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Enhancement of Red Pigment Production by *Monascus purpureus* MTCC 410 under submerged Fermentation by Regulating RSM coefficient through Plackett Burman Analysis

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ABSTRACT: In an attempt was initiated to explore red pigment production of *Monascus purpureus* strain MTCC 410, employing potato powder as a carbon source with combinations of different nitrogen sources. The physical and nutritional parameters were optimized under submerged condition utilizing Plackett Burman analysis to determine the limiting parameters which affect mainly the formation of red pigment of *Monascus purpureus*. The present study illustrated that among all the parameters three most important parameters were selected temperature, potato powder and yeast extract. The optimum concentrations of nutritional parameters found to be potato powder (8.5 g %), yeast extract (2.75 g %) and the important physical parameter was selected at 30°C temperature using response surface central composite design. This computer assisted formulation of media optimization showing that *Monascus* pigments can be utilized as biocolorant in food and textile industry by submerged fermentation using potato powder-a low cost agro industrial material as carbon source.

Keywords: *Monascus purpureus*, submerged fermentation, polyketide, optimization, biocolorant.

INTRODUCTION

Biocolorants are used as food additives from ancient times to enhance the acceptability of pigments in food, extracted from different biological sources like vegetables, fruits, leaves, microorganisms etc. Generally chemical colours are harmful in nature because of their carcinogenic and teratogenic properties, so nowadays peoples are getting inclined towards natural colorants (Jung *et al.*, 2003). Microbial pigments, which are mostly made from agro-based product, have recently drawn a lot of attention because of their numerous therapeutic characteristics, nutritional impacts, and controllable and predictable yield (Li *et al.*, 2023). Microbial food colorant gain a favorable acceptance to other color supplements extracted from plants or animals, because they can be produced in good amount hence less production cost. Now a days, microorganisms like bacteria and fungi specially filamentous fungi gain a wide acceptance as an alternative options of naturally found colorants (Ghoochani *et al.*, 2015; Zheng *et al.*, 2023).

The filamentous fungi are adapted industrially for the fermentative production of several compounds also the biocolorant for their use in cosmetics, foods and medicines. Among the fermentative production of pigments, one of the most promising pigment from *Monascus*, that has been used widely as food colorant in eastern centuries. Traditionally the mold *Monascus* is grown on rice, formed red rice or ang-kak and consumed with added nutrient value. Anka or red rice possesses different kinds of therapeutic benefits like antioxidative, anticarcinogenic, antimutagenic, hypolipidemic activities (Yanli & Xiang 2020; Lai *et al.*, 2021). Pigments possess vast array of biological pursuit possess like anticancer and antimutagenic properties (Su *et al.*, 2003), potential antiobesity properties (Lai *et al.*, 2021), antimicrobial properties (Zhang *et al.*, 2021), anti-inflammation properties (Wu *et al.*, 2021). The reported documents of *Monascus* has described the different uses of its color in verities food items like fish, hams, sausage, tomato ketchup etc (Jung *et al.*, 2005). Although *Monascus* is capable to grow in different solid and liquid media but rice is common natural substrate for pigment production. *Monascus* pigment consist of a combination of six important polyketide pigments; two red pigments (monascorubramine and rubropunctamine), two orange pigments (monascorubrin and rubropunctatin) and two yellow pigments (monascin and ankaflavin) (Huang *et al.*, 2023). Few important features of pigments are generally they formed in the state of cell-bound condition, less solubility in water and light and heat sensitive in nature (He *et al.*, 2022). The culture substrates of *Monascus* sp. started to appear as red color because of the production of the pigments rubropunctamine and monascorubramine that are azoto analogues in orange pigments, the orange pigments monascorubrin and rubropunctatin containing different side chains with in ozolactone ring chemically modified and produced red pigments, therefore the rubropunctatin and monascorubrin are produced from acetyl Co-A by enzyme polyketide synthase in cytosol (Ali *et al*., 2022). The other two kinds of yellow pigments monascin and ankaflavin are reduced form of orange pigments (Zheng *et al.*, 2009). Apart from above described six well known pigments, so many different kinds of pigments within *Monascus* sp. are still unexplored (Jůzlová *et al.,* 1996). Among them few are identified and marked as ankalactone, monasfluore, rubrupunctin, monascolidone, monascopyridine, monaspurpurone, monascusone, monarubrin etc. (Loret & Morel 2010).

Apart from pigments, the fermented red rice is also important for different metabolites like monacolin K and L (antihypercholesterolemic agents), Gama-aminobutyric acid (a neurotransmitter), citrinin (a mycotoxin) are found (Su *et al*. 2003). Monacolin K, functions as an effective competitive inhibitor against HMG-CoA reductase, commonly known as a cholesterol-reducing drug (Lin *et al.,* 2023). Monacolin K is a common drug used for medicine of hypercholesterolemia symptom (Chen & Johns 1993). During the fungal cultivation, apart from secondary metabolites ethanol, carbon dioxide and acetate are also produced (Rosenblitt *et al.,* 2000). *Monascus* pigments maintain it's stability nearly temperature up to 60°C and in pH levels low acidic to basic range (Carvalho *et al*., 2005).

Traditionally in oriental countries *Monascus* was grown on rice to prepare anka or red colored rice, though different kinds of solid or submerged media have been applied to produce colour. Previously solid-state fermentation (SSF) was used as a conventional process of fermentation but nowadays most of the industry used submerged or liquid fermentation for industrial fermentative processes (Lin & Demain 1993). Though it is possible in modification on cultural conditions in *Monascus* sp. positively have an effect on pigment constituents (Hajjaj *et al.,* 2012). This study emphasizes the formation of red color from *M. purpureus* in liquid fermentation by chemically defined media.

Maximum reports on pigment production was done by using conventional method of one variable at a time basis which have facing many disadvantages such as, this method cannot determine optimal parameters and failed to show the interaction between them (Filer *et al.,* 2000). To overcome this problem Response Surface Methodology can be utilized to select parameters. This process has some advantages over conventional methods. The conventional method changes only single independent factor at a time while remaining variables kept in a constant position. This technique determines the optimal parameters interplay within the nutrients at different concentrations and also limited the experiment numbers (Ravuri & Shivakumar 2020). RSM is a mathematical method used in statistical analysis to calculate the ideal values of significant process variables and to ascertain how various variables affect the desired outcomes. The construction of models and the solution of multivariate equations both use quantitative data from the planned experiments. Numerous applications of the statistical method can be found in the fields of drug formulation and development as well as in the enhancement of current product designs. Central composite design (CCD), which is based on a second-order polynomial equation, is used in response surface approach.

Process factors and the impact of test variables on the response are described by the second-order equation (Haji & Rahimi 2022). RSM is frequently employed in a variety of fermentation processes to improve the production of enzymes with industrial importance. In this study initially we employed AVOT approach for selection of different parameters for red pigment estimation. Next approach was employed in our current study where PB analysis designed for screening the initial components of *Monascus purpureus* for red pigment production. Next further level of optimization was carried out by response surface methodology to understand significances to form the product by using different combination of ingredients to identify significant physical and nutritional variables which influence the pigment estimation of *M. purpureus* MTCC 410 in liquid fermentation.

MATERIAL AND METHODS

Microorganisms and media. The organism *Monascus purpureus* (MTCC 410) is exploited as experimental microorganism and it is routinely maintained in PDA medium at 4°C in Refrigerator. Malt extract/yeast extract (MY) medium is used as culture medium. MY media contained (g/l): glucose 10.0, yeast extract 3.0, malt extract 5.0 and peptone 5.0; pH 7.0. Fermentation medium is composed of different carbon sources and nitrogen sources (were given below) with NaCl as salt solution (Soccol & Vandenberghe 2003).

Inoculum preparation. *M. purpureus* strain was transferred from stock culture to PDA slant and allow for incubation at 30±2° C for 6-7 days. After 7 days the spores were collected with sterile medium consisting 0.9 % NaCl and 0.2 % tween 80 (Singh *et al.,* 2015). One ml of spore suspension (5×10^5 CFU/ml) was taken from it and allowed to inoculate the suspension (20 ml MY broth) and allow to incubated at 30±2° C for 4 days for seed preparation. Prepared seed was poured to 100 ml of fermentation medium and allow incubating at 20-40° C up to 16 days in a rotary shaker at 120 rpm.

Selection of agro based raw material for red pigment production. Different complex growth substrates (potato powder, sugarcane bagasse, grape waste, rice hulls and potato peels) were collected from local market. The collected raw material were sliced into big portions (0.5 cm) and then dried into oven at 70ºC. Finally, completion of this step they milled with in mixer grinder into small pieces to make fine powder (2 mm). They were tested individually containing 1 % peptone into the culture medium for estimation of red pigment production.

Measurement of extracellular red pigment. In submerged fermentation extracellular red pigment yield estimation was measured using cultural filtrate. Mycelium was isolated from the broth by membrane filter (0.8 mm pore size). Filtrate was allowed to centrifuge at 10000×g for 15 minutes. After collection of supernatant the broth was estimated at 500 nm with UV-VIS spectrophotometer of relative absorbency (Shimadzu, Model No. UV-2101 PC) using uninoculated specimen considering as colorimetric blank sample. The absorbance values were transformed into pigment concentration (unit of absorbance UA) was measured by multiplied with absorbance value with specific dilution factor (Ravuri & Shivakumar 2020).

Optimization of physical and nutritional parameters of red pigment production from *M. purpureus*:

Determination of variables using one factor at a time process in submerged fermentation. The red pigment production of *M. purpureus* MTCC 410 was optimized under submerged fermentation condition using three consequential step of statistical strategy (OVAT, PBD, and CCD). Typically, OVAT (One variable at a time) technique was used to look for the important physicochemical factors that could have some sequential effect on red pigment yield of *Monascus* sp. All agro- industrial based raw materials (i.e., potato powder, sugarcane bagasse, grape waste, rice hulls and potato peels) were added individually with 1 % peptone (nitrogen source) in 250 mL Erlenmeyer flask of fermentation medium. Prepared seed was poured to 100 ml of fermentation medium. In order to determine the involvement of various physical parameters like pH affects on formation of the red pigment, previously mentioned substrates were combined with a modified fermentation medium that was adjusted to various pH levels (ranging from pH 4.0 to 9.0) in 250 ml Erlenmeyer flasks. By varying the incubation period (ranging from 4.0-16.0) and temperature (ranging from 20°C - 40° C) of the fermentation media, the impact of the initial incubation period and temperature on the formation of red pigment was examined. To explore the role of nutritional parameters as inducers of the synthesis of red pigment, the concentration of carbon sources (1 g, %- 16 g,%) and nitrogen sources (0.5-5.0%) and were measured in the cultures.

Selection and identification of different significant variables by using the process of Plackett-Burman (PB). This present study was experimented by using nine sets of experiments was assigned by using Plackett–Burman analysis in Design expert (version 13) software (Stat-Ease Corporation, USA). Our current study was emphasis to identify the crucial factors for optimum red pigment production based on two factorial design and screen on n variables number in *n*+ 1 number of experiments (Plackett & Burman 1946). Total nine variables were selected for this experiment were designated as A: temperature, B: fermentation days, C: pH, D: potato powder concentration (%, g), E: Tryptophan concentration (%, g), F: Peptone concentration (%, g), G: Yeast extract (%,g), H: Monosodium glutamate (%, g), I: NaCl concentration (%, g) under submerged fermentation (Table 1). All the experimental works were carried out in triplicate and pigment production was measured as mean values of these triplicate values. The following equation reveals the effect of individual parameter on red pigment yield:

$$E(Xi)=2(\sum M^{+}-\sum M^{-})/N \qquad (1)$$

Here E indicating outcome of input variables, M^{+} and M^{-} representing the effect of red pigment estimation of experiments where different parameters were considering at highest and lowest values, accordingly and N is considering the numeral of experimental assessments.

ANOVA calculated to proof significances of experimental model. Pareto chart was prepared to find out the positive and negative effects of different factors and the factors which show the maximum positive effects were calculated for optimizing the red pigment production by using Central Composite Design (CCD) of Response Surface Methodology.

Optimization of pigment production using response surface methodology (RSM):

Response surface central composite design (CCD) was used to analyze and optimize the levels of the significant parameters as shown by the Plackett-Burman experiment and their interaction effects that may considerably affect the synthesis of red pigment. RSM works well for small numbers of variables (up to five), but it is impracticable for large numbers of variables because it requires a lot of experimental runs.

Current study was done by Central composite design (CCD) of RSM was created by the software Design expert (version 13, Statease Inc., Minneapolis, USA). Response surface data were analyzed by using the design expert software for construction of 3D graphs defining optimal parameters and connection within them. The significant level of three major variables temperature, potato powder and yeast extract were chosen. The mean values of red pigment yield capability (OD U/ml) were estimated by three experimental values by 20 numbers of different experimental runs. The second-degree polynomial equation was used for estimation of red pigment yield by using multiple regression technique.

The quadratic model equation is shown below as:

$$Y= \delta_0 + \Sigma\ \delta_i V_i + \Sigma\ \delta_{ii} V_{i2} + \Sigma\ \delta_{ij} V_i V_j \qquad (2)$$

Here, Y denotes anticipated response or red pigment yield (U/ml), δ_0, δi, δ_{ii}, δ_{ij} are fixed term, linear coefficients, squared coefficients and interaction coefficients. Utilizing the coefficient of determination R^2, the polynomial model equation's degree of fitting was assessed. Equation (2) was used for construction of three dimensional plots.

Validation experiments and data analysis: The Design expert software used for optimizing pigment production by utilizing different numerical option. The criteria for each dependent and independent variables were established, with the response being modified to the maximum level and the independent factors being taken into account within the experimental set up's range. Predicted values of independent variables and maximum amount of red pigment yield served as the solution. Following all experiments of the work was carried out in triplicate and mean were calculated from the triplicate values. Design Expert Version 13.0 (Stat-Ease Inc., Minneapolis, Minnesota, USA) software was applied to analyze and interpret all experimental design's outcomes.

RESULT AND DISCUSSION

This study was aimed to optimize extracellular red pigment production by *Monascus purpureus* by submerged fermentation. The pigment production capability of *Monascus purpureus* rest mainly on the physical and nutritional parameters and their appropriate concentration. The nutritional parameters carbon and nitrogen concentration was majorly take part in vital bit pat in production of red pigment of *Monascus purpureus*.

Selection of significant parameters using one factor at a time (AVOT) analysis: Temperature usually plays an important function in cell division and secondary metabolites production. The graphical representation of effect on different temperature (ranging from 20° C to 40°C) on red pigment production is presented in Figure 3 a. In this experimental condition variation in temperature affected the yield of red pigment production. The experimental result was found that red pigment production from *M. purpureus* reduced significantly at below 20°C and above 40°C. This is because the adverse temperature interfere the reaction of biochemical process and the different enzyme activity on biochemical reaction of pigment synthesis. In this experiment maximum red pigmentation was observed at room temperature (28°C - 32°C). It is found experimentally that the red pigment production was enhanced maximally at 30° C temperature (Fig. 1a). Different experimental reports found on effect of temperature on red pigment yield of *Monascus* sp. like *M. purpureus* can grow best at a temperature between 28° C - 32° C when fed on broken rice, wheat flour and different other grains (Dikshit & Tallapragada 2011). An optimal condition on 8-day incubation period, 30°C and pH 6.0 in the medium including rice and cassava bagasse found that *Monascus* sp. thrived best at pigment production (Carvalho *et al.,* 2007). 30°C temperature during incubation period showed the stimulation of most pigment formation, with substantial variances from other temperatures in pigment formation in *M. purpureus* (Nimnoi & Lumyong 2011). The best optimized pigment production of *M. purpureus* MTCC 369 was found at 32° C temperature under solid substrate fermentation (Sehrawat *et al.,* 2017). Our current experimental result was corroborated with previous work done on pigment production of *M. purpureus*.

Experimentally, the fermentation was carried for different time period up to 16th day and assessed and accordingly the pigment production of *Monascus purpureus* was measured. Since, 4th day of fermentation, sign of pigment production started and continued to accumulate in the fermentation broth throughout the fermentation period but the yield reached to its maximum level at 14th day (Fig. 3b). The measurement of pigment estimation was showed 5.60 U/ml on 14th day. Chatterjee *et al*. (2009) reported that *M. purpureus* become reddish within 3 days of incubation and started to diffuse the pigment there after, mature mycelia and aerial conidia are also visualized under microscope.

The pH of the fermentation medium has been reported to perform a major function in the conidial development and production of pigments (Orozco & Kilikian 2008) *Monascus* was grown at various pH values (4·0 – 9.0) in submerged conditions in shaking condition to find out the efficacy of pH on fermentation. The quality and quantity of pigment notably varied with the change of pH. In this study (Fig. 3 c) indicated that maximum red pigment was observed at pH 8.0 (6.63 U/ml). The results showed that maximum red pigment yield were observed at neutral to alkaline pH. The variation of pH of the fermentation medium from pH 4.0 to pH 9.0 influenced red pigment yield but the best red pigment production was found at alkaline condition in pH 8.

Among the nutritional parameters, carbon plays a very crucial role in secondary metabolites production. Therefore to determine the suitable carbon source for the production of red pigment, *Monascus* was cultivated in the fermentation medium containing various agro based carbon compounds (potato powder, sugarcane bagasse, grape waste, rice hulls and potato peels). Within these five carbon sources potato powder was showed higher pigment yield (Fig: 2). The maximum pigment red production was achieved at 8 g, % (6.26 U/ml) (Fig. 2 a), above this concentration the pigment production was decreased, perhaps due to respirofermentative metabolism (Lin & Demain 1991; Chen & Johns 1993).

Monascus sp. is able to grow in both organic and inorganic nitrogen sources. However in this experimental approach it is found that all the organic and inorganic nitrogen sources have positive effect on red pigment production of *M. purpureus*. Maximum effect was found on yeast extract (Fig. 4b) but other nitrogenous compounds like peptone, tryptone and monosodium glutamate affect on red pigment production. The 1 g, % concentration of yeast extract showed maximum pigment yield (5.73 U/ml) (Fig. 4c).

Significant parameters selection by Plackett Burman analysis: The goal of the current study was to use Plackett-Burman design to screen the significant medium components with regard to their primary effects. By screening n variables in n + 1 tests, this two-factorial experimental design was employed to determine the crucial factors needed for the best red pigment production. Plackett Burman analysis significantly plays an impact on every variable which is considered under investigation to the experimental research. The Plackett Burman analysis helps to differentiate the variables that effect favorably and unfavorably on red pigment yield. The parameters which chosen for the current study previously selected on our previous done lab work where different physical and nutritional sources were investigated according to their impact on pigment production by *M. purpureus* applying one variable at a time (OVAT) technique also selected to future investigation (PB analysis). The bigger data set in PB analysis is required to investigate high quality of information on the importance of every real parameter. To formulate the maximum red pigment estimation of *M. purpureus* during broth fermentation, a statistical technique has been employed to investigate the most prominent physical and chemical components. Therefore, different variables were investigated according to the previous reports available in various literature then Plackett Burman analysis was done to select the fruitful variables among them (Table 1). Generally, to carry out any fermentation technique, initially screening of different parameters should be done to recognize their impact and effect on product formulation. Then better components are chosen for further optimization of the product. PB analysis was employed to investigate nine individual parameters of physical (temperature, fermentation days, pH) including different nutritional parameters with standard NaCl solution in 12 run experiment. The experimental analysis of Plackett–Burman in pigment formation, all the components like the effect, standard error, t(xi), p and confidence level were summarized in the Tables 3. Pareto chart (Fig. 1) graphically arranged the outcome of various variables on yield. The column length significantly represented the outcome of various parameters on the production.

Here circumstances for *Monascus* fermentation are mostly vary from species to species, but ideal temperature was confined within the value between 28–32° C in colour formation of majority of the strain (Lin *et al.,* 1992); (Carvalho *et al.,* 2005). Temperature usually takes part in a crucial role in cell division and synthesis. In this experiment variant range of temperatures did affect much to the red pigment yield (de Carvalho *et al.,* 2005) observed a change in absorbance of pigment production with temperature (Dikshit & Tallapragada 2013) explained the polyketide synthase enzymatic activity appeared optimum effect on red pigment yield on *M. purpureus* between 32°C - 35°C. In this study also consequent significant effect of temperature was found in red pigment production.

Carbon takes part in a very important character in cell function. It also affects growth and pigment yield of the cell. So to determine the best source of carbon in red pigment formation, *Monascus* was grown on the submerged medium including different carbon compounds of agro-industrial substances like potato powder, sugarcane bagasse, grape waste, rice hulls and potato pills in one variable at a time approach. Within five different carbon sources potato powder showed relatively highest pigment yield therefore potato powder was selected as a parameter in Plackett-Burman analysis. Potato powder act as a source of energy during the formation of secondary metabolites. Utilization and concentration of carbon sources for pigment production of *Monascus* is varying from species to species and reports are also found (Lin and Demain 1993; Yoshimura *et al.,* 1975).

Monascus sp. is capable to utilize both the organic and inorganic nitrogen compounds. Nitrogen source affected both the growth of the organism and also pigment production (Chen and Johns 1993). Babitha *et al.* (2007) showed that nitrogenous compounds carried a influensive effect as additive on pigment yield of *Monascus* sp. It is observed that organic nitrogenous compound support positively comparing to inorganic nitrogenous compound except monosodium glutamate (MSG), for red pigment production. Various experimental reports showed that addition of inorganic substances like MSG appeared best for optimum red pigment production (de Carvalho *et al.,* 2008). But it our study we found yeast extract was showed maximum red pigment production and considered for further experiments as organic nitrogen source. The red pigment production of *M. purpureus* also vary of the culture conditions and quantity of nutritional parameters of the media and their proportion also play very important (Carels, M., & Shepherd; Wong *et al.,* , 1981). However in this study it is found that presence of yeast extract is found best for fungal red pigment production. The formation of red pigment by *Monascus purpureus* in submerged culture was examined (Sharmila *et al.,* 2013) utilizing potato powder as a cheap source of carbon. In this case, RSM was used to optimise the concentrations of potato powder, K_2HPO_4, $ZnSO_4$, $7H_2O$, and monosodium glutamate (MSG). The outcomes demonstrated that the ideal concentrations of potato powder, K_2HPO_4, $ZnSO_4$, $7H_2O$, and MSG were 2.50% (w/v), 0.480% (w/v), 0.0013% (w/v), and 0.60% (w/v), respectively. The results also demonstrated that the maximal pigment production was 7.18 ODU/ml.

Among the all parameters, only three parameters were adversely influence the production, apart from that all the other variables have positive effect on red pigment production. The output received from Pareto chart which significantly analyzed by ANOVA calculation (Table 2). The significant model was established with the data $p < 0.05$. From the major three influential parameters three (temperature, carbon source and nitrogen source) were selected for further study to optimize their concentration requirement and interaction effect to each other. Plackett–Burman experiment was only used for screening of significant parameters but fail to determine the degree of significances of the parameters. Finally, three parameters were chosen for further experiment to maximize the red pigment production yield by RSM technique.

Effect of significant variables by Response surface methodology: Three positive influencing important variables were used in CCD technique to estimate the degree of effect within them and concentration of optimal levels. The present work was done by multiple regression analysis to determine a quadratic model. To analysis this model, different combinations of these variables twenty experimental run was conducted. The central composite model with observed values of every experimental run is observed in the Table 4. A prognosticative quadratic polynomial equation was summarized and ANOVA result was calculated in estimation of secondary metabolites yield.

Pigment yield (OD/ml) = $+8.62 + (0.2938*A) + (0.5346*B) + (0.5858*C) - (0.1433*A*B) + (0.1433*A*C) + (0.1433*B*C) - (1.10*A^2) - (0.4184*B^2) - (0.5132*C^2)$ (3)

A= Temperature (°C), B= Glucose (g %) and C= Yeast extract (g %).

The second-order model equation's statistical significance was assessed by F-test ANOVA analysis was shown in the Table 4. It showed that the regression highly significant statistically for the estimation of pigment yield in *M. purpureus*. F-value of this chart showing that 7.57 which indicate that the statistically signification of the model. The bigger mode of F-value indicates there is noise and the chances indicate only 0.20%. The P-value below the number 0.0500 detect that the model is significant. Therefore, in our current study all the parameters are showing statistically significant values (Table 4). Negligible lack of fit is advantageous for the model's fitness. Here 0.35 F-value indicating the lack of fit is non- significant in comparison with pure error. Higher coherence between the noticed values and anticipated values is determined by closer determination coefficient value (R^2 value=0.8720) to 1. The Predicted R^2 is 0.6042, which having truthful assignment with Adjusted R^2 of 0.7567; i.e. the differentiation between them is less than 0.2. Adeq Precision value is used to signal to noise ratio. The value higher than 4 is required. Our experimental ratio of 7.543 indicates proper signal. So, all the interpretation indicating that the model can be used to navigate the design.

CV value or coefficient of variation designates intensity of precision with comparison to the experiment. The more value of CV is the indication of the more ratability of the experiment. The experiment by lower value of CV % (9.32) indicates that the experiment was fruitfully conducted.

Red pigment yield of effect of interaction parameters were observed through changing the intensity of two independent parameters while kept the other independent parameter in constant. Two and three dimensional response surface graphs were used for prediction of the optimal range of all test parameters. Thereafter, three different contour plots were drawn by mixing all the probable combinations. The interaction between temperature and potato powder on pigment yield that shows both the parameters on their initial range having no valuable effect but enhancement of temperature and potato powder concentration while in much higher levels both shows decreasing in the red pigment yield. Both temperature plus potato powder at optimum range showed positive impact on red pigment yield (Fig. 6). Higher temperature range and lower yeast extract concentration reveal that maximum red pigment yield but both maximum temperature plus yeast extract reveal turn town the pigment yield. So, both the variables on the optimum range were found promising on pigment production. The interconnections between potato powder along with yeast extract concentration (Fig. 5) also take part in a principal role in pigment estimation. The increased potato powder concentration and decreased rate of yeast extract was established as an important variable in pigment measurement. Yeast extract concentration at specific range enhances the pigment yield mean while at enhanced range it adversely influences the pigment estimation.

The three dimensional plots were showing the interconnection among two parameters while third variable was considering in optimum range. These plots are also explore that how pigment production is influenced by changing the two variables in a fixed time while the third one kept in optimal range. Red pigment yield enhanced with increment of temperature range and potato powder concentration separately (Fig. 5a and b) while the two parameters in combination of higher potato powder and yeast extract concentration resulted in reduction in red pigment estimation (Fig. 7a and b).

As per analysis of response surface graph, concentration of potato powder of 8.5 g %, yeast extract of 2.75 g % with 30º C temperature of fermentation medium shows maximum production of red pigment yield i.e. 9.14 OD U/ml. In the CCD chart we find that predicted value of maximum production of red pigment yield was found 8.62 OD U/ml which is quite nearer to actual value.

Analysis of the statistically validation of the model: Regular plot for variables helped to assess the validation of the model. Approximate linear data points should be required. Generally a linear data point is the indication of validity of the model where non linear data point (like an S-shaped curve) is the indication of the non-normality within error term. The current study showing normal distribution of red pigment production (Fig. 8a). The arrangement in the graphical line suggesting that there are no deformalities in the data. The perturbation graph shows how one factor at constant position while others factor showing movement from the chosen reference point. The graph indicates that red pigment production was maximally influenced by temperature, and then next to potato powder, yeast extracts concentration respectively. Increasing the concentration of potato powder concentration showed that enhancement of red pigment production while enhancement of the range of temperature upto the coded reference point enhance red pigment production. It also suggesting that increment more than coded point reduces red pigment production.

Table 1: Placket-Burman analysis to select different important variables for red pigment production under submerged fermentation using *M. purpureus* MTCC 410.

Std	Run	Factor1 A:Temperature (^{0}C)	Factor 2 B:Fermentation period	Factor 3 C:pH	Factor 4 D:Potato-powder conc (%)	Factor 5 E:Tryptone Conc. (%)	Factor 6 F:Peptone (%)	Factor 7 G:Yeast extract (%)	Factor 8 H:MSG (%)	Factor 9 I:NaCl concentration (%)	Response 1 Pigment yield U/ml
1	1	40	16	4	16	0.5	2.5	0.5	1	1	6.25111
2	2	20	16	9	1	0.5	2.5	5	1	1	5.69
12	3	20	4	4	1	0.1	0.5	0.5	1	1	5.57222
3	4	40	4	9	16	0.1	2.5	5	5	1	6.52333
7	5	40	4	4	1	0.5	0.5	5	5	1	5.21556
6	6	20	4	4	16	0.1	2.5	5	1	6	5.94889
11	7	40	4	9	16	0.5	0.5	0.5	1	6	5.45222
10	8	20	16	9	16	0.1	0.5	0.5	5	1	6.34778
9	9	40	16	9	1	0.1	0.5	5	1	6	6.35889
4	10	20	16	4	16	0.5	0.5	5	5	6	5.41667
5	11	20	4	9	1	0.5	2.5	0.5	5	6	4.49778
8	12	40	16	4	1	0.1	2.5	0.5	5	6	5.96556

Table 2: Analysis of variance (ANOVA) of Placket-Burman response of red pigment production using *M. purpureus* MTCC 410.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	3.60	8	0.4506	9.50	0.0453	(significant)
A-Temperature	0.4383	1	0.4383	9.24	0.0559	
B-Fermentation period	0.6627	1	0.6627	13.97	0.0334	
C-pH	0.0208	1	0.0208	0.4393	0.5548	
D-Potato powder conc.	0.5808	1	0.5808	12.25	0.0395	
E-Tryptone	1.47	1	1.47	30.90	0.0115	
F-Peptone	0.0220	1	0.0220	0.4630	0.5450	
G-Yeast extract	0.0948	1	0.0948	2.00	0.2523	
I-NaCl concentration	0.3201	1	0.3201	6.75	0.0805	

Std. Dev. 0.2178 **R^2**0.9620
Mean 5.77**Adjusted R^2** 0.8608
C.V% 3.77 **Adeq Precision** 10.7399

Table 3: Experimental and predicted values of red pigment yield in submerged fermentation using *M. purpureus* MTCC 410 of full experimental Central composite rotary design matrix.

Std	Run	Factor 1 A: Temperature (°C)	Factor 2 B: Potato powder conc. (g, %)	Factor 3 C: Yeast extract (g, %)	Response Pigment yield (U/ml)
10	1	46.8179	8.5	2.75	5.77667
16	2	30	8.5	2.75	8.89667
7	3	20	16	5	7.02667
20	4	30	8.5	2.75	8.89667
19	5	30	8.5	2.75	8.89667
14	6	30	8.5	6.53403	8.65667
12	7	30	21.1134	2.75	8.46333
2	8	40	1	0.5	6.32
3	9	20	16	0.5	6.49333
15	10	30	8.5	2.75	8.9
9	11	13.1821	8.5	2.75	5.43667
4	12	40	16	0.5	6.49333
11	13	30	-4.11345	2.75	6.58
13	14	30	8.5	-1.03403	5.885
17	15	30	8.5	2.75	9.14
18	16	30	8.5	2.75	6.95
1	17	20	1	0.5	5.17333
6	18	40	1	5	6.85333
5	19	20	1	5	5.70667
8	20	40	16	5	8.17333

Table 4: Analysis of variance (ANOVA) for the Central composite design (CCD) of using *M. purpureus* MTCC 410 for optimization of red pigment production.

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	30.96	9	3.44	7.57	0.0020(significant)
A-Temperature	1.18	1	1.18	2.59	0.1384
B-Potato powder conc.	3.90	1	3.90	8.59	0.0150
C-Yeast Extract	4.62	1	4.62	10.26	0.0097
AB	0.1644	1	0.1644	0.3598	0.5610
AC	0.1644	1	0.1644	0.3598	0.5610
BC	0.1644	1	0.1644	0.3598	0.5610
A^2	17.33	1	17.33	37.85	0.0001
B^2	2.54	1	2.54	5.52	0.0398
C^2	3.72	1	3.72	8.31	0.0169
Residual	4.55	10	0.4546		
Lack of Fit	1.18	5	0.2357	0.3500	0.8630 (not significant)
Pure Error	3.37	5	0.6734		
Cor Total	35.50	19			

Std. Dev.	0.6742	**R^2**	0.8720
Mean	7.32	**Adjusted R^2**	0.7567
C.V%	9.32	**Predicted R^2**	0.6042
Adeq Precision	7.5430		

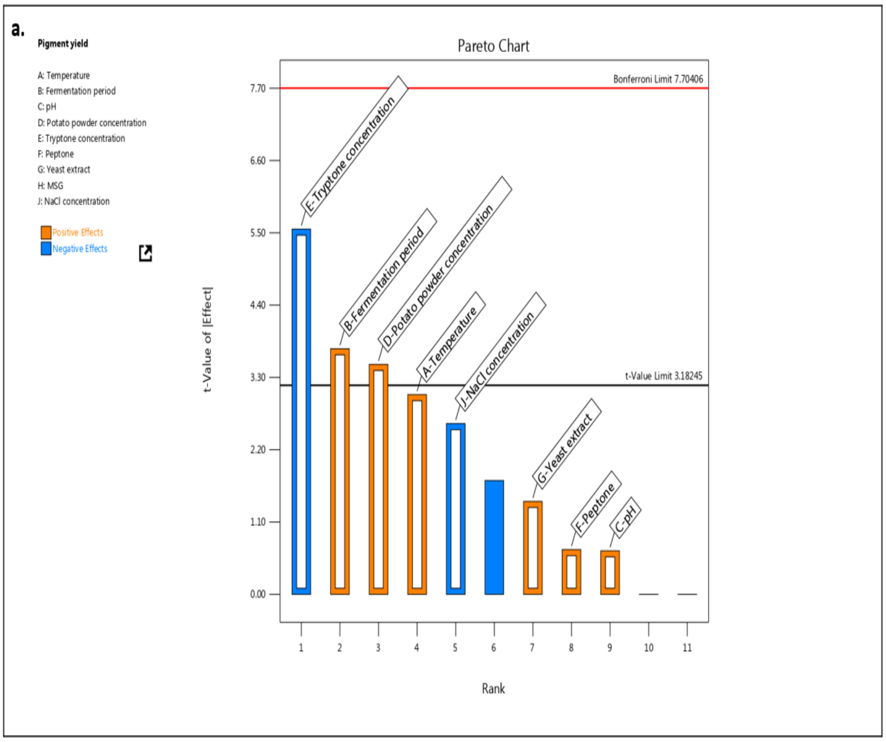

Fig 1a. Pareto chart are showing positive effects are in orange and negative effects are in blue, where 1: Tryptone concentration, 2: Fermentation period, 3: Potato powder concentration, 4: Temperature, 5: NaCl concentration, 6: MSG concentration, 7: Yeast extract, 8: Peptone, 9: pH.

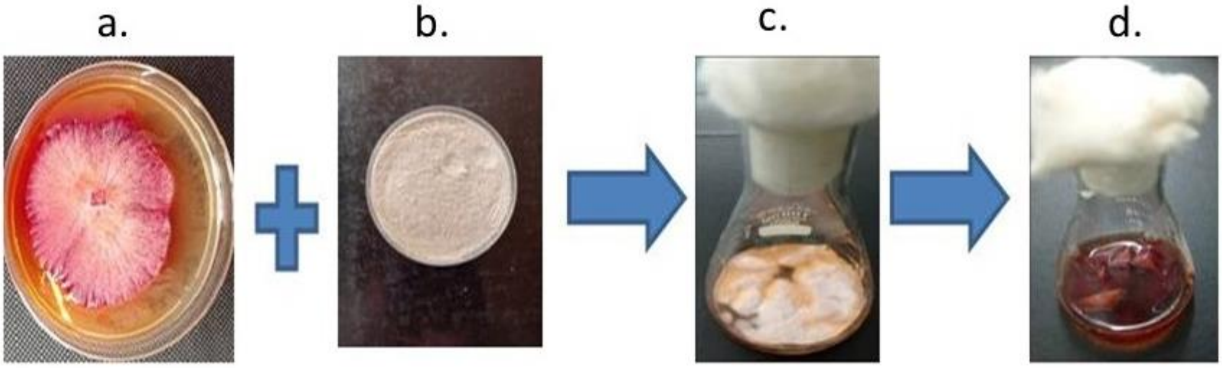

Fig. 2a. Showing growth of *M. purpureus* on MYA plate, **b.** showing potato powder was used as substrate. Next, **c.** and d. representing red pigment production of *M. purpureus* on 3 rd and 16 th days respectively.

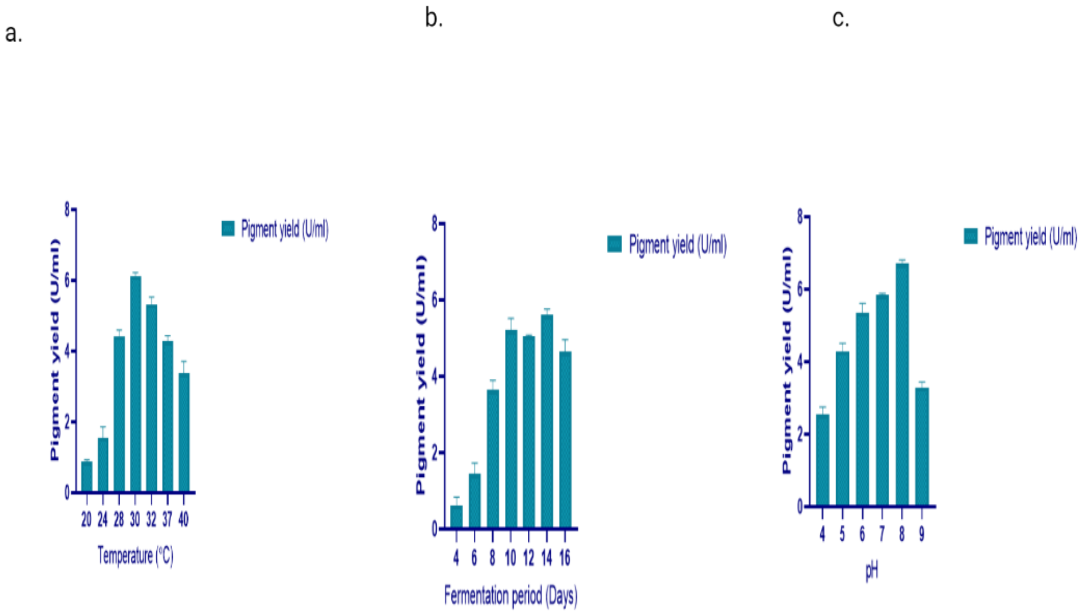

Fig. 3. Effect of different physical parameters like temperature **(a),** fermentation period **(b),** and pH **(c)** on red pigment production at AVOT approach.

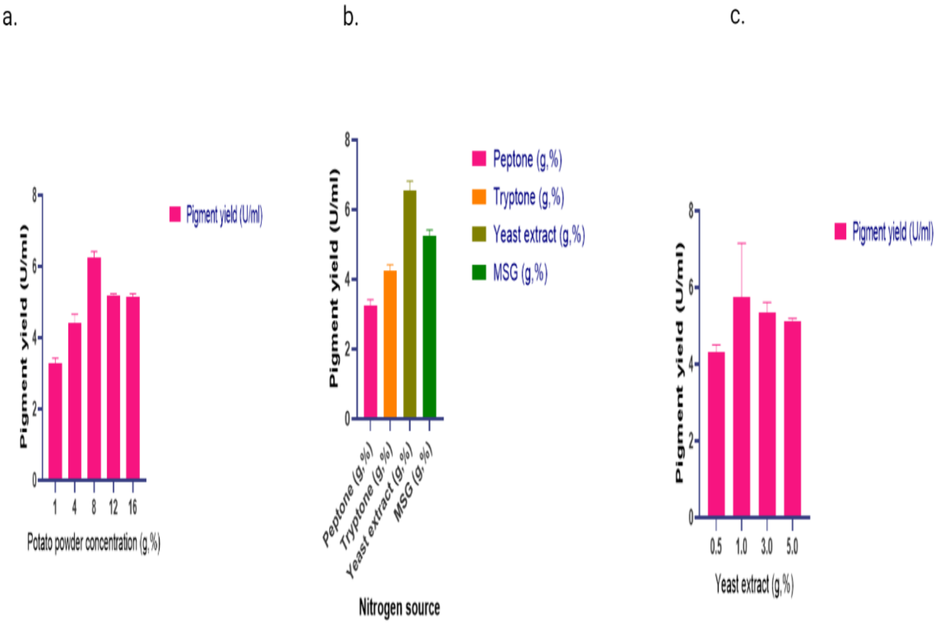

Fig. 4. Effect of different potato powder concentration **(a),** different nitrogen sources **(b),** and the different concentration of yeast extract **(c)** on red pigment production at AVOT approach.

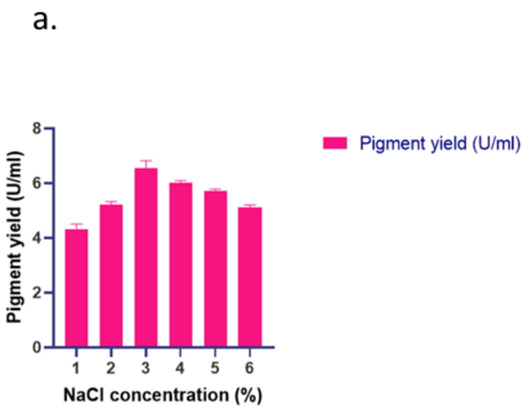

Fig. 5a. Effect of different concentration of NaCl on red pigment production at AVOT approach.

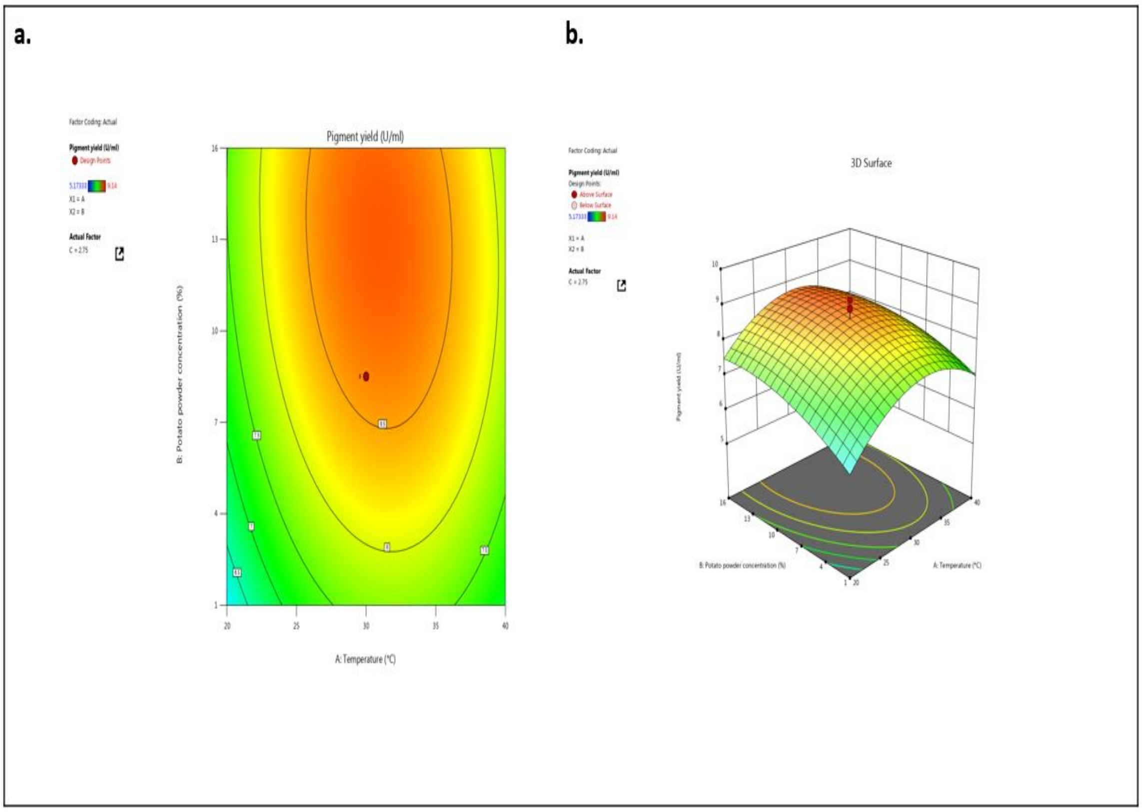

Fig. 6. Two-dimensional **(a)** and three-dimensional **(b)** response surface curves indicating interconnection among potato powder concentration in combination with temperature on red pigment yield of *M. purpureus*.

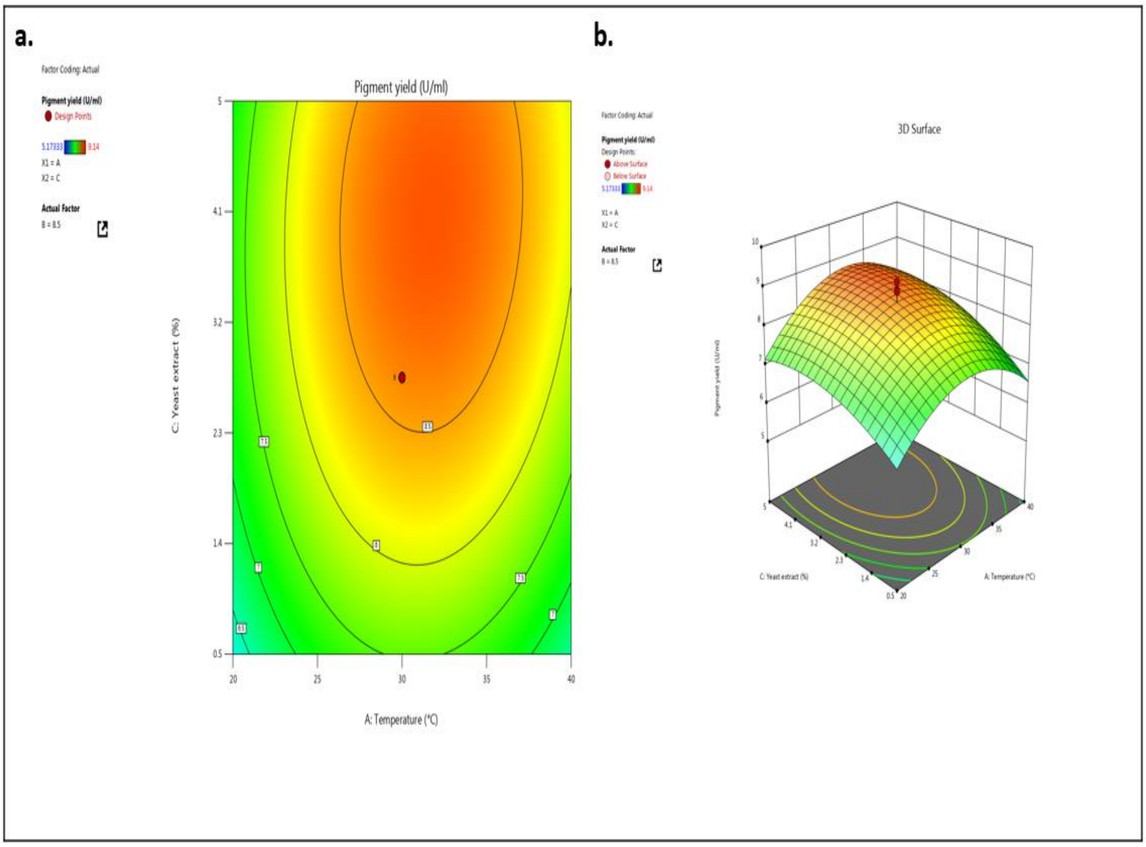

Fig. 7. Two-dimensional **(a)** and three-dimensional **(b)** response surface graph indicating interconnection between temperature in combination with yeast extract concentration on red pigment yield of *M. purpureus*.

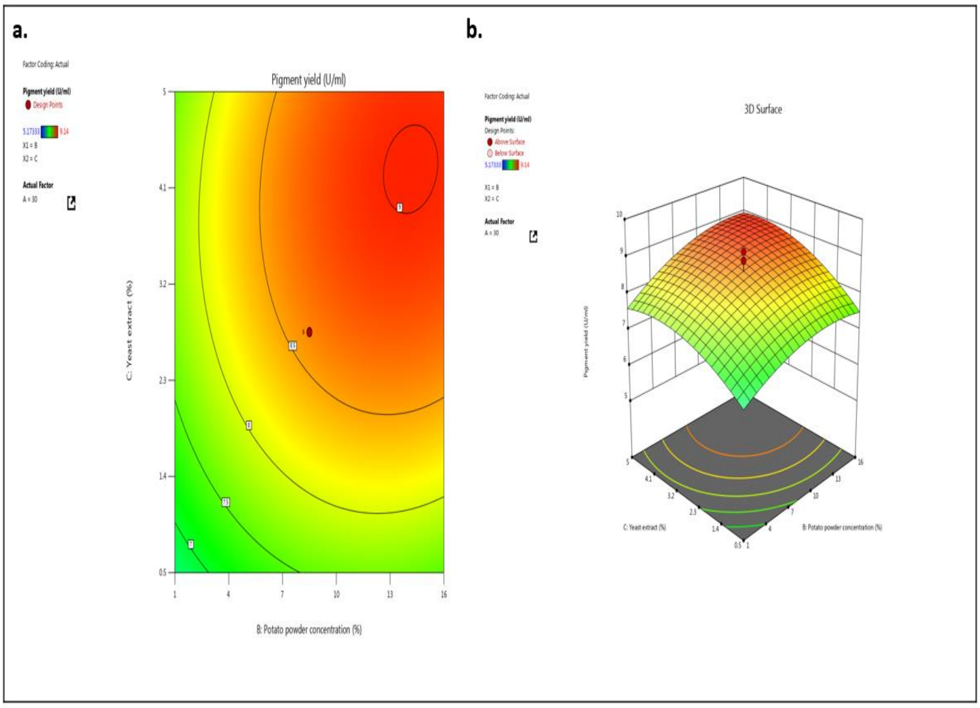

Fig. 8. Two-dimensional **(a)** and three-dimensional **(b)** response surface graph indicating the interconnection between potato powder in combination with yeast extract concentration on red pigment yield of *M. purpureus*.

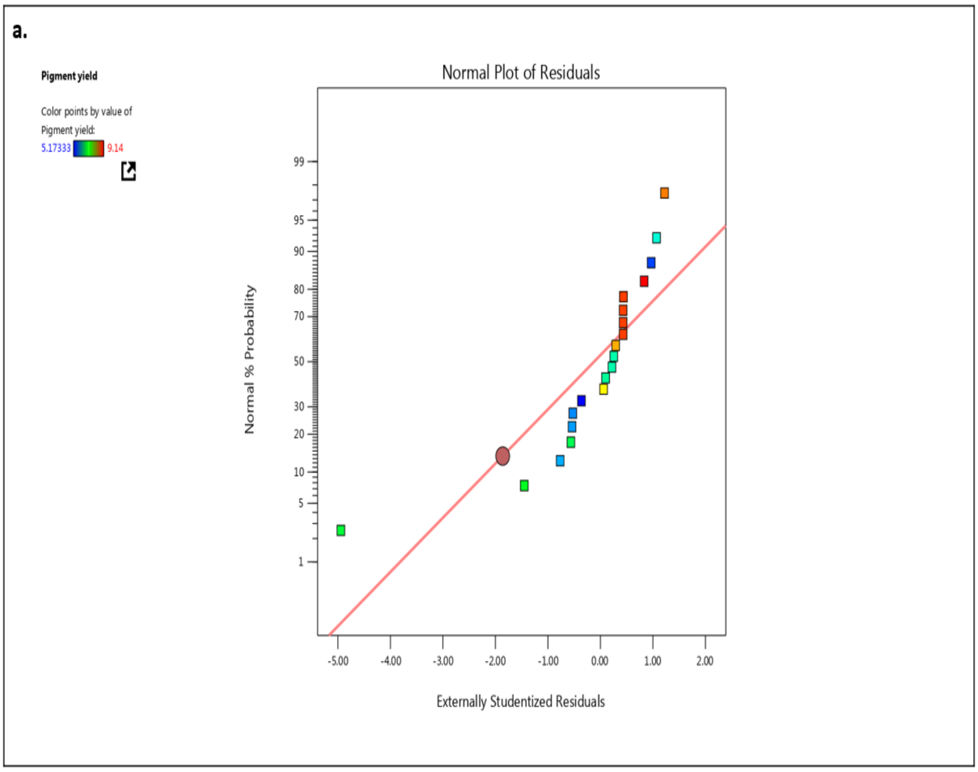

Fig. 9a. The perturbation graph showing that three factors affecting the yield of *M. purpureus* red pigments.

CONCLUSIONS

In comparison to other substrate previously utilised for the synthesis of pigment, potato powder is one of the cost-effective substrate in *M. purpureus*. Our study's goal was to create a fermentation method using submerged fermentation for the synthesis of *M. purpureus* red pigment. To maximise pigment production, we optimised important parameters. This study shows a steady way out of optimization of red pigment yield with *Monascus purpureus.* Nowadays, the tendency of the usage of natural substances in the food industry, gaining attention of the purchaser comparing to artificial man made products. Limited experimental reports were obtainable in the optimization process of red pigment formation by broth fermentation of *Monascus purpureus*. Current experimental investigation has showed that natural agro based substances along with different physical and nutritional parameters influencing red pigment production of a fungal organism. In future this experimental model can be used as a prospective great potential source of red pigment production in the food industry.

FUTURE SCOPE

Humans develop greed as a result of their constant need, which saturates their capacity for satisfaction. While consumers who care about their health often opt for natural products, the hefty cost of these products causes them to second-guess their choice. It demands the manufacturing of natural pigments at a reasonable price. Therefore, finding an organism like *Monascus purpureus* capable of producing bio pigments at a large scale in an inexpensive, simple medium, as well as process optimization for less energizing fermentation conditions and eco friendly substrate, are essential for the development of a viable process.

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Conflict of Interest. None.

REFERENCES

Ali, I., Al-Dalali, S., Hao, J., Ikram, A., Zhang, J., Xu, D. & Cao, Y. (2022). The stabilization of Monascus pigment by formation of Monascus pigment-sodium caseinate complex. *Food Chemistry, 384*, 132480.

Babitha, S., Soccol, C. R., & Pandey, A. J. B. T. (2007). Solid-state fermentation for the production of Monascus pigments from jackfruit seed. *98*(8), 1554-1560.

Carels, M., & Shepherd, D. (1977). The effect of different nitrogen sources on pigment production and sporulation of *Monascus* species in submerged, shaken culture. *Canadian Journal of Microbiology*, *23*(10), 1360-1372.

Carvalho, J. C., Oishi, B. O., Woiciechowski, A. L., Pandey, A., Babitha, S. & Socco, C. R. (2007). Effect of substrates on the production of Monascus biopigments by solid-state fermentation and pigment extraction using different solvents.

Carvalho, J. C. D., Oishi, B. O., Pandey, A. & Soccol, C. R. (2005). Biopigments from Monascus: strains selection, citrinin production and color stability. *Brazilian Archives of Biology and Technology, 48*, 885-894.

Chatterjee, S., Maity, S., Chattopadhyay, P., Sarkar, A., Laskar, S. & Sen, S. K. (2009). Characterization of red pigment from Monascus in submerged culture red pigment from *Monascus purpureus*. *Journal of Applied Sciences Research, 5*(12), 2102-2108.

Chen, M. H. & Johns, M. R. (1993). Effect of pH and nitrogen source on pigment production by *Monascus purpureus*. *Applied Microbiology and Biotechnology, 40*, 132-138.

de Carvalho, J. C., Soccol, C. R., Babitha, S., Pandey, A., & Wojciechowski, A. L. J. C. D. i. S.-s. F. (2008). Production of pigments. 337-355.

Dikshit, R., & Tallapragada, P. (2011). *Monascus purpureus*: A potential source for natural pigment production. *J Microbiol Biotechnol Res., 1*(4), 164-174.

Dikshit, R., & Tallapragada, P. (2013). Comparative study of *Monascus sanguineus* and *Monascus purpureus* for red pigment production under stress condition. *International Food Research Journal, 20*(3), 1235.

Filer, C., Dubash, N. K., & Kalit, K. (2000). *The thin green line: World Bank leverage and forest policy reform in Papua New Guinea*: Boroko, PNG: National Research Institute.

Ghoochani, R., Riasat, M., Rahimi, S., & Rahmani, A. (2015). Biochemical and physiological characteristics changes of wheat cultivars under Arbuscular Mycorrhizal symbiosis and salinity stress. *Biological Forum – An International Journal* (Research Trend), *7*(2), 370-378.

Haji, A. & Rahimi, M. (2022). RSM optimization of wool dyeing with *Berberis thunbergii* DC leaves as a new source of natural dye. *Journal of Natural Fibers, 19*(8), 2785-2798.

Hajjaj, H., François, J. M., Goma, G. & Blanc, P. J. (2012). Effect of amino acids on red pigments and citrinin production in *Monascus ruber*. *Journal of Food Science, 77*(3), M156-M159.

He, J., Jia, M., Li, W., Deng, J., Ren, J., Luo, F. & Liu, J. (2022). Toward improvements for enhancement the productivity and color value of Monascus pigments: A critical review with recent updates. *Critical Reviews in Food Science and Nutrition, 62*(26), 7139-7153.

Huang, Z., Hu, T., Yang, S., Tian, X. & Wu, Z. (2023). Genetic responses to adding nitrates to improve hydrophilic yellow pigment in Monascus fermentation. *Applied Microbiology and Biotechnology, 107*(4), 1341-1359.

Jung, H., Kim, C., Kim, K. & Shin, C. S. (2003). Color characteristics of Monascus pigments derived by fermentation with various amino acids. *Journal of agricultural and food chemistry, 51*(5), 1302-1306.

Jung, H., Kim, C. & Shin, C. S. (2005). Enhanced photostability of Monascus pigments derived with various amino acids via fermentation. *Journal of agricultural and food chemistry, 53*(18), 7108-7114.

Jůzlová, P., Martinkova, L. & Křen, V. (1996). Secondary metabolites of the fungus Monascus: a review. *Journal of industrial microbiology, 16*, 163-170.

Lai, J. R., Hsu, Y. W., Pan, T. M., & Lee, C. L. (2021). Monascin and ankaflavin of *Monascus purpureus* prevent alcoholic liver disease through regulating AMPK-mediated lipid metabolism and enhancing both anti-inflammatory and anti-oxidative systems. *Molecules*, *26*(20), 6301.

Li, T., Zhao, W., Wang, C., Shi, K. & Chen, G. (2023). Regulated synthesis and metabolism of Monascus pigments in a unique environment. *World Journal of Microbiology and Biotechnology, 39*(2), 46.

Lin, T. S., Chiu, S. H., Chen, C. C., & Lin, C. H. (2023). Investigation of monacolin K, yellow pigments, and citrinin production capabilities of *Monascus purpureus* and *Monascus ruber* (*Monascus pilosus*). *Journal of Food and Drug Analysis, 31*(1), 85.

Lin, T. & Demain, A. (1993). Resting cell studies on formation of water-soluble red pigments by *Monascus* sp. *Journal of industrial microbiology, 12*, 361-367.

Lin, T., Yakushijin, K., Büchi, G. & Demain, A. (1992). Formation of water-soluble Monascus red pigments by biological and semi-synthetic processes. *Journal of industrial microbiology, 9*, 173-179.

Lin, T. F. & Demain, A. L. (1991). Effect of nutrition of *Monascus* sp. on formation of red pigments. *Applied Microbiology and Biotechnology, 36*, 70-75.

Loret, M. O. & Morel, S. (2010). Isolation and structural characterization of two new metabolites from

Monascus. *Journal of agricultural and food chemistry, 58*(3), 1800-1803.

Nimnoi, P., & Lumyong, S. (2011). Improving solid-state fermentation of *Monascus purpureus* on agricultural products for pigment production. *Food and Bioprocess Technology, 4*, 1384-1390.

Orozco, S. F. B. & Kilikian, B. V. (2008). Effect of pH on citrinin and red pigments production by *Monascus purpureus* CCT3802. *World Journal of Microbiology and Biotechnology, 24*, 263-268.

Plackett, R. L. & Burman, J. P. (1946). The design of optimum multifactorial experiments. *Biometrika, 33*(4), 305-325.

Ravuri, M. & Shivakumar, S. (2020). Optimization of conditions for production of lovastatin, a cholesterol lowering agent, from a novel endophytic producer *Meyerozyma guilliermondii*. *Journal of Biologically Active Products from Nature, 10*(3), 192-203.

Sehrawat, R., Panesar, P. S., Panesar, R., & Kumar, A. (2017). Biopigment produced by *Monascus purpureus* MTCC 369 in submerged and solid state fermentation: a comparative study. *Pigment & Resin Technology, 46*(6), 425-432.

Sharmila, G., Nidhi, B. & Muthukumaran, C. (2013). Sequential statistical optimization of red pigment production by *Monascus purpureus* (MTCC 369) using potato powder. *Industrial crops and products, 44*, 158-164.

Singh, N., Goel, G., Singh, N., Pathak, B. K. & Kaushik, D. (2015). Modeling the red pigment production by *Monascus purpureus* MTCC 369 by Artificial Neural Network using rice water based medium. *Food Bioscience, 11*, 17-22.

Soccol, C. R. & Vandenberghe, L. P. (2003). Overview of applied solid-state fermentation in Brazil. *Biochemical Engineering Journal, 13*(2-3), 205-218.

Su, Y. C., Wang, J. J., Lin, T. T. & Pan, T. M. (2003). Production of the secondary metabolites γ-aminobutyric acid and monacolin K by Monascus. *Journal of industrial microbiology and biotechnology, 30*(1), 41-46.

Wong, H., Lin, Y., & Koehler, P. J. M. (1981). Regulation of growth and pigmentation of Monascus purpureus by carbon and nitrogen concentrations. *73*(4), 649-654.

Wu, H. C., Chen, Y. F., Cheng, M. J., Wu, M. D., Chen, Y. L. & Chang, H. S. (2021). Different types of components obtained from *Monascus purpureus* with neuroprotective and anti-inflammatory potentials. *Food & function, 12*(18), 8694-8703.

Yanli, F., & Xiang, Y. J. F. i. M. (2020). Perspectives on functional red mold rice: functional ingredients, production, and application. *11*, 606959.

Zhang, R., Yu, J., Guo, X., Li, W., Xing, Y. & Wang, Y. (2021). Monascus pigment-mediated green synthesis of silver nanoparticles: Catalytic, antioxidant, and antibacterial activity. *Applied Organometallic Chemistry, 35*(3), e6120.

Zheng, B., Li, X., Hao, J., & Xu, D. (2022). Meat systems produced with Monascus pigment water-in-oil-in-water multiple emulsion as pork fat replacers. *Food Chemistry, 402*, 134080.

Zheng, Y., Xin, Y. & Guo, Y. (2009). Study on the fingerprint profile of Monascus products with HPLC–FD, PAD and MS. *Food Chemistry, 113*(2), 705-711.

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