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Thermal and pH stability of yellow and red pigments produced by *Thermomyces* sp and *Penicillium purpurogenum* using response surface methodology

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Abstract
The thermal stability of Yellow and red pigments produced by *Thermomyces* sp and *Penicillium purpurogenum* in submerged fermentation were analyzed. The pigments were evaluated under different temperature and pH conditions in glass bottles viz., 58.8º C to 101ºC and 3.5 to 10.4 respectively. The Central composite design involving the variables pH (X1) and temperature (X2), a response surface methodology for the stability of yellow and red standardized. Samples of yellow and red pigments were collected and submitted to measurement of the absorbance at 410 and 510 nm, respectively. The thermal degradation of the yellow and red pigments followed a first-order kinetic reaction. The response surface models and empirical results described the behavior of the responses of color degradation of the pigments, and the temperature dependence of the degradation constants.

Keywords: natural pigments, penicillium, submerged fermentation, thermomyces thermal stability

1. Introduction
Colour affects every moments of our lives, strongly influencing the clothes we wear, the furnishings in our homes and gardens, and the appeal of foods. Most of our colour choices are unconscious but much research has identified that the colour of our surroundings affects our moods and perception of quality. Colour plays an important role in our enjoyment of life. It is one of the characteristics perceived by the senses and is indispensible for rapid identification and ultimate acceptance of the product [1].
Demand for natural instead of synthetic pigments for colouring fabrics, foods and cosmetics is increasing. Unlike pigments that are synthetic, those from natural sources allow subtle differences in tone because such pigments generally comprise various colour components. Natural dyes and pigments was emerged as an important alternative to potentially harmful synthetic dyes. These natural dyes and pigments were applied in dyeing of cotton, silk and wool samples [2, 3]. However, the main disadvantage of these natural dyes or pigments lies in the order of magnitude of their extraction yield factors (a few grams of pigment per kg of dried raw material).
Microorganisms produce a variety of pigments that includes polyketides, carotenoids, phenazines, acylphenols, pyrones, sclertiorins, anthroquinones but most of these pigments are toxic to humans except for carotenoids and polyketides. There are number of micro-organisms which have the ability to produce pigments in high yields, including species of *Monascus, Paecilomyces, Serratia, Cordyceps, Streptomyces* and yellow-red and blue compounds produced by *Penicillium herquei* and *Penicillium atrovenetum.* Amongst them, many species of fungus have attracted special attention because they have the capability of producing different coloured pigments showing high chemical stability [4]. For industrial applications of microbial pigments, higher production of pigment yield, chemical and light stability are essential features. Isolation of new strain is still of particular interest because of necessity to obtain microorganisms with suitable characteristics for submerged cultivation.
To minimise colour degradation in food and fabrics, the optimisation of pH and temperature parameters is required. Response surface methodology (RSM) may be employed to optimise critical parameters by estimating interactive and quadratic effects. A further benefit of using RSM is the reduction in the number of experiments needed as compared to a full experimental design. The aim of this work was to investigate and to present new data on the effects of temperature and pH on the thermal stability of yellow and red pigments produced by *Thermomyces* sp and *Penicillium purpurogenum* in submerged fermentation, using response surface methodology.

2. Materials and methods
2.1 Microorganisms and culture conditions
The microorganism used in this study is *Thermomyces sp*, and *Penicillium purpurogenum* which was isolated from soil. Stock cultures maintained on Potato dextrose agar slants, which contained potato extract and dextrose and sub cultivated periodically. The slants were incubated at 28 ± 2 0C for 7 days. After cultivation of 5-7 days, spores were collected with 5 mL sterilized water, and the spore suspension corrected was used as inoculums preparation. 0.5 mL of spores suspension was inoculated in 50 mL of submerged culture medium in 250 mL Erlenmeyer flasks, whose ingredients include (g/L) Yeast extract-5, Sucrose - 30, $NaNO_3$ - 3, KCl- 0.5, K_2HPO_4-1, and $MgSO_4$ -1. The submerged culture medium (initial pH 6.0) was cultivated at 28 ± 2 ºC for 6-7 days in a incubator.

2.2 Growth and culture conditions
A loop full of *Thermomyces* sp and *Penicillium purpurogenum* from the PDA slants was inoculated into 10 ml of broth. After 2 or 3 days of growth the inoculum was

transferred to 3 lit and 5 lit flasks containing the Czapek yeast extract broth and Potato dextrose broth. The extracellular pigments that are excreted in the broth after 5 days of growth were harvested by filtration using Whatman No 1 filter paper.

2.3 Pigment extraction

To the filtrate, one volume of 95% (v/v) methanol was added and kept on a rotary shaker for 30 min at 150 rpm at 35°C and was centrifuged at 5000 rpm for 15 min. The same process was repeated for removal of fungal biomass and the filtrate was filtered through a preweighed what man filter paper (47 mm). Next, the absorption spectrum was observed at 410 nm and 520 nm for Thermomyces and Penicillium respectively using spectrometer. The absorbance values were converted to colour units [5].

2.4 Heat treatment

The yellow and red pigments were diluted in citrate phosphate buffers ($C_6H_8O_7$—0.1M citric acid and Na_2HPO_4—0.2 M sodium phosphate dibasic anhydrous) to the desired pH values. The pH Values of the pigment and buffer solutions were measured using pH meter calibrated with pH 4.0 and7.0buffer solutions. The effects of the temperature and pH on the heat degradation of the yellow and red pigments produced by *Thermomyces* sp and *Penicillium purpurogenum* were determined in a glass bottle. The yellow or red pigments were added to 300mL of buffer solution and the initial absorbance adjusted to approximately 1UA 410nm and 1UA 510 nm, respectively, in a Spectrometer. The pigment solution was transferred to a 400 mL glass flask with water circulation at the temperature of the assay. The temperature was controlled by circulating water from a water bath through the glass flask. The flask was maintained at the selected temperatures and periodically agitated with a magnetic stirrer to ensure a uniform temperature throughout the bulk of the sample. The pigment solutions were heated for approximately 120 min, and samples collected after pre-determined time intervals.

The first sample was collected after attaining constant temperature and immediately transferred to an ice water bath. The absorbance was measured in a spectrophotometer.

2.5 Experimental design

The effect of the significant independent variables temperature (pH and heat treatment time) were evaluated in terms of degradation constant (DC) of the yellow and red pigments by response surface methodology (RSM) in a temperature range from 53. 8 to 96.2 1C and pH from 4.08 to 6.91. All the experiments were performed in triplicate.Table1 shows the matrix for the coded and real variables of the RSM. For the calculation, the factors were transformed into non-dimensional factors, varying from −1 to +1. If the distance from the Center of the design space to a factorial point is ± unit for each factor, the distance from the center of the design space to a star point is ±α [6, 7, 8]. The Relation between the coded forms of the input variable and the actual value of the temperature and Ph are described by

Li = Vi−V0 / ΔV.

Where Li is a coded value and VI the actual value of the temperature or pH, V0 the actual value of the same variable at the center point, and ΔV the step change of the variable. For a scientific or engineering investigation concerning a processor system response Y, which depends on the input factors (also called in put variables) X1, X2, X3… XK, the relationship between response and variables

Y= f (X1, X2, X3,.....Kn)+ ε;

The response variables DC were fitted to a second order mathematical model in order to correlate the response variables to the independent variables temperature and pH. From the experimental data for the heat degradation constant (DC) and the half-life (t1/2) as a function of temperature and pH, a second-order polynomial model was obtained

$$Y = \beta_0 + \sum_{i=1}^{2} \beta_i X_i + \sum_{i=1}^{2} \beta_{ii} X_i^2 + \sum_{i} \sum_{j=i+1} \beta_{ij} X_i X_j$$

Where Y is the predicted response; β0 is a constant, βi is the linear coefficient; βii is the quadratic coefficient; βij is the product of the coefficients and Xi and Xj are the independent variables. For each variable, the quadratic models are represented as response surface plots, and the Student's t-test permitted checking of the statistical significance of the regression coefficients. The analysis of variance (ANOVA) was then applied to the statistically significant experimental terms using the Statistica 6.0 software. The response model was expressed using the coded variables, taking into account only these terms.

3. Results

The pH of the medium had a major influence on colouration of the pigment solution inducing sometimes a modification in their structure. In the present study *Thermomyces* sp and *Penicillium Purpurogenum* extract was introduced in to solutions with different pH, greater sensitivity was observed for yellow and red pigments at acidic pH.

3.1 pH and Thermal stability of yellow fungal pigments

There was greater degradation of the yellow pigment when subjected to heat treatments at 101ºC and pH 6.5 and at 95ºC and pH 8.5, indicating that higher pH values increased the degradation of this pigment. Higher pigment stability was observed at low pH values, since the yellow pigment was produced in cultures with a pH of 5.5. These results clearly demonstarted that the degradation constant of the yellow pigment increased as the pH decreased from 5.0 to 3.05. Fig. 1a shows that the response surface had a maximum point. Larger DC values were obtained at higher temperatures and higher pH values (Table 2). The application of response surface methodology resulted in the following empirical relationship between DC as a function of the independent variables temperature and pH, which both had a significant effect on the heat stability of the yellow pigment. The most significant parameter was clearly the pH (X2) followed by its quadratic effect (X2 2). The linear (X1) and quadratic (X12) effects of temperature were significant but less important.

3.2 pH and Thermal stability of red fungal pigments

The behavior of the red pigment was the contrary of that observed for the yellow one. Higher pH values reduced color degradation, whereas lower pH values increased it. The degradation constant for the degradation of the red pigment increased from 0.013 to 0.62 as the pH decreased from 8.0 to 3.5 at 80ºC. In general, the yellow pigment

presented high resistance to low pH values whereas the red pigment is more resistant to high pH values.

The fit of the model was also expressed by the coefficient of determination R^2, which was found to be 0.9545, indicating that 95.45% of the variability in the response could be explained by the model. The closer the R^2 value is 1, the better the model is fit to experimental data, the less is the distance between the predicted and the observed values (Table 3 and 4). Three-dimensional graphs were generated for the pair-wise combination of the two factors, while keeping the other two at their center point levels. Graphs are given here to highlight the roles played by various factors (Fig.1 b).

4. Discussion

The thermal degradation of the yellow and red pigments followed a first-order kinetic reaction. The response surface models and empirical results described the behavior of the responses of color degradation of the pigments, and the temperature dependence of the degradation constants.

Stability of natural colourants towards formulations, different processing conditions, additives, pH, uniformity in distribution in products and availability of raw materials, cost economics of preparation, *etc*., are the major factors affecting the selection and application of natural food colours in processed food products [9].

Thermal treatment is one of the most important methods of food preservation. However, excessive heating produces considerable loss in the quality and particularly in the sensory properties of foods [10]. During processing, deterioration reactions contribute to the formation of brown pigments, which is undesirable with respect to the color, flavor and market value [11, 12].

The monacolin K produced in Monascus-fermented products was stable over a pH range from 3 to 9.However, as the pH increased to 11, so the content of monacolin K decreased rapidly and thus the authors concluded that monacolin K was more stable at lower temperatures, shorter times and lower pH values, such as the pasteurization of acidic food products [12]. For other natural pigments, The t1/2 values b for the degradation of anthocyanins were 54.3, 22.5 and 8.1 h in sour cherry juice at 60, 70 and 80 1C, respectively [13].

The same results was obtained by Response surface methodology and the empirical results obtained described the behaviour of the responses DC and $t^{1/2}$ of the pigments produced by Monascus ruber CCT 3802 in submerged fermentation, indicating that the kinetic equations obtained for the color variables represented the color changes in the monascus pigments well, and could therefore be used to describe color degradation during heat processing and storage [14].

In this study, the thermal stabilities of the yellow and red pigments produced by *Thermomyces* sp and *P.purpurogenum* in submerged fermentation were estimated. The heat treatment at different temperatures and pH values resulted in significant color degradation. The pigments showed different behaviors to variations in the temperature and pH value. The heat degradation constants of the red pigment decreased with increasing pH and temperature, indicating that the red color was retained at neutral pH values, and the yellow pigment showed greater stability in the lower pH and high temperature range

Table 1: Experimental range and levels of the independent variables

Factors	Coded symbols	Coded levels				
		-α	-1	0	+1	α
Temperature	T	58.8	65	80	95	101
pH	P	3.5	5.0	6.5	8.0	10.4

Table 2: Heat degradation constants (DC) for different heat treatments of the natural pigments produced by Yellow and red pigments by Thermomyces and Penicillium in submerged fermentation.

Pigment	Coded variable		Real variable		Response of yellow pigments		Response of red pigments	
					Observed	Predicted	Observed	Predicted
1	-1	-1	58.8	5.0	0.015	0.033	0.013	0.029
2	+1	-1	95	8.0	0.047	0.042	0.073	0.043
3	-1	+1	65	8.0	0.061	0.063	0.25	0.29
4	+1	+1	95	8.0	0.48	0.57	0.53	0.52
5	-1.41	0	58.8	6.5	0.047	0.042	0.047	0.01
6	+1.41	0	101	6.5	0.85	0.80	0.62	0.6
7	0	-1.41	80	3.5	0.25	0.17	0.07	0.08
8	0	+1.41	80	10.4	0.14	0.042	0.16	0.19
9	0	0	80	6.5	0.57	0.55	0.048	0.037
10	0	0	80	6.5	0.042	0.042	0.036	0.037
11	0	0	80	6.5	0.051	0.042	0.031	0.037
12	0	0	80	6.5	0.071	0.16	0.034	0.037
13	0	0	80	6.5	0.024	0.042	0.035	0.037

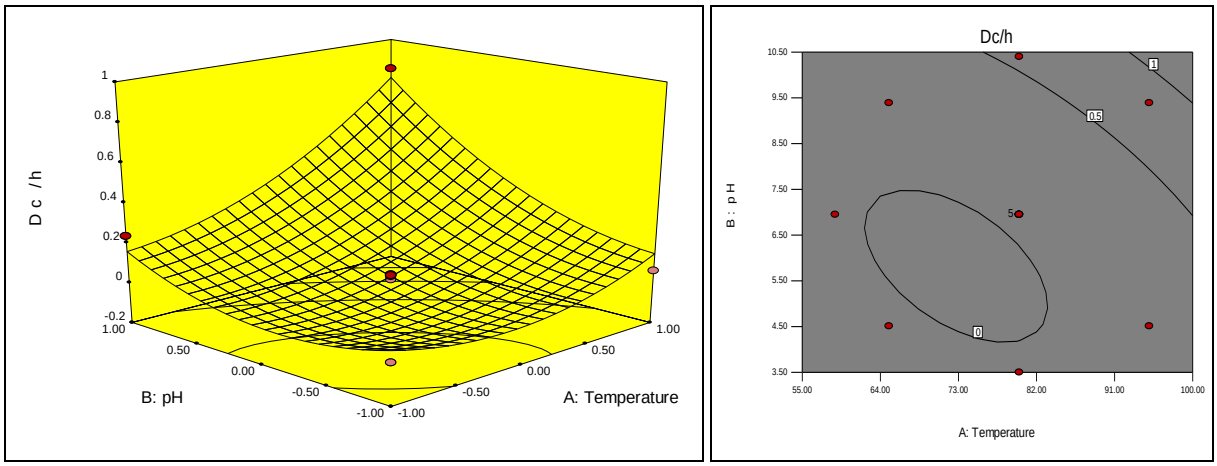
Table 3: Estimates of the effects of temperature and pH and their interactions on the heat degradation constants of the Yellow and red pigments produced by Thermomyces and Penicillium

Factor	Regression Co efficient	Standard error	P value
Yellow pigment			
Intercept	0.042	0.033	< 0.0001
A-Temp	0.18	0.026	<0.0001
B-pH	0.19	0.026	0.0002

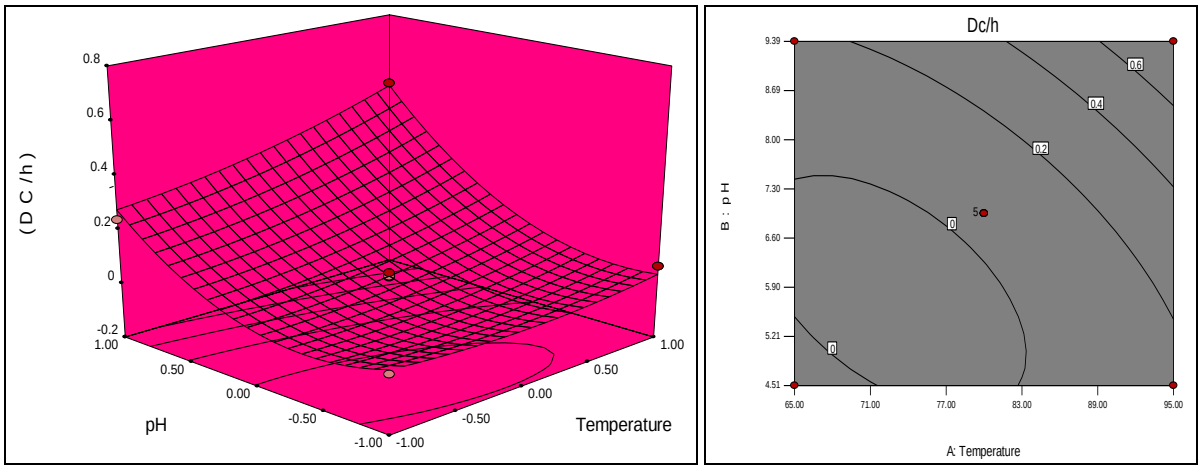
AB	0.13	0.037	0.0089
A^2	0.12	0.028	0.0032
B^2	0.13	0.028	0.0023
Red pigment			
Intercept	0.037	0.012	< 0.0001
A-Temp	0.063	9.863E -003	<0.0004
B-pH	0.18	9.863E-003	0.0001
AB	0.056	0.014	0.0051
A^2	0.031	0.011	< 0.021
B^2	0.15	0.011	<0.0001

Table 4: Regression coefficient for thermal stability of Yellow and red pigments produced by Thermomyces and Penicillium

Source	Sum of Squares	Degree of Freedom	Mean Square	*F*-value	Significance-*F*
Yellow pigment					
Regression	0.82	5	0.16	29.38	<0.0001
Residual	0.039	7	5.56E-003	-	-
Total Model	0.86	12	-	-	-
R^2 = 0.9545, adjusted R^2 = 0.922					
Red pigment					
Regression	0.48	5	0.096	122.83	<0.0001
Residual	5.49E -008	7	7.783E-004	-	-
Total Model	0.48	12	-	-	-
R^2 = 0.9887, adjusted R^2 = 0.9807					



(a) Yellow pigment



(b) Red pigment

Fig 1: Response surfaces illustrating the effect of temperature and pH on the DC of the natural pigments produced by *Thermomyces* sp and *Penicillium purpurogenum* in submerged fermentation (a) yellow pigment and (b) red pigment

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