



## Research Article

# Purification and optimization of pink pigment produced by newly isolated bacterial strain *Enterobacter* sp. PWN1



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Received: 2 October 2020 / Accepted: 4 January 2021 / Published online: 11 January 2021

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## Abstract

Pigment-producing bacteria were isolated from kitchen wastewaters of the National Institute of Technology, Rourkela. A pink non-virulent bacterial strain PWN1 was selected based on the India Ink Broth and Coomassie Brilliant Blue (R-250) dye assay. According to morphological and biochemical characterization, the strain PWN1 was a Gram-negative, rod-shaped, motile, non-coliform bacterium and could utilize only glucose and adonitol as sole carbon source. The pigment was found to be a growth-associated product, and the pigment production was accelerated after 40 h of bacterial culture. Further, 16S rRNA gene-based molecular identification showed its similarity with *Enterobacter* sp. The pigments were extracted by the solvent extraction method using chloroform and ethanol (3:1). The extracted pigments were then purified through thin-layer chromatography and column chromatography. To maximize pigment production, the culture condition was optimized for maximum biomass production using statistical software Design Expert v13. A quadratic model was structured describing the process efficiently and it suggested a moderate temperature, pH, and a high inoculum concentration which generated biomass of  $3.81 \pm 0.02$  g/L. At optimized condition, 1 L of cell culture produced 3.77 g of biomass which produced a crude pigment of 0.234 g after solvent extraction and 0.131 g after column chromatography, implying a yield of 6.2% for crude pigment and 3.47% for purified pigment from biomass. The yield of the obtained pigment was high enough to draw interest for industrial production, although the application of the pigment is considerable for further study.

**Keywords** Bacterial pigment · Solvent extraction · Chromatography · Optimization

## 1 Introduction

Pigments are colorful secondary metabolites produced by bacteria for survival advantage. Bacteria thriving in harsh and extreme environments suffer from stress and damage to bacterial cells which force them to come up with a mechanism for cell adaptation; pigment production is one such mechanism [1]. For instance, bacteria in the cold region such as the Antarctic suffer from both low

temperatures as well as constant UV exposure, therefore bacteria tend to produce pigments like carotenoids which protect against cellular damage due to UV exposure, allowing bacteria to survive through the environment [2]. It has also been shown that colored bacteria have a better chance of survival when exposed to ozone and peroxide than non-pigment-producing bacteria [3]. Pigment production in bacteria not only provides protection but also serves many other functions such as resistance to other

**Supplementary information** The online version contains supplementary material available at (<https://doi.org/10.1007/s42452-021-04146-x>).

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SN Applied Sciences (2021) 3:105 | <https://doi.org/10.1007/s42452-021-04146-x>

microbes and phagocytosis and light-harvesting molecules for photosynthesis. It also serves as a basis for the taxonomic classification of bacteria.

Bacteria producing pigments can be found in a wide variety of habitats ranging from oceans to rhizospheric soil [4], Antarctic regions [2] to desert areas [5] from tree bark to insect gut [6]. The bacterial strains responsible for the production of pigments are as vast and diverse as their niche. Some pigment-producing bacterial strains are listed in Table 1. The most common bacterial genus responsible for the production of pigments includes *Streptomyces* spp., *Staphylococcus* spp., *Arthrobacter* spp., *Pseudomonas* spp., and *Chryseobacterium* spp. producing diverse pigments including carotenoids, flexirubin, prodigiosin, pyocyanin, violacein, staphyloxanthin, melanin, flavonoids, etc. [7, 8].

Pigments obtained from bacteria find applications in various industries such as textiles, food, pharmaceuticals, cosmetics, and others. In the textile industry, pigments like prodigiosin (red) and pyocyanin (blue) have been used to dye fabrics that curbed the growth of microorganisms like *Staphylococcus aureus* and *Escherichia coli* [18, 16]. Similarly, violacein pigment obtained from *Janthinobacterium lividum* can be used for dyeing fabric. The antimicrobial, antioxidant, antileishmanial, anticancer, antiviral properties of bacterial pigments prove their potential in pharmaceutical applications. Carotenoids produced by bacteria of *Arthrobacter* sp. and *Staphylococcus* sp. have shown anticancer properties against cancer cell lines and thus can be used as anticancer drugs. Wound healing activities are also observed in certain bacterial pigments such as pyranone [6, 10, 15]. Apart from industrial and pharmaceutical use, bacterial pigments can also be used for application in solar cells as dye sensitizers [2].

Bacteria are preferred over other sources for pigment production because of several advantages. A shorter life cycle and compatibility with environmental changes make

bacterial source ideal for pigment extraction. Apart from this, bacterial cultures are easy to maintain and scalable for industrial use and can be grown using cheap, easily available, renewable sources that reduce the cost of production. Besides being natural, bacterial pigments also possess various properties such as antibacterial, antimicrobial, antioxidant, antileishmanial, anticancer, and antitubercular activities which increases their potential use in industries such as textile, food, cosmetics, and pharmaceuticals. To replace the hazardous synthetic pigment industry and meet the market demand, different colors and shades of bacterial pigments need to be discovered. Kitchen wastewater is a nutrient-rich microbial reservoir with previous reports suggesting it as a source of much resourceful bacterial communities. In that context, the present study was focused on isolation, purification, and optimization of bacterial pigment from kitchen wastewater.

## 2 Materials and methods

### 2.1 Screening and isolation of pigment-producing bacteria

Kitchen wastewater samples from the National Institute of Technology Rourkela, India (22.2514° N, 84.9115° E) were collected in clean, sterile containers and the temperature of the sample at the time of collection was measured to be  $\sim 30 \pm 0.4$  °C with a pH of about  $7.5 \pm 0.2$ . The water sample was serially diluted at  $10^{-7}$  dilution rates, using autoclaved distilled water, and 250  $\mu$ L of the diluted samples were spread on sterile R-2A (M1687, Himedia, India) agar plates with media composition for 1000 ml: Casein acid hydrolysate: 0.5 g, Yeast extract: 0.5 g, Protease peptone: 0.5 g, Dextrose: 0.5 g, Starch, soluble: 0.5 g, Dipotassium phosphate: 0.3 g, Magnesium sulfate: 0.024 g, Sodium

**Table 1** Application of different pigments obtained from bacteria

No.	Bacteria	Pigment	Application	References
1.	<i>Hymenobacterium</i> sp. <i>Chryseobacterium</i> sp.	Carotenoids	Solar cells	[2]
2.	<i>Serratia</i> sp.	Red pigment	Antimicrobial	[9]
3.	<i>Arthrobacter</i> sp.	Carotenoids	Anticancer activity	[10]
4.	<i>Streptomyces</i> sp.	Undecylprodigiosin	Pharmaceutical activities	[11]
5.	<i>Streptomyces bellus</i>	Unknown	Antioxidant activity	[12]
6.	<i>Cellulophaga fucicola</i>	Carotenoid	Antioxidant activity	[13]
7.	<i>Serratia marcescens</i>	Prodigiosin	Textile dye	[14]
8.	<i>Vibrio</i> sp.	Pyranone	Wound healing and anti-bacterial activity	[15]
9.	<i>Pseudomonas aeruginosa</i>	Pyocyanin	Textile dye	[16]
10.	<i>Staphylococcus gallinarum</i>	Staphyloxanthin	Anticancer activity	[6]
11.	<i>Janthinobacterium lividum</i>	Violacein	Fabric dye	[17]

pyruvate: 0.3 g, Agar powder: 1.5%. The plates were then incubated at 30 °C for 3 days. Pure cultures of the pigment-producing bacterial strains were obtained by subsequent plating and culturing of the colonies from culture media to fresh media.

## 2.2 Virulence assay

The pigment-producing bacterial colonies were screened based on their inherited virulence. The cultures were primarily examined by filtered India Ink (CAMLI-SOL 100, Kokuyo Camlin Ltd.) using 0.22 µm syringe driven filters (SF-126, Himedia, India) in R-2A broth [19]. R-2A broth of 5 ml containing 1% of the filtered ink was inoculated with 250 µl of freshly grown bacterial cultures and incubated at 30 °C for 48 h. The absorption of the ink from the culture broth by the bacterial cells was measured by evaluating the transmittance of the cell-free culture broth using UV 3200 UV-Vis Spectrophotometer (Lab India, India) at 600 nm wavelength. The pathogenic trait of the bacterial strains was further confirmed by streaking the fresh bacterial cultures on 0.01% Coomassie Brilliant Blue (CBB) R-250 (MB153, Himedia, India) containing R-2A agar plates incubated at 30 °C for a period of 36 h [20].

## 2.3 Characterization of pigment-producing strain

### 2.3.1 Biochemical characterization

The bacteria were characterized by Gram staining using Gram Staining Kit (K001) from Himedia, India [21]. The spore-forming nature of the isolates was identified by the standard procedure using malachite green for 10 min on the pre-heat-fixed smear that stains up the endospores produced by the bacteria on exposure to stress [22]. The motility of the bacteria was identified by the standard hanging drop method, using fresh cultures on concavity slides and their visualization under 1000X microscopic magnification [23]. The microstructure was examined under Quanta 600 FEG Environmental Scanning Electron Microscope (ESEM) (FEI, Japan) to identify the cellular dimensions. Fresh cultured cells were smeared on carbon tape to observe under 15 kV power and 300 Pa pressure at a magnification of 50000X [24]. The coliform nature of the isolated strain was determined using the IMViC Test Kit (Himedia, India) which included indole, methyl red (MR), Voges-Proskauer (VP), and citrate utilization tests. The different forms of carbohydrate compounds utilized by the strains were separately examined, which included glucose, adonitol, arabinose, lactose, sorbitol, mannitol, rhamnose, and sucrose [25, 26].

### 2.3.2 Molecular identification and phylogenetic analysis

The molecular identification of the bacterial isolate was done by partial sequencing of the 16S rRNA gene. For this purpose, the genomic DNA of the bacterial strains was isolated following the phenol-chloroform-isoamyl (PCI) alcohol extraction method using a standard protocol. The 16S rRNA gene from the genomic DNA was extracted out and amplified using hot-start PCR (Veriti 96-Well Thermal Cycler, Applied Biosystems, Thermo Fischer Scientific, Singapore). For this purpose, 27F forward and 1492R reverse universal degenerative primers were used [27]. The initial hot stage was conducted by maintaining 94 °C for 5 min which is followed by 35 cycles comprising a denaturation stage of 94 °C for 15 s, an annealing stage of 52 °C for 50 s, and an elongation stage of 72 °C for 90 s. At the end of the 35 cycles, a concluding extension step was incorporated for 10 min. The PCR amplified DNA was further purified using Gel Extraction Kit (Genetix Biotech Asia Pvt Ltd., India) before outsourcing for the sequencing from Eurofins Sequencing Services (Eurofins Genomics India Pvt Ltd., Bangalore, India). The BioEdit tool (v7.0.5) was used to remove the noise and low signals from the obtained sequencing results. The homology analysis was conducted to find out related strains using the BLASTN tool of the National Centre for Biotechnology Information (NCBI) database. A phylogenetic tree was composed considering the neighbor-joining method using the sequences obtained from the BLAST analysis after a multiple alignment analysis using the CLUSTALW algorithm in MEGA-7 [28]. The identified sequence of the non-virulent pigment-producing strain was submitted to GenBank with Accession number MK943548.

### 2.4 Production and extraction of pigment

The non-pathogenic pigment-producing PWN1 strain was cultured in sterile 100 ml R-2A broth at 30 °C for 7 days with 140 rpm rotational shaking. The extraction of the intracellular pigments from the broth was carried out using a modified solvent extraction procedure [29]. To determine the best solvent for the extraction of intracellular pigment, different polar solvents viz. ethanol, methanol, and acetone, and non-polar solvents viz. diethyl ether, hexane, and chloroform were examined. For each solvent initially, 15 ml of 7 days old culture was centrifuged at 7800 rpm at 30 °C for 15 min to obtain the cell pellet followed by dissolving the pellets in 5 ml of the solvent. The cells in the solvent were then subjected to ultrasonication treatment digital ultrasonic cleaner (LCMU-2, Labman Scientific Instruments, India) till the cells turn white. The mixture was then centrifuged at 4 °C, 7800 rpm for 15 min to extract the pigment from the cell debris.

## 2.5 Purification of pigment

The methanol extracted pigment was eluted using different solvent mixtures on pre-coated thin-layer chromatography (TLC) plates from TLC Silica gel 60 F<sub>254</sub> (Merck, India). Different solvent mixtures with varying proportions like methanol:acetone:water (4:4:2); ethanol:water:chloroform (4:2:4) and (4:4:2); hexane:acetone (3:2) and (4:1); petroleum ether:ethyl acetate (9:1) and (1:9); chloroform:ethanol (2:1) and (3:1) were examined as the mobile phase to separate the different compounds of the pigment [29, 30]. The sample was loaded onto the plate at a height of 1 cm from the bottom, and the plate was then placed in the different developing chambers containing the solvent ratios. After the solvent front reached the marked line, the TLC plates were visualized under UV light. The retention factor ( $R_f$ ) was calculated from the distances traveled by the solvent and the pigment compound following Eq. 1 [31].

$$\text{Retention factor } (R_f) = \frac{\text{Distance traveled by compound}}{\text{Distance traveled by the solvent}} \quad (1)$$

The pigment was further purified by column chromatography to separate the different fractions using Silica Gel G (GRM7480, Himedia, India) suspended in hexane. The elution of the target band was obtained by the best performing elution mixture obtained from the TLC analysis. As the elution proceeded, readily separable bands of the pigment were collected in separate test tubes from the outlet. TLC was performed for each elutes produced to check the homogeneity and similarity of the compound purified out from the column. The purified compound was then collected in a round bottle and dried in a rotary evaporator to form a powder.

## 2.6 Optimization of cell growth

The pigment produced by isolated bacterial strain was found to be intracellular, hence to maximize the pigment production the biomass production had to be maximized. To achieve this, the biomass production was optimized considering the 3 most important process parameters which included, temperature coded as A, pH coded as B, and inoculum percentage coded as C. As changing the pH at minute level is very difficult to achieve and hard to maintain, the pH was considered as hard-to-change (HTC) factor. The range of the temperature for the optimization study was considered 25–40 °C, pH 5–9, and inoculum percentage 1.5–3.5%. A starter culture with cell concentration 0.28 g/L (OD=0.52) was used as inoculum culture. Design Expert trial version (v13) was used to construct the central composite design (CCD) which suggested 23

runs. The runs were performed randomly in triplicates for 3 days. After analyzing the response, a model equation was suggested by the software. The accuracy of the suggested model was statistically examined by evaluating the different parameters which included, SSR (regression sum of squares), SSE (error sum of squares), and SST (corrected sum of squares). Moreover, the significance of the model was statistically evaluated by Fisher's *F*-test, and the fit quality was determined by the coefficient of determination ( $R^2$ ). The model showing a minimum 95% level of confidence was considered significant. Response surface methodology (RSM) analysis was performed to construct the contour graph representing the interactions between the different process parameters and used to determine the optimum region to get the maximum biomass yield. Based on this analysis, the software suggested a probable solution for the highest biomass yield as a particular temperature, pH, and inoculum concentration. The practical biomass yield in the suggested physiological condition was determined and analyzed to establish the acceptance and reliability of the suggested process model.

## 2.7 Kinetic study and pigment yield calculation

Kinetic study on cell growth and pigment production of bacterial strain *Enterobacter* sp. PWN1 was conducted in 3 L of R-2A broth at optimized conditions [32]. Hundred ml of samples were collected at certain time intervals, and cell mass was obtained by centrifugation. From the collected cell mass, the crude pigment was extracted and weighed. The cell mass and pigment recovered data were converted to g/L and plot against time (h). The specific growth rate and specific pigment production rate were calculated. Moreover, a separate 1 L of culture was maintained for 3 days at the optimized condition to calculate pigment yield from biomass.

## 2.8 Statistical analysis

All the experiments were conducted in triplicates, and the obtained results were represented as the mean value. Different individual experiments were considered significant showing a relative standard deviation of less than 0.5%.

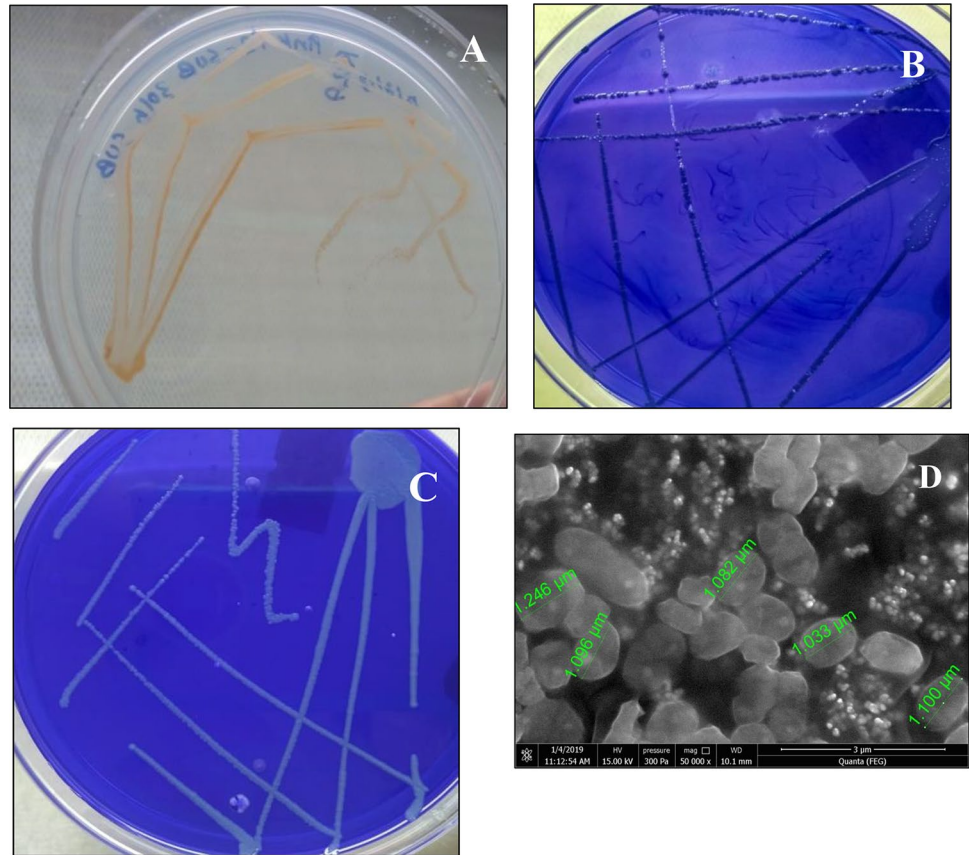
## 3 Results and discussion

### 3.1 Isolation and characterization of pigment-producing bacteria

From the spread plate, 2 different pigment-producing bacterial colonies (PWN1 and PWp1) were identified and subsequently streaked repeatedly to obtain a pure culture



**Fig. 1** Isolated pigment-producing strain. **a** Pigmented colonies produced by isolated bacterial strain *Enterobacter* sp. PWN1 on R-2A agar plate. **b** Pathogenic strain PWP1 producing blue-colored colonies. **c** Non-pathogenic strain PWN1 producing white-colored colonies. **d** Environmental Scanning Electron Microscopic (ESEM) image of PWN1 with cell length in a range of 1–1.3  $\mu\text{m}$



of the strains (Fig. 1a). The result from the India Ink experiment showed a higher transmittance value of the cell-free culture broth for strain PWP1 and also exhibited blue colonies in the CBB agar plate which indicated the presence of an A-layer (protective protein layer outside the cell wall). The presence of this A-layer is a trait of a virulent bacterial strain [19]. Hence, the strain PWP1 was suspected with inherent pathogenicity (Fig. 1b). On the other hand, the cell-free culture broth of the strain PWN1 showed lower transmittance value in the India Ink test and also produced white colonies in CBB signifying the absence of any such protein layer outside the cell wall (Fig. 1c) [20]. Therefore, the PWN1 strain was considered to be non-pathogenic and safe for further use. Giving importance to the laboratory safety issue, further experiments were carried out only with the PWN1 strain. The bacterial morphological study revealed that the strain PWN1 was Gram-negative, oval-shaped, and non-endospore forming. The strain was found motile when observed under 1000X microscopic magnification during the hanging drop analysis. Environmental scanning electron microscope analysis of strain PWN1 showed that the isolated cells were short oval-shaped with an average cell length of 1.11  $\mu\text{m}$  which was similar to the cellular morphology of *Enterobacter* sp. where the length is measured in the range of 0.8–2  $\mu\text{m}$  (Fig. 1d)

[33]. The culture broth of the bacterial strain showed no color change in the indole production test indicating its inability to produce indole from tryptophan. A similar negative result was obtained in the methyl red test as the color of the culture broth remained yellowish-orange. It signifies that the bacterial strain was not producing acid. In the Voges-Proskauer test, the color of the culture broth remained colorless indicating no production of acetoin by glucose fermentation. The strain was also found unable to utilize citrate as a carbohydrate source since no color change was observed in the citrate utilization assay. Moreover, the bacterial strain exhibited a narrow preference for carbohydrates as it was observed that it could survive only in the presence of glucose and adonitol, whereas other carbohydrate sources which included arabinose, lactose, sorbitol, mannitol, rhamnose, and sucrose-containing media was incapable to support the bacterial growth.

The obtained partial 16S rRNA sequence of the strain PWN1 was initially analyzed in BLAST. The report suggested its high similarity with *Enterobacter* sp. 90-A1 (KC920583), *Pantoea agglomerans* FL85 (KY818994), *Enterobacter cancerogenus* UW11 (KP209255), and other bacteria from *Enterobacteriaceae* family. But phylogenetic analysis using MEGA7 suggested that the PWN1 strain was situated in the same clad with *Enterobacter* sp.

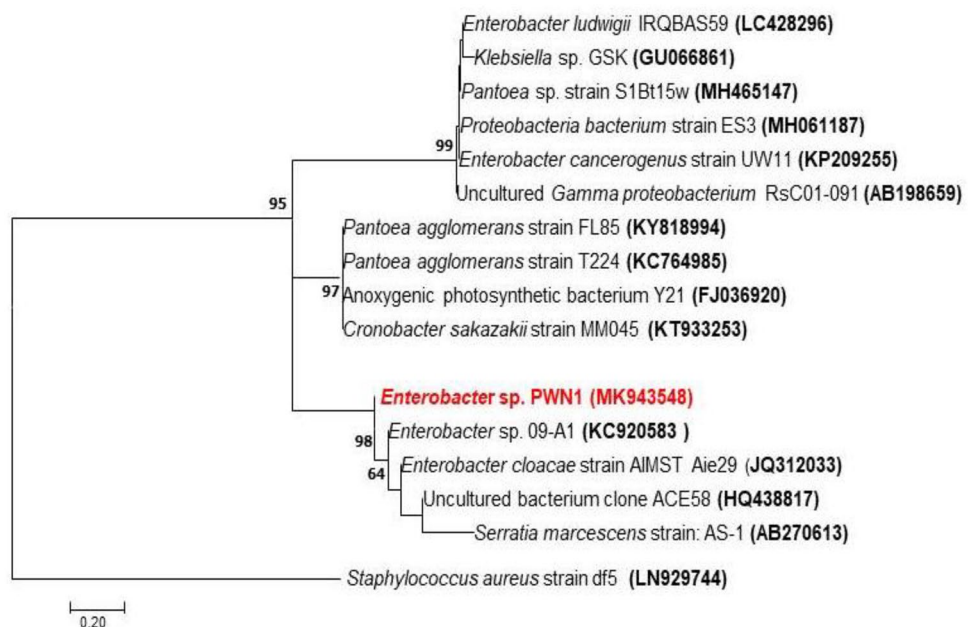
90-A1 (KC920583), and *Enterobacter cloacae* AMST Aie29 (JQ312033) with a bootstrap value of 98 and 64%, respectively (Fig. 2). Another residence of the clad was *Serratia marcescens* strain AS-1 (AB270613). Among these bacteria, *E. cloacae* and *S. marcescens* already have reports of yellow and pink pigment production, respectively [34].

### 3.2 Extraction and purification of bacterial pigment

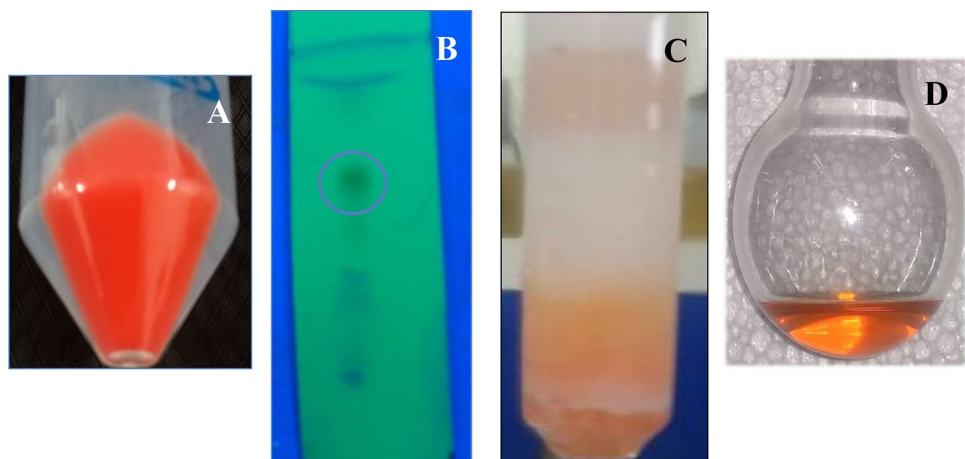
The solvent extraction process revealed methanol to be the best extraction solvent for the intercellular pigment as, after the cell lysis, the cell debris turns completely white and the solvent acquired the pink color of the pigment. On the other hand, other tried solvents like ethanol, diethyl ether, chloroform, hexane, and acetone did not acquire the color of the pigment and the cell debris

remained pink indicating the lower extraction efficiency of the solvents (Fig. 3a). Moreover, the extracted pigment was found able to resolve best with the solvent mixture of chloroform:ethanol = 3:1 in the thin-layer chromatography experiment. Comparative representation of resolving efficiency of other tried solvent mixtures has been presented in Table 2. The chloroform:ethanol mixture was able to resolve the extracted pigment in 4 separate bands based on the polarity of the compound present in the crude pigment. The most polar particle present in the crude pigment moved slowest with ethanol, while non-polar components of the crude pigment moved fast with chloroform in the TLC plate. The intermediate bands were formed based on their polarity. The second band which was showing the brightest presence of the color was slightly polar and was considered to be the purified pigment (Fig. 3b).

**Fig. 2** Phylogenetic analysis of strain *Enterobacter* sp. PWN1 using neighbor-joining method



**Fig. 3** Pigment extraction and purification. **a** Separation of pigment-producing cells from culture broth through centrifugation. **b** Thin-layer chromatographic separation of the different compounds in the pigment. The retention factor ( $R_f$ ) value of the target compound was calculated as 0.6. **c** Column chromatographic separation of the target pigment compound using chloroform and ethanol in the solvent phase. **d** Pigment compound obtained after purification



**Table 2** Response of the pigment to different solvents in TLC

No.	Solvent	Ratio of solvents	Response
1.	Methanol: Acetone: Water	4:4:2	–
2.	Ethanol: Water: Chloroform	4:4:2	+
		4:2:4	+
3.	Hexane:Acetone	3:2	–
		4:1	–
4.	Petroleum ether: Ethylacetate	9:1	–
		1:9	–
5.	Ethanol: Chloroform	1:1	+
		1:2	++
		1:3	+++

The  $R_f$  value of the targeted band was calculated as 0.66. From the result obtained in the TLC plate, column chromatography was conducted, and the first eluted band was discarded. Elutes from the second ring, a total of 17 different fractions were collected and each was tested in TLC for the presence of a single targeted band with retention factor 0.66 (Fig. 3c). All the purified pigment fractions giving positive results in TLC were collected in a round bottom flask (Fig. 3d). The purified pigment was dried in a vacuum evaporator and collected in powder form. A similar kind of intracellular pink-colored bacterial pigment, Prodigiosin was reported from *Serratia marcescens* using methanol as the extraction solvent. This pigment was also reported with anticancer and antibacterial activity [35, 36].

### 3.3 Biomass production optimization

To optimize the process parameters intending to maximize the biomass yield, 23 random runs were performed, and the obtained response (biomass concentration) is listed in Table 3. Based on the responses, the software suggested a quadratic model representing the process to be best suited for the representation of the process of biomass production shown as coded Eq. 2.

$$\text{Obtained biomass concentration} = 1.30 + 0.1639A + 0.1528B - 0.1172AB + 0.0951AC - 0.8525A^2 - 0.7055B^2 - 0.2627C^2 \quad (2)$$

Where, A represents the coded value of temperature, B is the coded value for pH, and C is the coded value of inoculum concentration. The ANOVA analysis of the equation suggested the model be significant as most of the model terms had a  $P$  value less than 0.05. The  $F$ -value of the whole model was found to be 140.55 which is significantly high indicating the statistical significance of the model. The  $F$ -value of the lack of fit of the model was significantly low. The ANOVA analysis also revealed that there was very little noise in the model as the lack of fit was found to be

**Table 3** Run table for biomass optimization of PWN1. The coded values for the factors have been mentioned in brackets

Run	Factor 1 A: Temperature (°C)	Factor 2 B: pH	Factor 3 C: Inoculum (%)	Response 1 Biomass concentration (g/L)
1	25 (–1)	5 (–1)	2.34 (–0.16)	0.47
2	33.7 (+0.16)	5 (–1)	3.5 (+1)	1.21
3	40 (+1)	5 (–1)	1.5 (–1)	0.54
4	40 (+1)	9 (+1)	1.5 (–1)	0.61
5	31.825 (–0.09)	9 (+1)	2.6 (+0.1)	1.89
6	25 (–1)	9 (+1)	3.5 (+1)	0.59
7	25 (–1)	5 (–1)	3.5 (+1)	0.39
8	31.3 (–0.16)	5 (–1)	1.5 (–1)	1.19
9	40 (+1)	5 (–1)	2.64 (+0.14)	0.97
10	25 (–1)	6.9 (–0.05)	3.5 (+1)	1.04
11	40 (+1)	6.9 (–0.05)	3.5 (+1)	1.78
12	25 (–1)	6.9 (–0.05)	1.5 (–1)	0.95
13	32.5 (0)	7 (0)	2.5 (0)	3.89
14	32.5 (0)	7 (0)	2.5 (0)	3.54
15	25 (–1)	9 (+1)	1.5 (–1)	0.75
16	33.1 (+0.09)	9 (+1)	2.41 (–0.09)	2.55
17	40 (+1)	9 (+1)	3.5 (+1)	0.79
18	32.5 (0)	7 (0)	2.5 (0)	3.64
19	32.5 (0)	7 (0)	2.5 (0)	3.94
20	32.5 (0)	7 (0)	2.5 (0)	2.98
21	32.5 (0)	7 (0)	2.5 (0)	3.74
22	32.5 (0)	7 (0)	2.5 (0)	3.64
23	32.5 (0)	7 (0)	2.5 (0)	3.81

non-significant (Table 4). On the other hand, the fit statistics of the model suggested that the difference between the predicted  $R^2$  value (0.9576) and adjusted  $R^2$  value (0.9780) was less than 0.2 indicating the colinearity of the predicted and actual values. Adequate precision, which is the ratio of signal to noise was found to be 33.5420 which is significantly high suggesting the model is very accurate to use as a navigation tool in the design space. The RSM

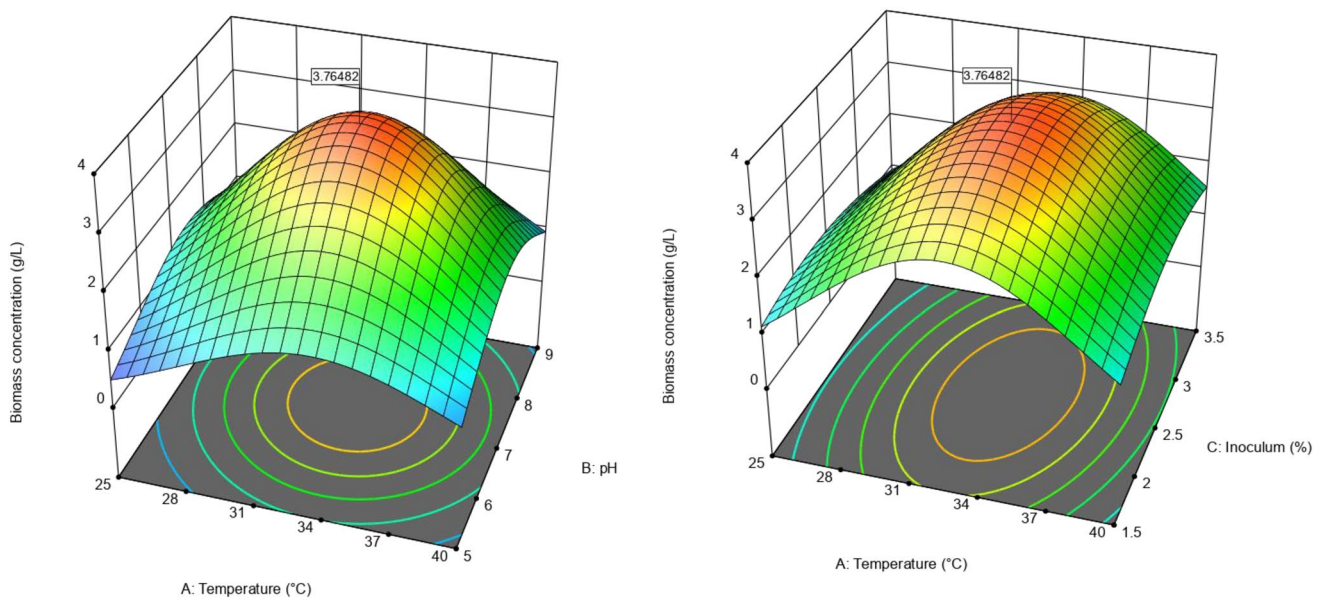
analysis revealed the interaction between the considered process parameters and had been represented as contour graphs (Fig. 4). However, the interaction between pH and inoculum concentration was found to be statistically non-significant and was not considered for the analysis.

The obtained model was solved for a solution to achieve maximum cell growth while setting the targeted temperature, pH, and inoculum concentration in the given range. The software suggested a temperature of 33.18 °C, pH 7.20, and inoculum concentration of 2.52% would be



**Table 4.** ANOVA analysis for optimum biomass production by PWN1

Source	Sum of Squares	df	Mean square	F-value	p value	
Model	13.88	7	1.98	140.55	<0.0001	Significant
A-Temperature	0.2876	1	0.2876	20.38	0.0004	
B-pH	0.2754	1	0.2754	19.52	0.0005	
AB	0.1108	1	0.1108	7.85	0.0134	
AC	0.0777	1	0.0777	5.50	0.0331	
A <sup>2</sup>	2.21	1	2.21	156.51	<0.0001	
B <sup>2</sup>	2.21	1	2.21	156.72	<0.0001	
C <sup>2</sup>	0.2098	1	0.2098	14.87	0.0016	
Residual	0.2116	15	0.0141			
Lack of Fit	0.1573	8	0.0197	2.54	0.1188	Not significant
Pure Error	0.0543	7	0.0078			
Cor Total	14.09	22				



**Fig. 4** Growth pattern of strain *Enterobacter* sp. PWN1 under effect of (a) Temperature and pH. b Temperature and Inoculum percentage

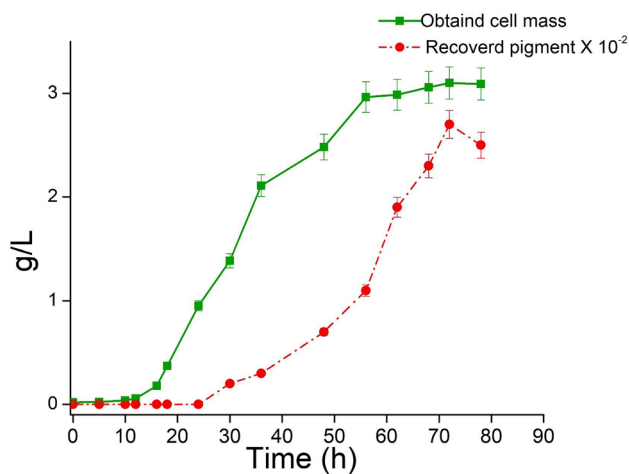
best to produce a biomass concentration of 3.77 g/L. The experimental run in these suggested culture conditions produced a biomass concentration of 3.81 g/L which implied a 1.06% error in the prediction. This error may be caused by the small amount of noise of the model as well as the limitation of maintaining exact culture conditions. The optimized condition for the biomass production had been deduced as a temperature of 33.18 °C, pH of 7.20, and 2.52% inoculum concentration after 3 days.

### 3.4 Growth kinetics and pigment yield

The growth kinetics curve between cell mass obtained versus time revealed that the log phase for the bacterial strain started after 15 h of culture and the same

ends at around 55 h and the stationary phase lasts up to 27 h after which the biomass started to decline. From the production kinetic study, it was observed that the pigment production started after 25 h and the highest pigment was obtained at 72 h. The nature of the curve indicates that the pigment production is growth associated, although the acceleration in the pigment production was slower than the cell growth (Fig. 5). From the kinetic analysis, the specific growth rate of the bacterial strain was found to be 0.069/h implying that the strain is very slow growing in nature. The cell mass productivity was also found to be very low as 0.043 g/h. The specific pigment production rate has been calculated as  $1.18 \times 10^{-4}$ /h. The yield was obtained as 8.7 mg of pigment from 1 g of biomass.





**Fig. 5** Growth kinetics of *Enterobacter* sp. PWN1 and pigment production kinetics

During the yield analysis, 1 L culture produced a total of 3.77 g of biomass after 3 days of incubation at the optimized conditions. The solvent extraction process using methanol recovered a total of 0.234 g of crude pigment upon the vaporization of the methanol from the extraction mixture. This implied 6.2% of crude pigment recovery yield from biomass. After the TLC, a total of 0.131 g purified pigment was recovered with a recovery efficiency of 55.98% of purified pigment from crude pigment. The overall yield of purified pigment was deduced to be 3.47% of the biomass. The obtained yield is higher than many other reported intracellular bacterial pigments. For example, the yield of an intracellular yellow pigment collected from *Vibrio owensii* was reported to be 1.14% using butanol as an extraction solvent [37]. Another intracellular pigment, violacein was extracted from *Duganella violaceinigra* and *Chromobacterium violacein* showing a yield of 18.9 mg/L and 2.5 mg/L, respectively, which were lower than the current study [38, 39]. On the other hand, a carotenoid-like intracellular pigment was extracted from *Arthrobacter* sp. using solvent extraction showing a yield of 840 mg/L which is higher than the yield of the current study [10]. Another intracellular pigment, Prodigiosin from *Serratia marcescens* was also lower using freeze-thawing and homogenization [40]. The purification process of the current study achieving a significant yield was also useful as it is low energy involving, rapid, and cost-effective which makes the process of pigment extraction industrially feasible. Thus, the process of pink color intracellular bacterial pigment production stated in this current study is promising for industrial production and to fulfill the demand for the pink pigment in the market, although the application of the extracted pigment is a subject of further study and characterization of the isolated pigment.

## 4 Conclusions

A pink-colored intracellular bacterial pigment was extracted from bacterial strain PWN1 which was isolated from kitchen wastewater. The isolated strain was found to be non-pathogenic, Gram-negative, motile, and non-endospore forming in nature. The average cell size was observed to be 1.11  $\mu\text{m}$ . The 16S rRNA gene sequencing and phylogenetic analysis established the relationship of the strain PWN1 with the *Enterobacteriaceae* family. The purification of the pigment was achieved by solvent extraction using methanol followed by column chromatography using elute solvent as 3:1 mixture of chloroform:ethanol. To maximize the biomass production, the culture condition was optimized considering temperature, pH, and inoculum concentration as principal influencers. A quadratic model was established describing the process whose accuracy was evaluated with only 1.06% error. The kinetic analysis at the optimized condition revealed that after 3 days maximum pigment production was achieved. The final yield of purified pigment was found to be 3.47% from biomass which is relatively higher than most other intracellular bacterial pigments. The process of achieving a significant recovery of the purified pink pigment was cost-effective, sustainable, eco-friendly, and energy-efficient which makes it industrially feasible. However, more work should be done to identify and characterize the pigment to find out the industrial importance of it.

**Acknowledgements** The authors acknowledge the Biotechnology and Medical Engineering of the National Institute of Technology Rourkela, India for providing a suitable environment and technical facilities to conduct the research work.

**Authors contribution** The authors have contributed to the work and certify the originality of the work and that any similar material has not been submitted or published elsewhere.

**Funding** There have been no exclusive grants from any funding sources for the completion of this research.

**Data Availability** Any data would be made available on request.

## Compliance with ethical standards

**Conflict of interest** The authors of this manuscript declare no conflict of interest for the submission of this manuscript to the journal "SN Applied Sciences".

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