

# Carotenoid Production by *Bacillus clausii* Using Rice Powder as the Sole Substrate: Pigment Analyses and Optimization of Key Production Parameters

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## Abstract

Natural  $\beta$ -carotene, an orange–red pigment of carotenoid family is widely used as food colorant. In this study microbial method of pigment production is adopted to encompass the other expensive technologies. Influence of substrate (rice powder) on  $\beta$ -carotenoid production has been extensively studied at optimum process conditions such as pH, temperature etc. A  $\beta$ -carotenoid producing pigment *Bacillus clausii* was isolated and identified by physiological tests as well as 16s rDNA sequence analysis. The maximum yield of  $\beta$ -carotenoid 48.9% using *Bacillus clausii* was achieved at pH 7 and 35°C utilizing rice powder as a sole substrate. A statistical design technique, Taguchi method was applied to evaluate the optimal process conditions such as pH, temperature etc. for maximum pigment production.

**Keywords:** Microbial pigments, *Bacillus clausii*, carotenoid,  $\beta$ -carotene, Taguchi method.

## Introduction

Pigments are particulate solids that disperse into a medium without significant interaction in solutions (Zollinger 2003). Their wide applications in fields of food, cosmetics, paints, pharmaceuticals, textiles, glass etc. has contributed to its increasing demand (Babitha et al. 2006). Among all natural pigments, carotenoids represent the largest and most diverse class compounds known to mankind (Britton, Liaen-Jensen et al. 2004). Considerable research was focused on these pigments as nutritional supplements with some \$ 935 million to billion dollars market in 2005 (Park et al. 2005,

Marova et al. 2012). Carotenoids also play a significant role in human health as precursors of vitamin A, scavengers of active oxygen, enhancers of *in vitro* antibody production, anticancer agents and so on (Park et al. 2005, Cabral et al. 2011).

The present production method of choice for carotenoids is chemical synthesis (Wittig and Pommer 1959, Storebakken et al. 1987). However, the consumer interests for natural products, high costs, and the dependence on by-products derived from fossil fuels and the detrimental impact on the environment have collectively intensified efforts to identify alternative bio-resources (De Carvalho et al. 2014). Thus research dedicated to carotenoids from microbial origin has been increasing year after year and have found maximum application in the food and cosmetic industries. Microbial sources especially bacteria are commercially attractive and can produce a diverse range of carotenoids with both C40 and C30 backbones, while higher plants, algae and fungi produce C40 structures (Perez-Fons et al. 2011). Reported studies showed carotenoids production ability by *Serratia marcescens*, *Pseudomonas* sp, *Pseudoalteromonas* sp, *Alteromonas denitrificans*, *Hahell* sp, *Vibrio* sp, *Micrococcus roseus*, *Brevibacterium linens*, *Bradyrhizobium* sp, *Xanthomonas campestris*pv and so on (Venil et al. 2013). Whereas wide color range brown, black, yellow, orange, red carotenoids of industrial interest can be obtained by *Bacillus* sp alone. Best known examples are *B. atrophaeus* which produces a soluble black pigment and *B. cibi*, *B. jeotgali*, *B. indicus*, *B. clarkii*, *B. okuhidensis*, *B. vedderi*, *B. pseudofirmus* which are able to give yellow to orange color range (Duc et al. 2006). Recent reports also showed the potential of *B. clausii*, a probiotic class gram-positive soil bacterium (which was isolated in this study) has the ability to produce carotenoids (MetaCyc, 2010, BioCyc, 2012, Database, 2012).

In view of high cost of current technology there is a need to develop low cost processes for the production of pigments which could replace the synthetic pigments in industrial scale. Production of pigments essentially depends on the substrate upon which the microbe grows (Kagliwal et al. 2009). A variety of substrates are tested for carotenoids production using different microbes with special emphasis on cheaper substrates

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like maple sap (Wasserman 1965), coconut milk (Domínguez-Bocanegra and Torres-Muñoz 2004), sugar manufacture processing waste (Hayman et al. 1995), fermented radish brain (Malisorn and Suntornsuk 2008), corn meal, pea nut meal, soybean meal, coconut residue (Mussatto et al. 2012), whey waste (Marova et al. 2012), corn syrup (Buzzini 2001), grape must, beet molasses, soybean flour extract, maize flour extract (Buzzini and Martini 2000), jack fruit seed (Babitha et al. 2006), plant extract (Kim et al. 2007), prehydrolysed wood (Parajó et al. 1997) etc. In addition to the above, utilization of rice powder as carbon source for microbial carotenoid production has been effectively practiced (Lian et al. 2007, Lin et al. 2008) and various

new processes at different conditions were patented and reported (Carels and Shepherd, 1979, Gierhart, 1994, Yongsmith et al. 1994). Extensive research has been focused on cheaper methodologies and efforts have been made in order to reduce the production cost of microbial pigments compared to that of synthetic, plant and animal derived pigments (Buzzini and Martini 2000, Dufossé et al. 2005).

This paper outlines the following objectives. 1) Isolation of the pigment producing bacteria from garden soil, 2) Examine the potential of cheap natural substrate like rice powder for the production of pigment, 3) Extraction, purification and analyzing the chemical nature of the produced pigment, 4) Investigating the stability of the obtained pigment, and 5) Optimization of key production parameters, using Taguchi experimental design.

## Materials and methods

### Sampling, microscopy and strain characterization

Carotenoid producing microbial cultures was isolated from soil sample collected from the premises of NIT campus Rourkela. Screening procedure adopted was plating of 1 mL ( $10^{10}$ ) diluted soil samples on nutrient agar (Himedia Pvt Ltd, India) with inoculation at 25 °C. The orange colored colonies developed on the plates were observed. The isolate showing maximum diameter of was propagated on rice plates at 25 °C for 24 h. Characterization and identification of the isolate with pigment production ability was carried out both morphologically with optical microscope (Hund-H600) and for better understanding of the morphological characters i.e., size and shape of the microorganism, SEM (Scanning Electron Microscope) analysis was done. JEOL JSM-6480LV was employed to capture images at various magnifications. Further the strain was sent to Xcelris labs Ltd, Ahmedabad, India for 16s-rDNA characterization.

### Pigment production

A finely ground rice powder was used as a substrate for pigment production. Experiments were conducted in petriplates of 10 cm diameter containing 3 g of rice powder. The substrate was moistened with double distilled water (8 mL) of neutral pH - 7 and autoclaved as stated above to maintain sterile conditions throughout the study. On cooling to room temperature; moisture content of the substrate was analyzed using moisture analyzer (Sartorius MA-150) and was found to be 52.04 %. The substrate in plates was inoculated with 1 mL inoculum culture and incubated at ambient room temperature ~ 30 °C for 10 days. After observing intense coloration in rice plates, other batch of experiments at pH 6 and 8 are also tried for pigment production.

### Purification and stability of the pigment

Pigment was extracted using a modified method adapted from Babitha et al. (2007). To evaluate the solubility of the pigment a definite quantity of substrate (rice powder) 1g was extracted with fixed volume 5 mL of different solvents such as acetone, methanol, ethanol, chloroform, water and 10 % NaOH (Declair and Richard 1972). The pigment-solvent mixture was allowed to stand after shaking for 15 minutes. Based on high extraction of pigment and fair dispersion with 10 % NaOH, ethanol was selected as extracting solvent. About 1:5 rice powder (grams) to solvent (mL) ratio was used for pigment extraction by thorough dispersion. The mixture was centrifuged at 9000 rpm and the supernatant was collected. The above process was repeated until the substrate was colorless. Phase separation of the extracted pigment in the supernatant was achieved on adding equal volumes of diethyl ether and NaCl (0.1 M) to obtain pure pigment devoid of cellular disturbances (Domínguez-Bocanegra and Torres-Muñoz 2004). To obtain orange colored powdered pigment the supernatant was subjected to vacuum evaporation.

The extracted pigment was assessed on 0.50 g of dry pigment and adjusted to 50 ml constant volume of ethanol before measuring its absorbance at ( $\lambda_{max}$ ) using a UV-3600 Shimadzu UV-Visible spectrophotometer. The structural analysis of the extracted pigment was examined by FTIR (Bruker ATR-FTIR, USA) at room temperature. Stability of the purified pigment was also tested after thermal treatment at 45 °C for 8 h at ambient conditions in an oven. It was subjected to UV-visible, fluorescence and Attenuated total reflectance Fourier transform infrared (FTIR) spectroscopy analyses to observe the structural confirmations.

### Optimization of key parameters

Taguchi experimental design method was employed to evaluate the optimum conditions for pigment production. The key parameters such as pH, temperature and moisture content were studied. A standard L16 ( $4^3$ ) with 15 degree of freedom

**Table 1:** Factors and their levels studied by Taguchi method.

Factor	Level 1	Level 2	Level 3	Level 4
pH	6	6.5	7	7.5
Temperature (°C)	25	30	35	40
Moisture % (w/w)	40	45	50	55

**Table 2.** Levels of three different factors in each of sixteen trails and obtained results.

pH	Temperature	Moisture	Pigment yield - mg/3 g substrate
1	1	1	46.1
1	2	2	52.4
1	3	3	68.3
1	4	4	54.0
2	1	2	50.2
2	2	1	49.3
2	3	4	86.6
2	4	3	88.7
3	1	3	78.0
3	2	4	95.8
3	3	1	87.6
3	4	2	92.8
4	1	4	56.4
4	2	3	68.4
4	3	2	70.5
4	4	1	65.3

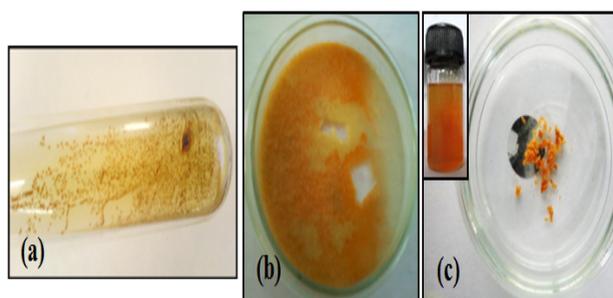
was used to examine three factors in four levels. The levels of

the factors studied and the layout of the L16 orthogonal array are represented in Table 1 and 2. ANOVA technique was adopted to analyze statistically and determine the factors that significantly affect the pigment production. The controlling factors were identified with the magnitude of effect quantified. Consequently, the optimal conditions were determined by combining the levels of factors that had the highest main effect value. All calculations were performed using MINITAB® Release 14.1 software.

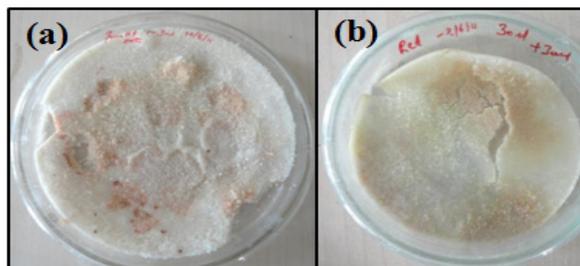
## Results and Discussion

### Colony Screening, pigment production

Distinct microbial colonies were observed on the agar plate after three weeks of incubation. An orange colored peculiar spot surrounded with cream white color colony was screened and cultured for the pigment production. After two days of incubation at 35 °C, visible cream white color colonies were identified on the agar slants. Incubation for another three weeks resulted in the production of evenly spaced orange-red spots (Figure 1a). These colonies were preserved using paraffin method as per the protocol (Aneja 2003). Previous investigations have revealed the utility of rice as a nutrient supplement in fungal pigment production (Lian et al. 2007, Kagliwal et al. 2009). In our study rice powder alone was used as a sole nutrient medium for orange colored pigment production through bacterial species. Using rice powder as nutrient medium an intense orange colored pigment was observed on the plate after 10 days of incubation at ambient temperature of 30 °C (Figure 1b). During the incubation, it was noted that the pigment intensity was more on the surface of the rice plate than the deeper layers of the plate. The pigmented rice material was suspended in ethanol for pigment extraction and concentrated by vacuum drying as shown in Figure 1c. At constant temperature (30 °C) and varying the pH (6 and 8) did not show notable color change on rice plates (Figure 2).



**Figure 1.** Microbial isolate with orange – red spotted colonies on nutrient agar slant (a), and orange pigment by the isolated soil microorganism on rice powder (b). Pigment concentrate in ethanol is shown in (c) and its vacuum dried form shown in the insert of (c).



**Figure 2.** Orange pigments by the isolated soil microorganism on rice powder plates at pH - 6 (a) and 8 (b) respectively.

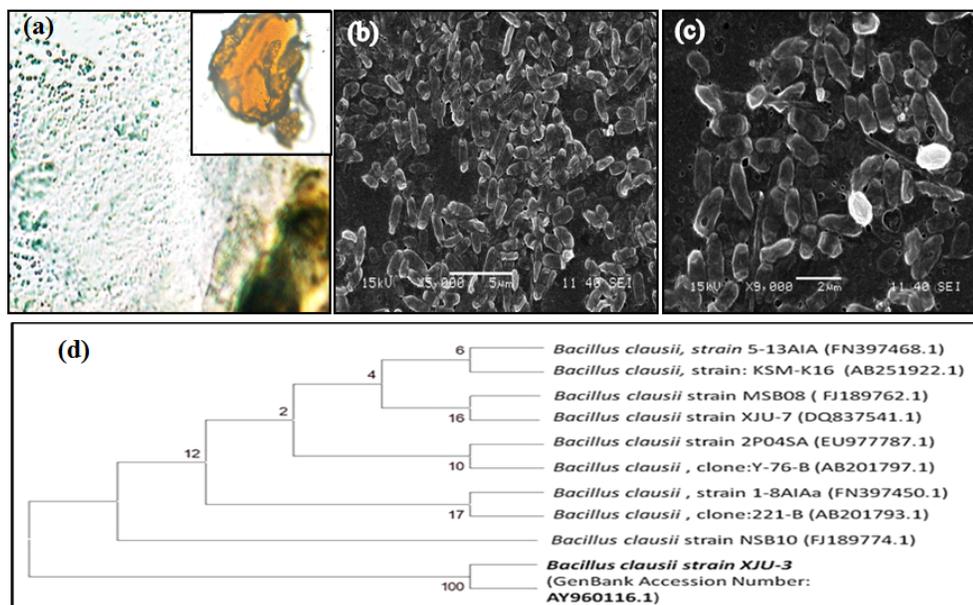
### Microscopy and microbial characterization

Figure 3a shows the optical microscopic image of the microbial isolate and the produced pigment. The nature of the obtained pigment was agglomerative and extracellular. It was also evident from the small size of the microbe involved that the bulky pigment clumps might be their possible metabolites. SEM analysis confined the morphology of the microbe (Figure 3b,c). It clearly suggests a rod shaped bacterial species of the size range 1.1 to 1.5 micron. These colored colonies based on morphology and phylogenic analysis through 16s rDNA sequencing were identified as *Bacillus clausii* XJU-3, This bacterium strain showed 100 % homology with *Bacillus clausii* (AY960116.1) and the phylogenic relationship is shown in Figure 3d. Reports on production of yellow, orange and pink carotenoids on a variety of substrates suggest that the identified species has potential for carotenoid pigment production (Duc et al. 2006, Khaneja et al. 2010).

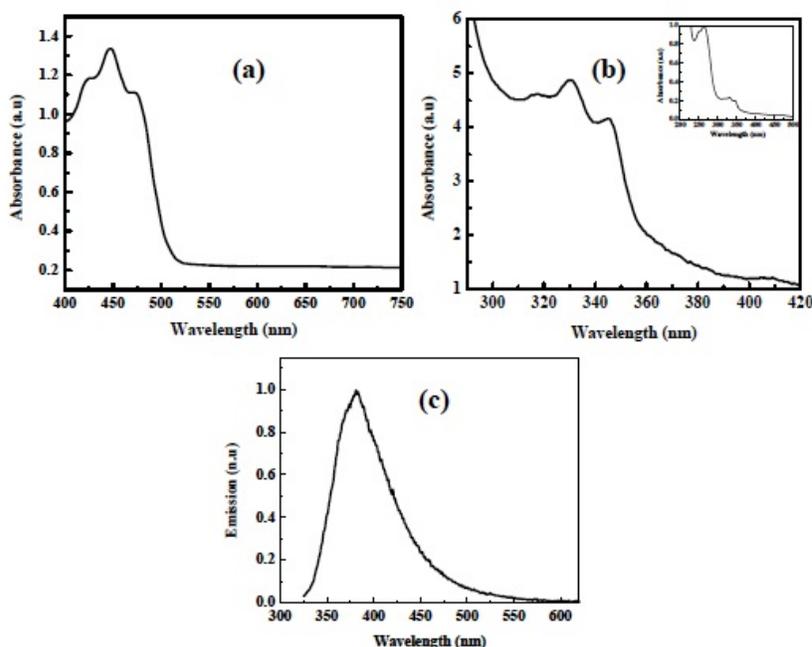
### UV-Visible/fluorescence spectrophotometry and stability of the pigment

Figure 4a shows the UV-Visible spectrum of pigment solution (extracted in ethanol) produced from the isolated *B. clausii* on the rice medium. The absorption maxima were observed at 447 and 472 nm and the spectral pattern were in close resemblance to the characteristic of  $\beta$ -carotene (Park et al. 2007). Literature shows that  $\beta$ -carotene type carotenoids are sensitive to oxidation, light and heat (Jacob et al. 2010). To confirm the pigment kind, pigment stability under different storage conditions was tested. In general,  $\beta$ -carotene is stored in aluminum foil bags under inert gas atmosphere at low temperatures < 15 °C to retain > 96% of the activity (European Food Safety 2012). To test the degradability and stability of the observed pigment it was subjected to thermal drying under aerobic conditions in a closed oven for 8 h at 45 °C. After incubation, the color of the pigment residue became slightly shaded from orange-red to pale yellow, which may be due to oxidation of the pigment. It was subjected to further analyses (UV/fluorescence).

Figure 4b shows the spectrum of the shaded pigment and the discrete difference in spectra of (a) and (b) might be due to oxidation of the produced pigment. The band maximum of spectrum in (b) was found to be at 330 nm with a shoulder peak at 345 nm, while for the diluted solution of the same, an additional absorption at 262 nm was observed (Figure 4b insert). The absorption of spectrum (b) at 330 nm of the electromagnetic spectrum indicates close spectral resemblance of vitamin A class retinol pigment (N'Soukpoe-Kossi et al. 2007). The additional peak split at ~ 340 nm and band at 262 nm specify that the extracted pigment was in its oxidized form (Martras et al. 2004). An interesting feature of these retinol class compounds absorption at shorter wavelength region is due to their highly twisted double-bond in their systems (Sporn 1984). The above evidence shows that the nature of the pigment obtained from *B. clausii* is of wholesome  $\beta$ -carotene and produced products like precursors of vitamin A as a result of aerobic thermal oxidation, which usually occur via chemical transformation (Adhikari et al. 2000). The evaluation of fluorescence property of the oxidized pigment also confirms the property of retinol type products (Adhikari et al. 2000). The maximum excitation was found to be at 315 nm and the  $\lambda_{emi}$  is observed at 382 nm as shown in Figure 4c. The fluorescence yield of pigment before thermal treatment has produced no or negligible spectral signal and was not reported.



**Figure 3.** Pigment producing bacterial species with an orange pigment (insert) magnified by an optical microscope at 400 X magnification (a). SEM images of the identified pigment producing bacteria with (b) 5000 X, (c) 9000 X magnifications and (d) Phylogenetic tree showing the position of the isolate XJU-3 with reference to related strains



**Figure 4.** (a) UV- visible spectrum of the extracted orange pigment in ethanol, (b) UV absorption of the oxidized pigment with narrow and wide (insert of (b)) spectral regions, and (c) Fluorescence emission spectra of the oxidized pigment at 382 nm.

#### FTIR analysis

In order to understand the pigment structure FTIR analysis was performed. The purified pigment was subjected to FTIR spectroscopy before and after oxidation steps (Figure 5a,b). Figure 5a shows the IR spectrum of the pigment with major peaks at 3320, 2943, 2832, 1652, 1448, 1405, 1100 and 1020  $\text{cm}^{-1}$ . The majority of the peaks from the obtained pigment show a close resemblance to a  $\beta$ -carotene structure (Baranska et al. 2006, Ammawath and Bin Che Man 2010). Peaks at 2943 and 2832  $\text{cm}^{-1}$  are due to asymmetric and symmetric stretching vibrations of  $\text{CH}_2$  and  $\text{CH}_3$  groups (Ammawath and Bin Che Man 2010). And peaks at 1020  $\text{cm}^{-1}$  is for

wagging vibration of  $(\text{RH})\text{C}=\text{C}(\text{RH})$  groups of the synthesized pigment. Peaks at 1405 and 1448  $\text{cm}^{-1}$  are due to symmetric deformation of  $\delta \text{CH}_3$  and deformation vibration of  $\delta \text{CH}_2$  groups. Moreover the peaks at 3320, 1652 and 1020  $\text{cm}^{-1}$  are due to vibrational modes of water interference in the analyzed pigment (Coates 2000, Baranska et al. 2006).

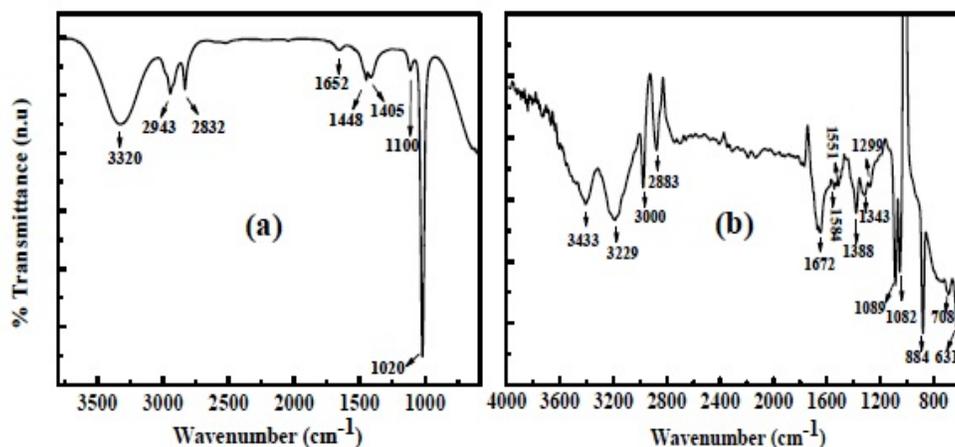
Figure 5b shows FTIR peaks after the oxidation of the pigment. The major peaks observed here are at 3433, 3229, 3000, 2883, 1672, 1388, 1343, 1089, 1082, 884, 708 and 631  $\text{cm}^{-1}$ . The spectral pattern highly resembles retinol compound of carotenoids

family (Lóránd et al. 2002). The absorptions in the region of 3200 - 2700  $\text{cm}^{-1}$  are normally characteristic of carbon and hydrogen containing species, and can be assigned to various forms of C-H stretching. The absorbance at and above 3000  $\text{cm}^{-1}$  suggest that the compound is likely to have unsaturated C=C. The absorption at 3433  $\text{cm}^{-1}$  which is in the region of 3650-3250  $\text{cm}^{-1}$  mainly occurs for hydroxy or amino groups. The additional intense bands in 1600-1300, 1200-1000 and 800-600  $\text{cm}^{-1}$  in Figure 5b confirms that the

function of three input parameters in this experiment was given below.

$$Y = -459 + 43.0(X_1) + 9.6(X_2) + 8.1(X_3) - 9.22(X_1 * X_1) - 0.119(X_2 * X_2) - 0.070(X_3 * X_3) + 0.902(X_1 * X_2) + 0.668(X_1 * X_3) - 0.106(X_2 * X_3)$$

where Y is the output (pigment yield) and  $X_1$ ,  $X_2$ ,  $X_3$



**Figure 5.** ATR-FTIR analysis of the extracted and purified orange pigment before (a) and after (b) oxidation process.

oxidized compound consists a simple hydroxy compound with O-H absorption and is likely to be an alcohol. The absorption at the low end and below 1700  $\text{cm}^{-1}$  was due to carboxylate (carboxylic acid salt). In our case the peak at 1672  $\text{cm}^{-1}$  may be due to conjugation at C=O. (may be our oxidized compound is a mixture of retinol and retinoic acid). Peaks at 3000  $\text{cm}^{-1}$  and 2986  $\text{cm}^{-1}$  which are just above and below 3000  $\text{cm}^{-1}$  may be due to unsaturated and saturated C-H absorptions in the compound. Bands at ~ 1000  $\text{cm}^{-1}$  and 880  $\text{cm}^{-1}$  indicate C-H out of plane bending which is usually seen for all *trans* retinol compounds. Further the olefinic double bond type unsaturation can be confirmed by a single peak around ~ 890  $\text{cm}^{-1}$  along with the absorptions at ~1650 and ~3000  $\text{cm}^{-1}$  (Coates 2000). The structural confirmations by IR analysis in concurrence with UV-Visible analysis infer that the produced pigment is exclusively close to  $\beta$ -carotene in all aspects. And the retinol derivatives of the obtained pigment on thermal treatment further confirm the instability of these  $\beta$ -carotene type pigments.

#### Optimization of key production parameters

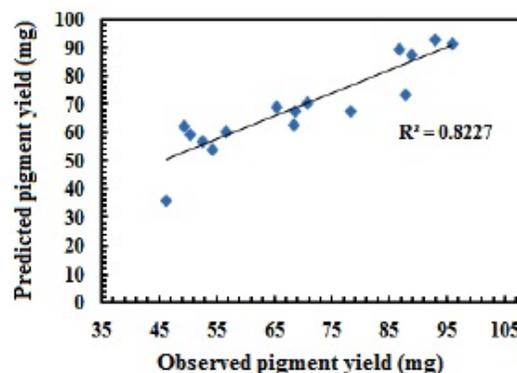
Extensive research focused on the optimization of the key process parameters for high yield carotenoid production by employing simple statistical procedures (Bhosale and Gadre 2001, Mantzouridou et al. 2002, Sarada et al. 2002). The above analysis inferred that the pigment produced by the bacterial isolate *B. clausii* needs proper attention while purification and preservation as it is thermally sensitive at normal atmospheric conditions. By following cautious steps and to know the influential factors for the pigment production in higher amounts; effect of key parameters like pH, temperature and moisture are studied by using a simplistic Taguchi method. This method of optimization offers ease in identifying the key process parameters and is a fractional factorial experimental design. This method can be effectively used in optimization of biotechnological processes, since it has the ability to include categorical factors also (Azin et al. 2007). Multiple regression technique is used to ascertain the relationships among variables of Table 2. The regression coefficients were obtained by experimental data and the regression equation for the response characteristics as a

corresponds to pH, temperature, moisture respectively.

The observed (experimental) results and the predicted values obtained using the model equation was shown in Figure 6. The predicted values and the observed data points, indicating a very good fit ( $R^2$  value of 0.823) of the response equation

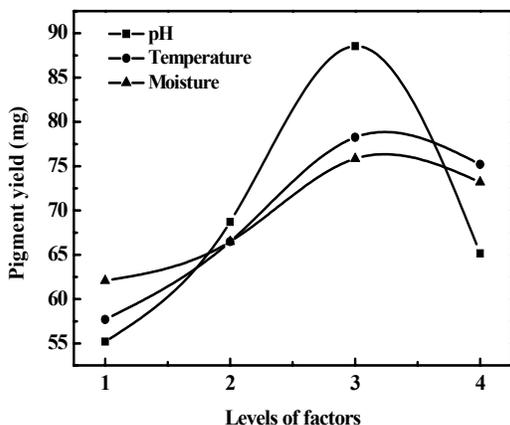
#### Validation test

The results of the experiment showed that the maximum yield of pigment was 95.8 mg/3 g rice powder, at optimum experimental condition (pH 7, temperature 30 °C and moisture 55 %) of Table 2. Figure 7 depicts the main effect of each factor (Table 2), the term 'main effects' considered as average of the obtained results in which each factor is at a given level. Results obtained using ANOVA software (Table 3) suggests the significance of the factor in the following order pH > temp with p - values of 0.011 and 0.065. Thus these p-values (on comparison with  $\alpha$  - level values) indicate that the parameter pH and temperature are significant at the levels 0.05 and 0.010  $\alpha$  respectively and are significantly related to the response.



**Figure 6.** Predicted pigment yield as opposed to observed pigment yield.

The average yield of the pigment was estimated as 69.4 mg/1g rice powder (Table 2). Table 4 shows the suggested conditions as per calculations. The statistical calculations as per the conditions suggested in Table 4 predicted that if the conditions were chosen as shown in Table 4, should produce 103.3 mg pigment per 1 g rice powder which is 48.9 % more yield. However, the experimental result showed  $107 \pm 1.2$  mg pigment production using 1 g rice powder since the deviation in actual and predicted value was only about 3.58 %, which is acceptable. Therefore on comparing with 95.8 mg pigment produced before, a further increase of about 11.6 % was achieved.



**Figure 7.** Main effects of factors or average of obtained results (pigment per 3 gm rice powder) in which each factor is at a given level. For detail about 'levels' refer to Table 1.

**Table 3.** Analysis of variance of main effects of factors. SS/MS indicate sum/mean of squares in the table.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	3	2347.7	2347.7	782.55	9.46	0.011
Temperature	3	1032.0	1032.0	343.99	4.16	0.065
Moisture	3	473.0	473.0	157.67	1.91	0.230
Residual error	6	496.3	496.3	82.72		
Total	15	4349.0				

**Table 4.** Optimum conditions suggested by statistical analysis after performing the tests

Factor	Level description	Level	Contribution greater than avg. yield in 'mg' - in '%'
pH	7	3	19.10 – 27.0
Temperature (°C)	35	3	8.85 – 12.7
Moisture % (w/w)	50	3	6.45 – 9.2

## Conclusions

The production of orange pigment was effectively done using a soil microorganism with rice powder as a sole substrate. Microscopic studies exposed the morphological characteristics and 16S rRNA sequence analysis confirmed the bacterial isolate as *B. clausii*. Upon purification, our observations by UV – visible absorption spectrum and ATR-FTIR analysis showed that the obtained pigment resemble the carotenoid class compound  $\beta$ -carotene. The thermal sensitivity of the obtained orange pigment was well explored by UV, fluorescence, ATR-FTIR analyses and the residual products were structurally close to  $\beta$ -carotene derivatives like retinol and retinoic acid. Maximum pigment production was achieved ( $107 \pm 1.2$  mg/3 g rice powder) by Taguchi method of optimization with conditions set

at pH - 7 and 35 °C temperature. The high sensitivity in yield with respect to close variation in key parameters (like temperature and pH) was not new and observed in several past studies by various research groups (Kagliwal et al., 2009; Decarvalho et al., 2006; Krairak et al., 2000; Orozco and Kilikian, 2007). The scope of this study can be extended by testing the isolated strain for pigment production with a variety of cheaper substrates. And exploration of rice powder as a sole substrate to obtain different carotenoids by various useful organisms.

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