25, 30 and 35°C with two agitation speeds of 100 and 150 rpm for 240 h. The whey media were adjusted to three different pH levels (5, 6.5 and 8) with 1 M HCl or NaOH, prior to sterilisation.

3.2. *Analytical methods*

After incubation, the entire fermented matter was mixed with 20 mL distilled water and filtered through a Whatman No. 1 filter followed by centrifugation at 3000 *g* (Sigma 3-30K, Osterode, Germany) for 15 min to separate the pigment from spores and other material. pH was determined

potentiometrically (pH/Ion-meter, Metrohm, Herisau, Switzerland) and then 100 mL ethyl acetate was added to an equal volume of supernatant pigment and the mixture was adjusted to pH 3.0 with 2 N HCL under vigorous hand mixing and then allowed to stand for 15 min until two layers were formed. The ethyl acetate layer (upper layer) was obtained by a funnel separating Pyrex pear s/c 100 mL and produced yellow pigment was measured at 445 nm by a spectrophotometer (WPA S2000UV/VIS, Cambridge, UK). The solution was evaporated using a rotary vacuum (Heidolph, Schwabach, Germany) at 50°C. The yellow pigment was collected and dried to a constant weight at 50°C for 48 h (Sopandi & Wardah 2012). Biomass was determined gravimetrically (dry weight) at the final time of incubation (Memmert, Schwabach, Germany) at 50°C for 48 h. Pigment yield coefficient ($Y_{P/X}$) calculated as the ratio of the amount of pigment produced in a certain time (t), into the biomass generated at the same time. Growth yield coefficient ($Y_{X/C}$) defined as the biomass produced per carbon source utilised (g/g) (Pisareva et al. 2005). Residual lactose concentration determined according to the method of Lane-Eynon.

3.3. *Infrared analysis*

An FT-IR spectrum of the pigment was recorded with a SHIMADZU spectrophotometer (SHIMADZU, Tokyo, Japan); spectral range: 400–4000 cm^{-1}; resolution: 0.8 cm^{-1}.

3.4. *Statistical analysis*

All experiments were conducted in a completely randomised factorial design with three replications. The data were compared using the mean Tukey's test ($P < 0.05$) and data analysis was performed using the statistical software, SAS.JMP.Statistical.Discovery. V8.0.1.Update. Only-NULL.

4. Conclusion

This study indicates that yellow pigment production is influenced by physical factors such as temperature, pH and agitation speed of the culture medium. This study showed that the effect of pH on yellow pigment production is most significant compared with temperature and agitation speed. It was possible to produce a water-soluble yellow pigment by *P. aculeatum* ATCC 10409 in a submerged culture using a whey media. Among the evaluated condition, maximum yellow pigment production (1.38 g/L) was obtained with a pH value of 6.5, a temperature of 30°C and an agitation speed of 150 rpm with statistical significance ($P < 0.05$). The combined effect of pH, temperature and agitation speed on yellow pigment production observed may be due to a stress in environmental conditions that favours the biosynthesis of yellow pigment. Results demonstrated that *P. aculeatum* ATCC 10409 is able to use lactose to subsequently produce yellow pigment and whey can be used as a suitable and economical substrate for pigment production.

Supplementary material

Supplementary material relating to this paper is available online, alongside Figures S1.

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