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Process Optimization of Red Pigment production from *Vibrio sp* Isolated from Marine Source

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ABSTRACT

Red pigment production pattern by isolated marine *Vibrio sp.* was investigated. The pigment yield was estimated by measuring the absorbance at 640 nm and 535 nm and pigment production was represented as pigment produced per unit cell. The pigment production yield was influenced by several physiological and nutritional parameters of the medium. Nutritional parameters especially carbon source played significant role compared to nitrogen. Among physiological parameters, sodium chloride concentration influenced the pigment yield and noticed that 100% increase compared to other. Evaluation of different parameters that influence the pigmentation process in this isolate revealed that sodium chloride concentration, incubation temperature and mannose as carbon source are essential for improved pigmentation. However, at individual level all the parameters revealed a positive influence on pigment production however the trend varied with parameter with increase in their concentration during fermentation. While at two factor interactive level incubation temperature did not show any interactive influence with carbon source, sodium chloride and pH of the medium and showed interaction with inoculum concentration. Up on optimization of culture conditions, the pigment production yield improved to more than seven folds (i.e. >30000 pigment produced unit/cell). This study suggests the potential of this marine microbe for industrial application especially in development bioprocess for red pigment production.

INTRODUCTION

Pigmentation is a characteristic nature of most of the bacterial species. Pigments are chemical components with light-absorbing character. Natural colours edge over chemical synthesized especially at their utility spectra in industrial sectors mainly due to their biocompatibility, safety to use and ecofriendly nature in addition to safety issues associated with many artificial synthetic colorants. Because of this biocompatibility nature and increasing awareness on environmental pollution, there is an increasing demand for natural colors in the food, pharmaceutical, cosmetics, textile, printing and dye industry (Kim et al., 1999). Nature is rich in pigment producing sources ranging from higher plants to microbial strains including animals, bacteria, fungi and yeast. They are generally extracted /produced from fruits, vegetables, roots & microorganisms hence often called bio-colours (Lauro 1991). In present era, utilization of natural pigments in food stuff, dye stuff, cosmetics & pharmaceutical manufacturing process has been enormously increased especially for natural pigments like carotenioids, melanins, flavins, quinines, etc in industrial and pharmaceutical sectors (Kim et al., 1999).

Commercial production of natural pigments can be either obtained mainly from plants (Bennett and Bentley, 2000) or from microorganisms (Williamson et al., 2006). Diverse groups of pigments are produced by microbial strains which play significant role in the survival of the organisms. For example, the pigment xanthomonadin protects Xanthomonas oryzae from damage due to light (photo damage). Among all natural pigmentation sources, microbial pigments are a promising alternative to colour additives extracted from vegetables and/or animals due microbe growth mediated advantages such as lower life span, higher productivity, controlled growth conditions etc. Several pigment producing micro-organisms belongs to genera *Monascus, Paecilomyces, Serratia, Cordyceps, Streptomyces, Penicillium herquei, Chromobacterium, Serratia marcescens, Vibrio psychroerythrus, Actinobacteria, Streptomyces coelicolor* and *Hahella chejuensis* (Williamson et al., 2006) have been isolated and studied in detail. Some of these pigments produced by microbial strains gained industrial as well as health sector importance due to their potential as antibacterial, antifungal, antiprotozoal, antimalarial, immunosuppressive and anticancer agents revealing their potential role in modern environment-friendly era. However, each pigment/colorant has specificity towards its biological functionality hence scientific community is always on their toes to explore the nature for novel pigment producing microbial strains. In view of the above, attention was focused to isolate and screen the efficient pigment producers as a potential role in pharmaceutical industry. To gain industrial application potential of any isolated strain, it needs to be evaluated for their production levels which are generally very low in quantities hence need a thorough study to improve the productivity levels of compound of interest to economically viable process. Microbial fermentation based metabolic linked product production processes, several factors such as physiological (pH, temperature, aeration and agitation), nutritional (carbon, nitrogen, other mineral salts) and biological (inoculum level, inoculum age and microbial cell physiology) factors play crucial role. These parameters influence the growth kinetics of microbial strain as well as regulate the metabolic processes leading to develop a process with high product yield. In the present study, a microbial strain having potential to produce a red pigment with antibiotic activities against bacterial pathogens was isolated and studied the influence of different nutritional factors such as carbon, nitrogen source influence and pH, NaCl concentration role in addition incubation temperature effect on production pigment was evaluated.

Sample collection

The marine water samples were collected from Nellore costal region, Krishna Patanam beach in Andhra Pradesh, India, during the month of October 2009.

Isolation of bacteria

10µl of sea water sample was spread over the surface of the marine agar (Zobell Marine agar) with composition of Peptone 5.0 g, Yeast Extract. 1.0 g, Ferric Citrate 0.1 g, Sodium Chloride 19.45 g, Magnesium Chloride 8.8 g, Sodium Sulfate 3.24 g, Calcium Chloride1.8g, Potassium Chloride 0.55 g, Sodium Bicarbonate 0.16g, Potassium Bromide. 0.08 g, Strontium Chloride 34.0 mg, Boric Acid 2.0 mg, Sodium Silicate 4.0 mg, Sodium Fluoride 2.4 mg, Ammonium Nitrate 1.6 mg, Disodium Phosphate 8.0 mg, Agar 15.0. The individual red colony was taken and maintained as stock culture at 30 ºC in marine agar test tubes slants. The selected strain was streaked on the Petri plate and continued for the further studies.

Production of the red pigment from *vibrio sp*

A loopful of culture was inoculated to the pre-sterilized medium 50 ml Zobell marine broth. The flask was kept incubation for 16 – 18 h at 28 °C at stationary conditions. After the incubation time is completed 1 ml of the innoculum is transferred to the production medium 100 ml Zobell marine broth in 250 ml Erlenmeyer flask. The flask was incubated at 28 °C for 72 h.

Estimation of Pigment

After the incubation time is completed the culture was harvested by centrifuging at 10,000 rpm for 10 min. The supernatant was discarded and pellet was re suspended in methanol. The mixture was shaken well and the suspension was centrifuged at 5000 rpm for 10 min. The supernatant was collected in fresh vial and observed under UV-Visible spectrophotometer at 535 nm.

Optimization studies of pigment production

To study the effect of innoculum concentration of the

production of pigment the flasks were sterilized with equal volumes of zobell marine broth. After sterilization the flasks were inoculated with different volumes of inoculum which is of 0.2 optical density viz., 1, 2, 3, 4, 5 ml respectively. The flasks were incubated at 28 °C for 48 h. The pigment production was estimated after incubation. The inoculum concentration at which maximum production of pigment was observed was chosen and maintained in the following studies.

Effect of initial pH on the production of pigment

To study the effect of initial pH on the production of pigment the flasks with medium was set with different initial pH viz., 4, 5, 6, 7, 8 and 9. Equal volumes of inoculum were added to each flask and incubate at 28 °C for 48 h. The pigment production was estimated after incubation. The initial pH at which maximum production of pigment was observed was chosen and maintained in the following studies.

Effect of % NaCl concentration on the production of pigment

Equal volumes of bacterial isolated was inoculated in zobell marine broth with various initial concentrations of % NaCl viz., 4, 5, 6, 7, 8 & 9. The flasks were incubated at 28 °C for 48 h. The pigment production was estimated after incubation. The initial % NaCl concentration at which maximum production of pigment was observed was chosen and maintained in the following studies.

Effect of Temperature on the production of pigment

To study the effect of temperature on the production of pigment equal volumes of culture inoculum was inoculated in zobell marine broth and incubated at various temperatures viz., 15, 20, 25, 30 and 35 °C respectively for 48 h. The pigment production was estimated after incubation. The temperature at which maximum production of pigment was observed was chosen and maintained in the following studies.

Effect of different carbon sources on the production of pigment

To study the effect of different carbon sources on the pigment production different carbon sources like glucose, maltose, sucrose, fructose, mannose, glycerol and xylose were taken at 1 % concentration along with zobell marine broth. Equal volumes of innoculum were inoculated in to each flask and the flasks were incubated at 28 °C for 48 h. The pigment production was estimated after incubation. The carbon source which produces maximum production of pigment was observed and that carbon source was further optimized at different concentrations.

Effect of different nitrogen sources on the production of pigment

To study the effect of different nitrogen sources on the pigment production different carbon sources like yeast extract, peptone, beef extract, soyabean meal, malt extract and urea were taken at 1 % concentration along with zobell marine broth. Equal volumes of innoculum were inoculated in to each flask and the flasks were incubated at 28 °C for 48 h. The pigment production was estimated after incubation. The nitrogen source which produces maximum production of pigment was observed and that nitrogen source was further optimized at different concentrations.

Statistical Design studies

Identification of suitable variables using Plackett – Burman design. The Plackett – Burman experimental design identifies thecritical physico-chemical parameters required for elevated prodigiosin production by screening n variables in n + 1 experiments (Plackett and Burman, 1946). The variables chosen for the present study were inoculum size, pH, % NaCl (g/L) concentration, mannose as sole carbon source and incubation temperature (°C) for improved production of pigment. The experimental design for the screening of the variables was presented in Table 1. All the variables were denoted as numerical factors and investigated at two widely spaced intervals designated as -1 (low level) and +1 (high level) (Table 1).

RESULTS AND DISCUSSION

Isolation and identification of red pigment producing marine microbial strain

In the present study, pigmented marine bacteria were enumerated for the isolation of antagonistic marine microbes. Different water samples collected from marine source are immediately transferred to laboratory and screening was performed by using marine agar medium. Several different colour producing microbial strains were noticed on agar plates, however those who have shown prominent colour producing colonies were picked up for further studies. Among 32 different microbial colonies, a few was characterized with yellow, orange, red, pink colour etc. For further studies, only 9 colonies were selected with red pigmentation. On secondary screening, one colony with bright red colour pigmentation and showing antagonestic activity against other microbial strains was further selected for studies. The strain was identified based on lipid profile of the isolated strain and identified as *Vibrio sp.* (Kim et al., 2000). Isolation of such marine microbial strains with bioactive substances has been reported by Jayanth et al., 2002.

Production and estimation of red pigment

The isolated strain was grown in Zobell marine broth for 48 hrs. Growth was monitored for every 6 hrs. The growth and pigment readings were taken at 640 nm and 535 nm respectively and pigment production was represented as pigment produced per unit cell according to Mekhael and Yousif (2008) based on the following formulae.

$$\text{Pigment unit/cell} = \frac{[OD535 - (1.381 \times OD640)] \times 1000}{OD640}$$

OD 535 – pigment absorbance
OD 640 – bacterial cell absorbance
1.381 – constant

. Generally, pigments are organic in nature and whose extraction is effective with organic solvents. Keeping this in view, different solvents such as ethanol, methanol, distilled water, chloroform, ethyl acetate, petroleum ether and acetone have been tested to find the most suitable solvent for pigment extraction (data not shown). The concentration of extracted pigment was estimated by measuring the absorbance at 535 nm. The data revealed that the solvent plays a significant role in extraction of pigment from isolated bacterial strain. Methanol was the most and petroleum ether the least efficient solvent for the extraction of the pigment. Little traces of the pigment were detected in distilled water further supporting the hydrophobicity of the pigment. A similar observation has been reported regarding the extraction of the red pigmented compound prodigiosin from *Serratia sp.* (Kim et al., 1999).

Initial observation revealed that pigment production by this isolate is associated with its growth and noticed that pigment production is maximum at 18 hrs of growth (Fig 1) indicating pigment production is growth associated in this strain. The observed low pigmentation in the initial fermentation i.e., 6 hours of growth may be attributed to the lag phase of isolated strain in which cell reproduction is hardly seen through the cells are metabolically very active. Subsequent increase in pigmentation by more than several times 12 hours of fermentation do suggest that the selected microbial strain attains log phase within 12 hours and pigmentation is an associated product of growth of the cell. Maximized pigmentation was noticed when the cells reached to 18 hours of growth and any further increase in incubation time rather resulted in 10 to 20% decrease in pigmentation. This type of variation in pigmentation process by active isolated microbial cell further confirm that the pigment production by this strain is growth dependent and maximized pigmentation occurs during log phase of the growth. A slow decrease in pigment quantity during fermentation beyond 24 hours of fermentation may be attributed to the stability of the pigment against the other metabolic products produced and released during the stationary phase of isolate.

Effect of inoculum level on the production of pigment

Growth associated any product production any living entity is the consequence of metabolism and the rate of the production of product is directly proportional to microbial cell number during fermentation process. It was observed that several growth mediated enzyme/industrial product production by microbial strains is influenced by several parameters including initial inoculum level. To understand the role of initial inoculum on pigmentation process by this isolated microbial strain, pigment production against the fermentation time was studied with the supplementation of different initial inoculum levels under constant growth conditions. Analysis of pigmentation at 18 hours of incubation revealed variation in yield level. A maximum of 4300 pigment per unit cell was observed with 2% inoculum supplementation. Any variation in inoculum levels revealed reduced pigment yield (Fig 2). This reduction in pigment yield was lower with 1% inoculum compared to higher inoculum levels than 2%. This could be evidenced from the data represented in Fig. 3 where, a 10% reduction in pigment yield was observed with 1% inoculum compared to 2% inoculum while more than 20% reduction in pigment yield noticed with the inoculum levels above 2%. This higher inoculum mediated decrease in pigment yield was increased with increase in inoculum level beyond 2%. This decrease in pigment yield at 1% inoculum may be attributed to the limitation at isolated cell source in the initial levels and availability of the other nutrient requirements during fermentation process. Observed lower yields with increase in inoculum level may be associated with nutrient limitation or may be attributed to reduced lag phase with increase in inoculum levels.

Effect of pH and % NaCl concentration on the production of pigment

Any microbial growth is effective when it maintain a cytoplasmic pH in order to compatible with optimal functional and structural integrity of the cytoplasmic proteins that support growth. Though most non-extremophilic microbes grow over a wide range of external pH values ranging from 5.5–9.0, however maintain a cytoplasmic pH that lies within the narrow range of pH 7.4–7.8 (I R Booth 1985). The effect of pH value of the liquid medium on growth and pigment production by isolated marine microbial strain was studied by evaluating the bacterial growth in zobell marine broth prepared with different initial pH values (4.0 to 9.0). The bacterial isolate showed remarkable ability to grow and produce red pigment over a wide range of medium pH of 4.0-.

-9.0. However it showed its maximum growth and pigmentation efficiency at pH value of 7.0 - 8.0 (Fig 3). The growth associated pigment yield was very limited in the pH range of 4.0 to 5.0 while it increased more than 100% when the pH of the medium was increased to 6.0 whereas further increase in medium pH drastically improved the pigment yield suggesting that the growth of the isolated marine microbial strain is neutrophilic and acidophillic conditions retard the growth and associated pigmentation process in this strain. Growing the isolated in alkaline conditions especially at pH 8.0 resulted in effective pigment yield compared all other pH environments. Such pH mediated growth variations were reported in several microbial strains producing pigments of different nature (Krause et al., 2012).

One of the specific characters of marine microbial strains is their growth tolerance or adaptability towards salt concentration. Sodium chloride regulates many physiological function of halophilic bacteria especially helps in protecting the bacterium from changes in osmotic pressure between the internal and external environments. The salt also prevents osmotic stress associated swelling, deformation, busting and lysis of bacteria. In order to determine the effect of different concentration of salt, the bacterial isolate was grown in zobell marine broth containing different concentrations of sodium chloride (4.0 to 9.0%). The isolate showed its ability to actively grow and produce red pigment in the range of 4.0 to 9.0 per cent NaCl concentration but optimum growth and pigmentation was observed at 7.0 to 9.0 (Fig 4). A maximum of more than 8000 pigment per unit cell volume indicating sodium chloride is one of the major requirements for effective pigment production in this isolated strain. Requirement of NaCl for growth and pigmentation by different pigmented bacteria including Serratia marcescens have already been reported by Allen et al., (1983) and Silverman and Munoz (1973). The observed gradual increase in pigment yield as a function of unit cell of bacterium may be attributed to the sodium ion mediated metabolic changes in this bacterial strain. This could be further confirmed based on the reports where sodium ions are known to stimulate the activity of halophilic enzymes such as permeases, lipases, amylases and cytochrome oxidase (Mudryk and Donderski, 1991). In addition, it was also reported that in marine microbes, limitation of sufficient sodium ion concentration during growth results in inactivation of several enzymes involved in oxidation of sugars, amino acids and organic acids as well as oxygen uptake (Mudryk and Donderski, 1991). In the present study too, when sodium chloride concentration increased beyond 8% resulted in decrease in pigment yield which may be attributed to sodium ion mediated inhibition of oxygen uptake by the microbial strain which is similar to that noticed with *Achromobacter, Bacillus* and *Pseudomonas* (Mudryk and Donderski, 1991)

Effect of temperature on pigment production

Temperature is one of the environmental factors thought to shape the abundance as well as geographic locations of different species. Past research has provided a strong theoretical framework for the relationship between temperature and phytoplankton growth and physiology. This becomes more prominent when salt is present in the medium as one of the components as salt concentration reduces the temperature of the medium more pronouncedly compared to medium without high salt concentration. Keeping these in view, the effect of temperature on the pigment production at different temperatures (15, 20, 25, 30 and 35 °C) was investigated. The data revealed that pigment yield varied with variation in incubation temperature and effective pigment yield was noticed with culture incubated at 25 °C (data not shown). Any variation in this incubation temperature resulted in reduction of pigment yield which further confirm that pigmentation by this isolate is associated to metabolic activity of bacterium.

Effect of carbon and nitrogen sources on the production of pigment
Carbon sources

The bacterial strain *Vibrio sps*, showed its ability to utilize all the different carbon sources for its growth and pigment production but to different extents. Mannose and maltose are most efficiently utilized carbon sources whereas glucose and xylose are the least utilized carbons fructose and sucrose are moderately utilized (Fig. 5). This study indicated that metabolism associated with mannose and maltose utilization rather than conventional carbon source metabolism i.e. glucose catabolic pathway is an important to produced more pigment. To evaluate further, the influence of mannose concentration mediated pigmentation process in this microbial strain, the pigment yield was measured during fermentation produced with the supplementation of different concentration of mannose as carbon source. Among different concentrations of mannose (4 % to 9 %) studied, 4 % mannose concentration supplementation is optimum for the maximum production of pigment. However, some other *S. marcescens* strains have also been reported which could utilize ethanol as sole carbon source for growth and pigment production better than glucose and could be attributed to difference amongst strains (Cang et al., 2000). Pigment production by *Serratia marcescens* has also been reported to be highly variable among its strains and is dependent on several cultural and nutritional parameters (Giri et al., 2004).

Plackett-Burman Design

The results (Table 2) indicated that there was a wide variation of total pigment yield in the thirteen trials (14266 -32643 pigment unit/cell) and maximum and minimum yield was noticed with 8th and 12 set of experiment. These variations reflected the importance of medium optimization to obtain higher prodigiosin yield. Less than 10% variation was noticed between experimental and predicted values (Table 2) suggesting the confidence of experimentation similar to noticed by Hymavathi et al., (2010). The medium components were screened and those with a p – value of < 0.1 using 90 % confident level were accepted as significant factors affecting the production of prodigiosin. From the pareto chart of standardized effects (Fig. 7) it was observed that factors C (% NaCl), D (mannose concentration) and E (Incubation temperature) are mainly effecting the production of pigment. Kim et al. (2008) selected five medium components ($CaCl_2$, $Na2SO_4$, $Na2SiO_3$, $NaHCO_3$ and $NH4NO_3$) through Plackett – Burman design for prodigiosin production by *Hahella chejuensis* KCTC 2396. These results indicated that the Plackett – Burman design is a powerful tool for identification of the variables that could significantly affect prodigiosin production. Further analysis of the selected factors and their levels denoted that all the parameters revealed a positive influence on pigment production at

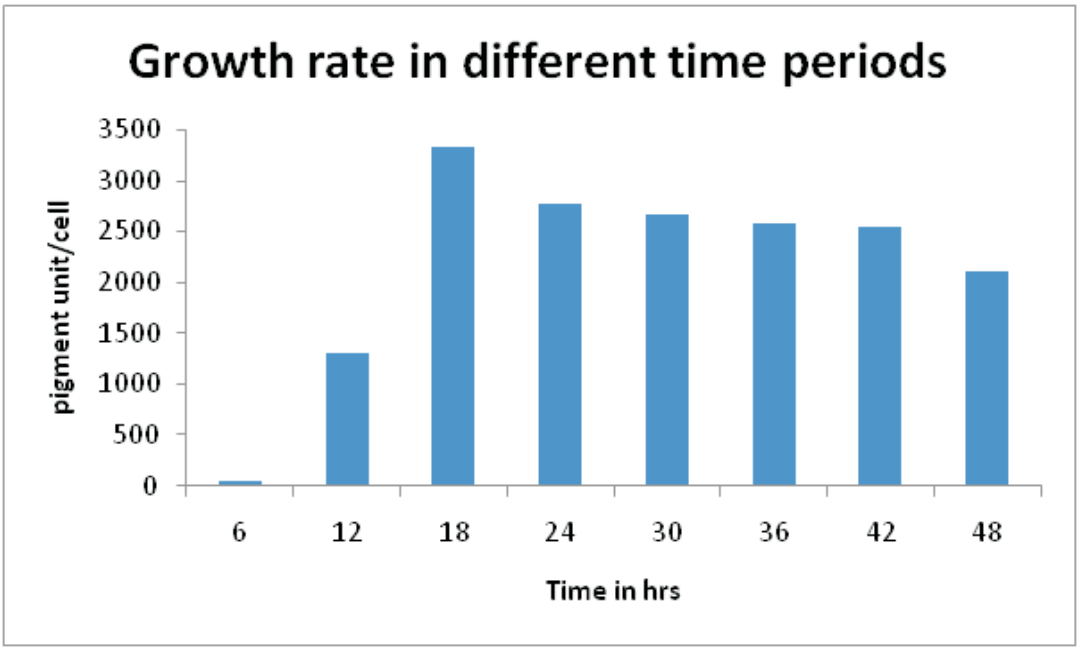

Fig 1: Isolated marine microbial strain growth and pigmentation pattern

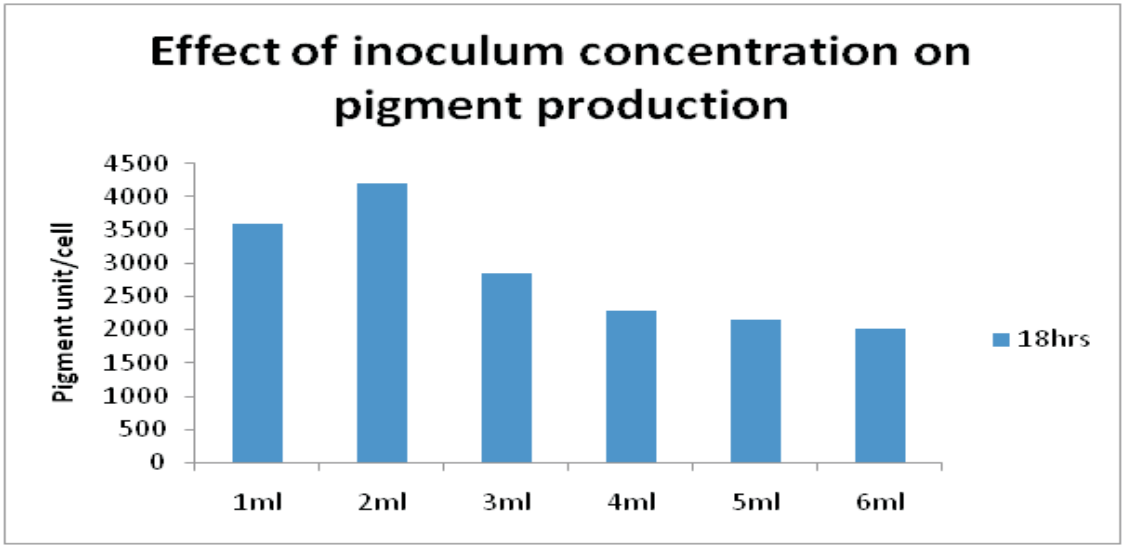

Fig 2: Influence of inoculum level on pigment yield by isolated marine microbial strain.

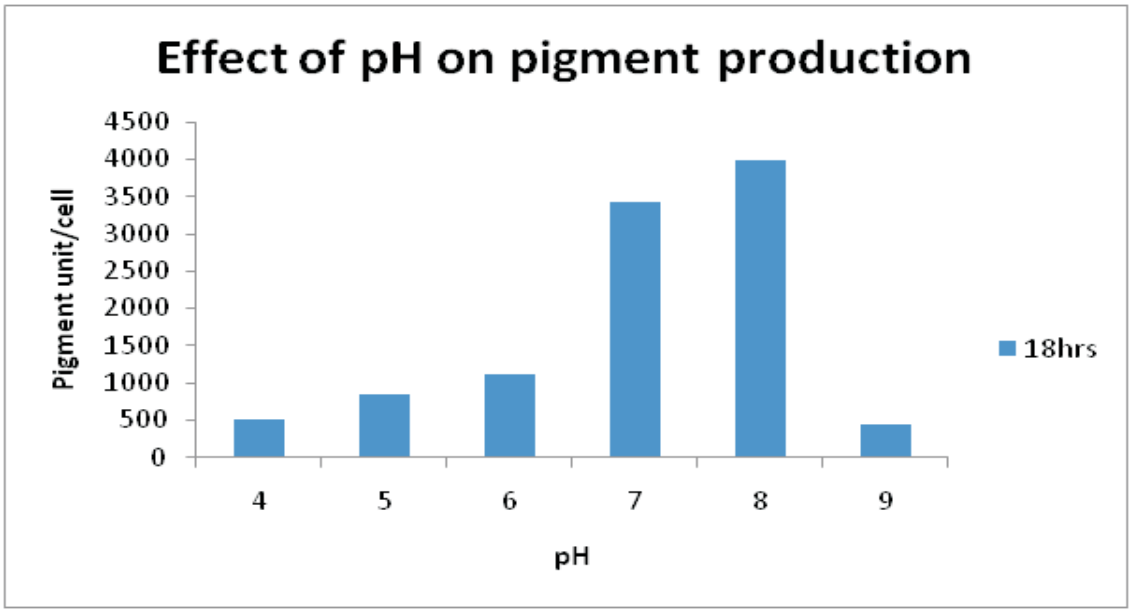

Fig 3: Effect of medium pH on pigment yield by isolated marine microbial strain

Table 1: Experiment layout for improved production of pigment by marine isolate

Alphabet	Factor	0	1	-1
A	Inoculum (ml)	2	3	1
B	pH	8	12	4
C	% NaCl (g/L)	8	12	4
D	Mannose (g/L)	4	7	1
E	Temperature (^{o}C)	28	38	18

individual level (Fig 7) however the trend varied with parameter with increase in their concentration during fermentation. This variation trend was in the order of temperature, mannose, sodium chloride concentration, inoculum and pH (Fig 7). This further confirm the pigment production metabolism influenced majorly by the nutritional parameters especially carbon source but not nitrogen source. Critical analysis of interactive influence at two factor level revealed that incubation temperature did not show any interactive influence with carbon source, sodium chloride and pH of the medium and showed interaction with inoculum concentration (Fig 8). This may be attributed to the fact that incubation temperature is one of the critical parameter

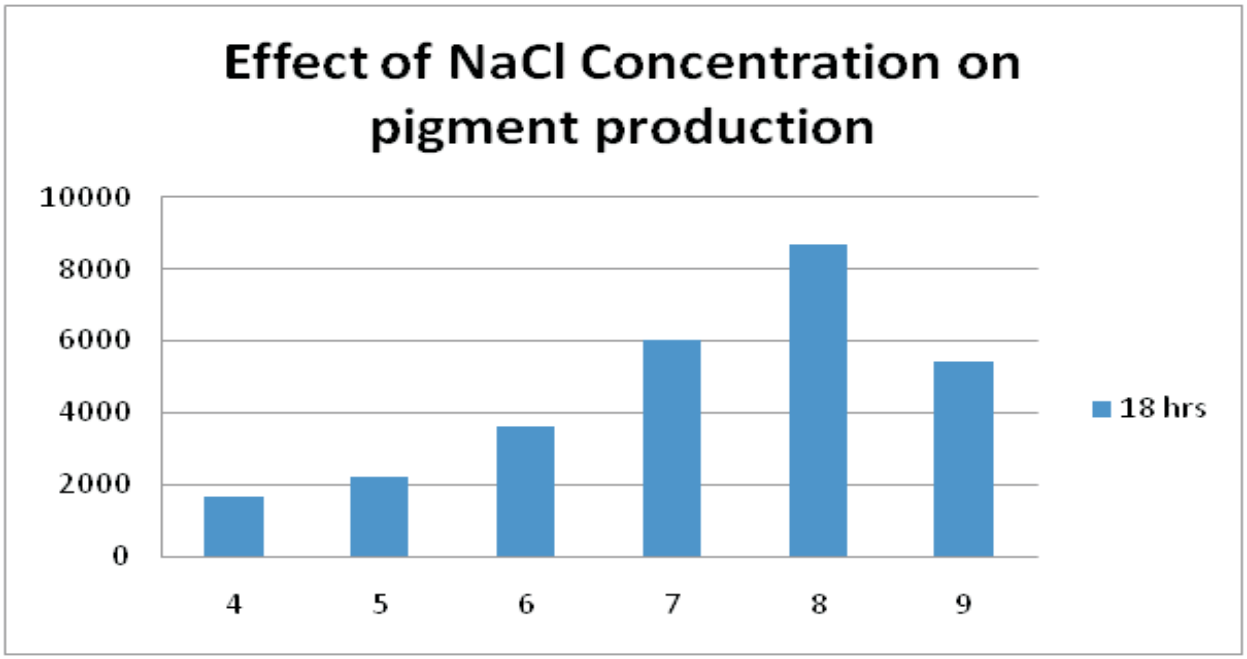

Fig 4: Effect NaCl concentration on pigment yield by isolated marine microbial strain

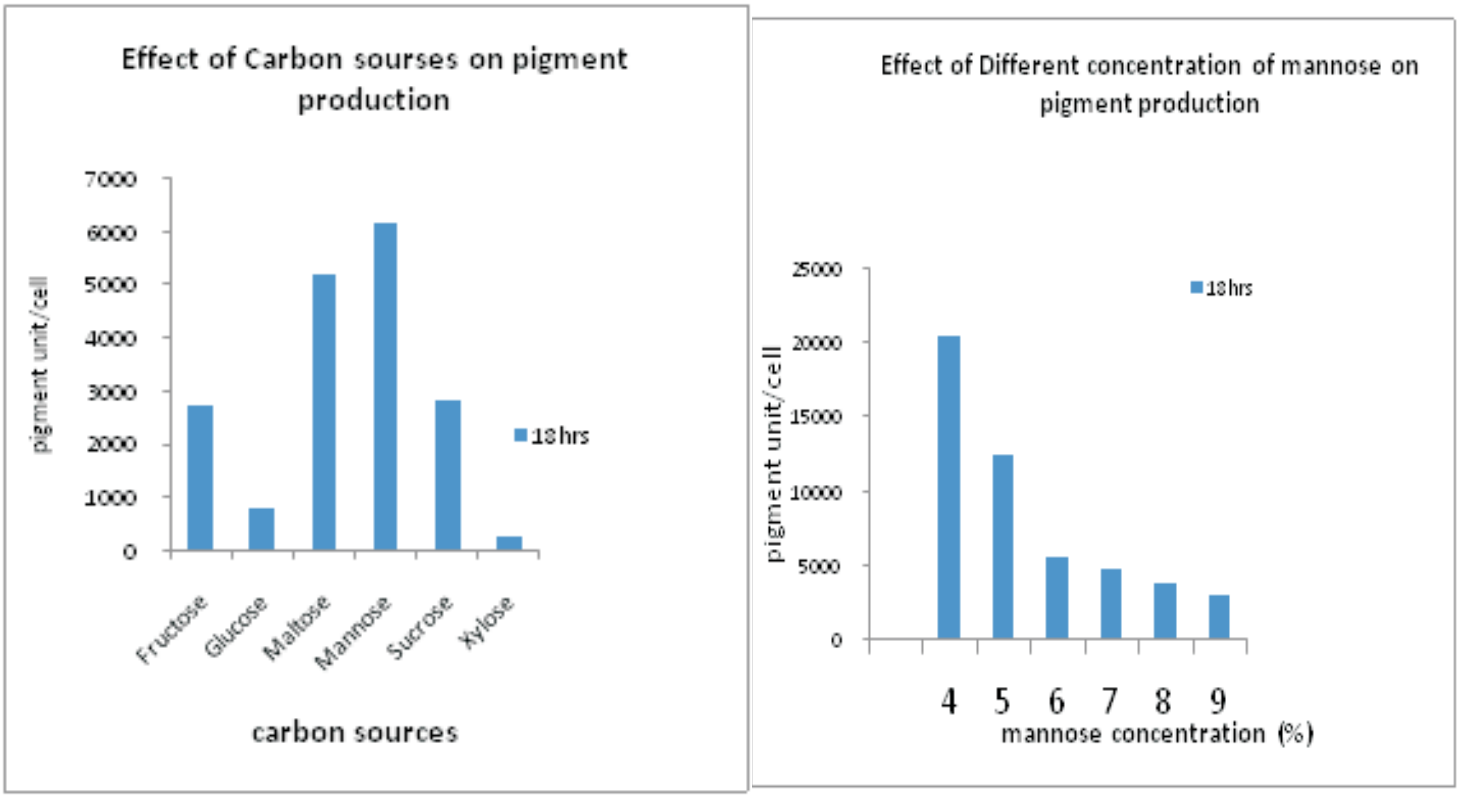

Fig 5: Effect of carbon sources on pigment yield by isolated marine microbial strain

which regulate the entire metabolism and optimized temperature is essential for survival of microbes. The observed increasing trend of pigment production by this isolate even at third level of incubation temperature further suggest that the pigment present in the intracellular level might have leached to fermentation medium due to fermentation at elevated temperature. Under selected parameter concentrations, carbon source (mannose) concentration revealed interactive role with all selected parameters except temperature (Fig 8). Similar trend was noticed with sodium chloride interaction with pH and inoculum. Overall, the interaction wherever observed was noticed above 20000 pigment unit/cell concentration level suggesting below 20000 pigment unit/cell level, all these parameters have little interference with one another at pigment production metabolism of this isolate.

CONCLUSIONS

The pigment yield by isolated marine microbial strain, *Vibrio sp*. was observed to be regulated mainly by un conventional carbon source and its type as common carbon source i.e. glucose showed little impact on pigment production while one fold increase in pigment yield was noticed with mannose supplementation. Natural habitat is another influential factor for effective pigment yield by this isolate. Sodium chloride played significant role in improving the pigment yield. Incubation temperature is most prominent physiological factor which regulated the pigmentation yield. Over all the combination these three factors resulted in improving the pigmentation yield more than seven folds indicating the imperative role of growth parameters and their concentrations in regulation of metabolically mediated pigment production in this isolate. The statistical approach applied in this study could be successfully applied to any bioprocess, where an analysis

Table 2: Screening of factors using Placket – Burman design for prodigiosin production by Vibrio species

RunOrder	A	B	C	D	E	Results	Predicted values
1	1	-1	1	-1	-1	20768	20128
2	1	1	-1	1	-1	23789	23294
3	-1	1	1	-1	1	27645	26078
4	1	-1	1	1	-1	26987	26404
5	1	1	-1	1	1	30749	30003
6	1	1	1	-1	1	25735	27497
7	-1	1	1	1	-1	23672	25645
8	-1	-1	1	1	1	32643	31694
9	-1	-1	-1	1	1	27127	27924
10	1	-1	-1	-1	1	22367	23067
11	-1	1	-1	-1	-1	16529	15599
12	-1	-1	-1	-1	-1	14266	14939
13	0	0	0	0	0	22373	22373

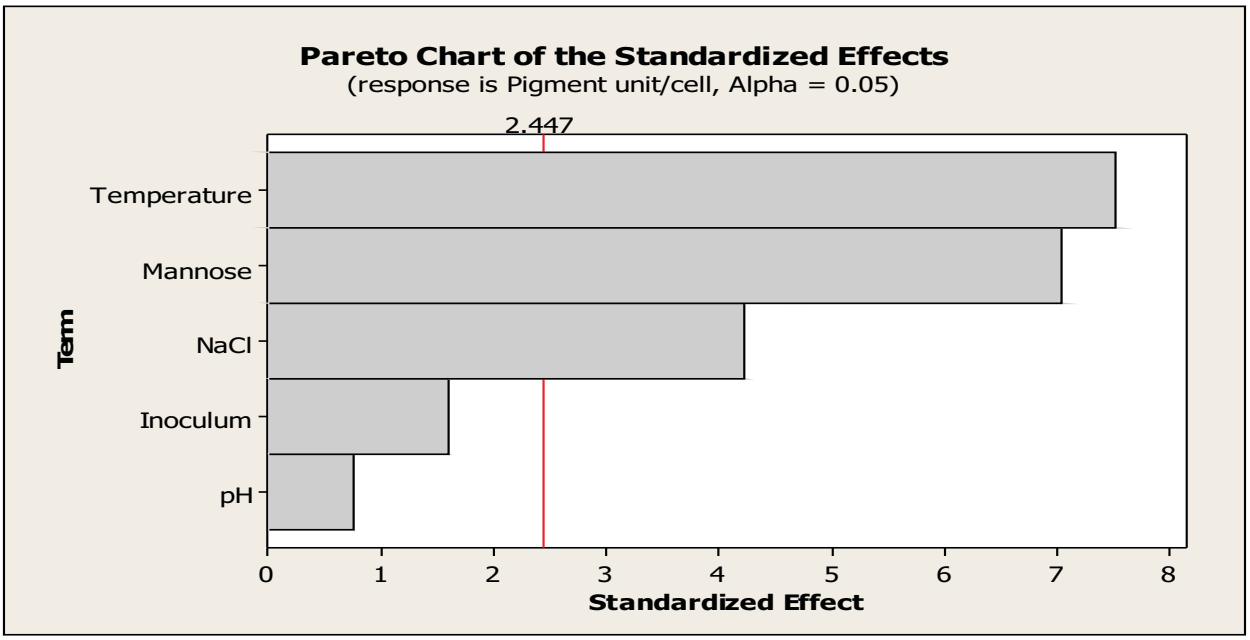

Fig 6: Pareto chart of standardized effects

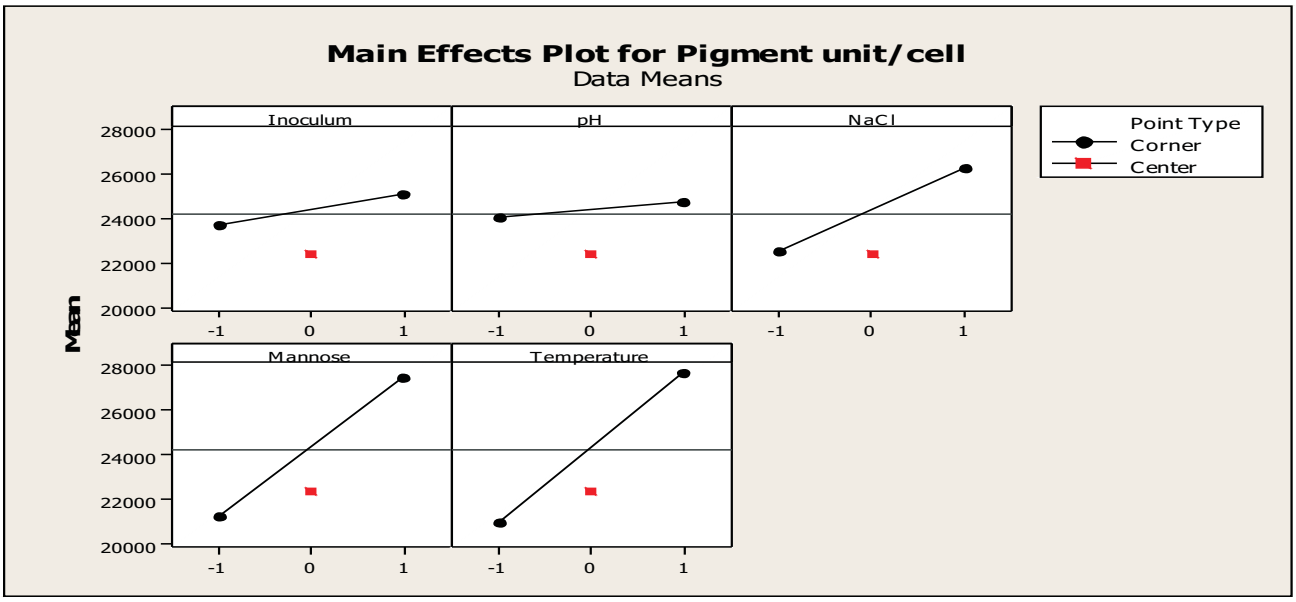

Fig 7: Influence of individual factors on the production of red pigment

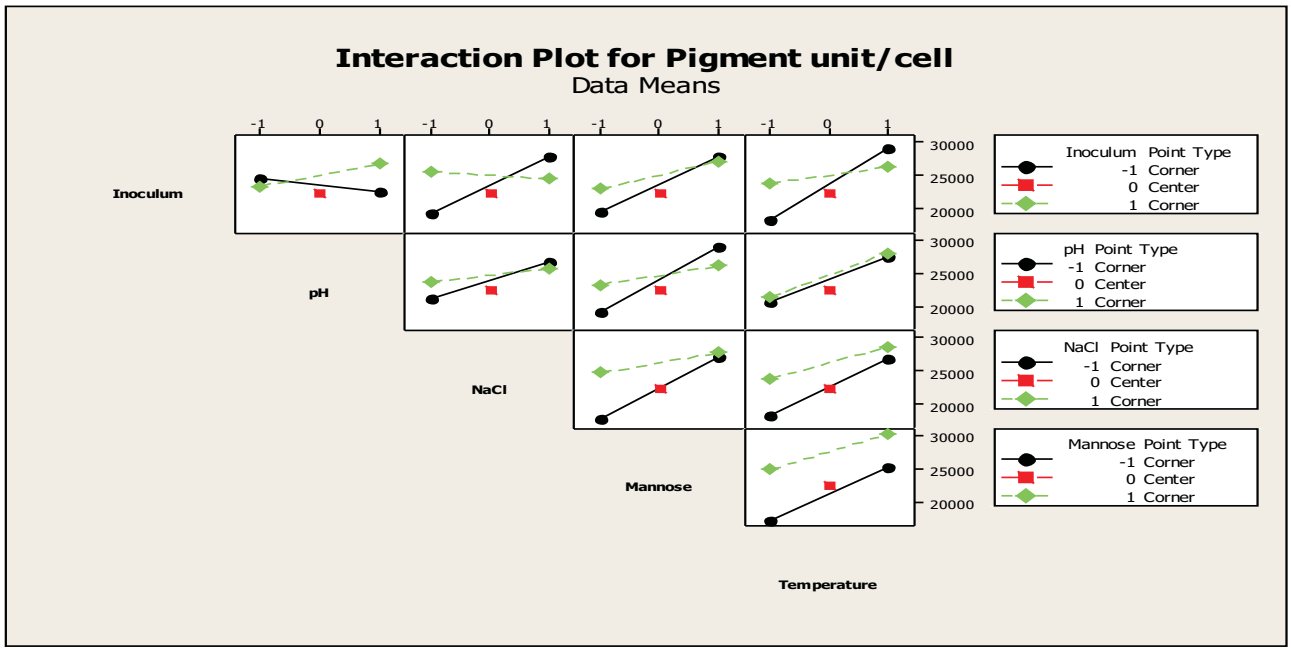

Fig 8: Influence of interaction parameters on the production of red pigment

of the effects and interactions of many experimental factors are mandatory. Software studies further improved the pigment yield and among selected parameters, incubation temperature, carbon source and sodium chloride played significant role than inoculum and pH of the medium at all selected concentration level.

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