# Isolation and Study of Prodigiosin Pigment from *Serratia marcescens* and its application as Temporary Hair Colour.

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## *Abstract*

*Prodigiosin is the vibrant red pigment with tripyrole ring structure containing the azo group and produced by the bacteria such as Serratia spp, Pseudomonas spp. and actinomycetes.*

*The current study evaluates the application of the prodigiosin pigment as a temporary hair colourant in hair care application. The red pigment producing bacterium was isolated from the industrial water and the extraction of the pigment was done by using the methanol and chloroform. The red pigment producing isolates was identified as Serratia marcescens using the 16S rRNA technique. The cytotoxicity study of the prodigiosin pigment was studied against the human keratinocytes cell lines using MTT assay. The concentration of prodigiosin used for the study was 100 µg/ml-1000 µg/ml and 100% viability was observed for the human keratinocytes cell lines at 1000 µg/ml. The 1000 µg/ml of dried prodigiosin pigment was incorporated in the temporary hair gel cream formulation. The formulation of the temporary hair gel cream consists of the 2% gum arabic, 4% propylene glycol, 0.1 % sodium gluconate 0.7 % carbopol 940, coconut oil 2%, liquid paraffin 5 % and 1% phenoxyethanol: Ethyl hexylglycerine (90:10). The performance was evaluated on the bleached hair traces with single, doble and multiple applications. The temporary hair gel cream showed colouration of the hair traces and generation of the warm vibrant colour on the hair traces. Thus, there is scope to use the temporary hair gel cream as highlighter for the hair.*

*Key words: Serratia marcescens, MTT assay, temporary hair gel cream, Prodigiosin, Human keratinocytes*

# 1: Introduction:

Colouring of the hair is very trendy and not gender specific, colouring of hair with a bright warm colour viz. red, green, or blue is style icon among youngers.

But the hair colouring dyes used are sometimes harmful for the end users as they cause allergic reaction such as rashes, and itching. The synthetic dyes present in the hair dyes penetrate the hair shaft and cortex and make the hair cortex porous resulting in hair damage. Whereas the natural dyes and pigments don't penetrate the hair cortex are not harmful to the hair shaft. These bio pigments possess various biological activities inclusive of antibacterial, antiviral, anticancerous and they are rich source of diet , because of their antioxidant property. Some bio pigments are vitamins and serves as important nutraceutical properties such as riboflavin, vitamin C. (Cuellar-Bermudez et al. 2015)

The red pigment producing bacterium was isolated from the water system and isolated on the media containing glycerol and histidine as substrates. The Prodigiosin is the bio pigment produced by microorganism viz,. *Serratia marcescens, Vibrio psychroerythrus, streptomycin griseoviridis* and *Hahela chejuensis*. (Rokade and Pethe 2017) .It is red pigment alkaloid and first identified and characterised in the *Serratia marcescens.* Prodigiosin is tripyrole ring pigment family contains common 4 methoxy2-2, bipyrrole ring system. The biosynthesis of prodigiosin is the bifurcated system in which mono and bi pyrrole precursors synthesized separately and then attaches to form a pigment molecule (Pore, Khanolkar, and Nadaf 2016). Prodigiosin is the UV resistant molecule, it can block the UV rays hence it has application to be used as commercial sunscreen.(Suryawanshi et al. 2015)

It is secondary metabolite of the bacterium and possess various distinct properties including antimicrobial, antioxidant, antiparasitic and anticancerous (Naveen Raj et al. 2009). The prodigiosin pigment is distinct vibrant reddish pink colour it can colour the fabrics. The application of the prodigiosin pigment was assessed for the hair colouring purpose. The hair colouring gel cream was formulated using the prodigiosin pigment and evaluated on the bleached hair traces. The stability of the formulation was assessed by using standard protocol of the stability study at 5 $^{O}$C, 25 $^{O}$C and 45 $^{O}$C. There is the further scope to accelerate the application of the prodigiosin pigment in hair care industry with respect to other applications.

# 2: Material and methods

### 2.1: Sample collection

The water sample was collected from the industrial water treatment plant located in the Panvel region of Maharashtra (19°4'4.53"N and 73°6'28.89"E). Industrial water treatment plants are based on the usage of different resins, chemical additives and filtration techniques. The flora and fauna of the water treatment plant varies as per the resins used, the frequent addition of the chemicals alters the change in microbial flora and development of the new characteristics in microbial flora. (Desai and Desai 2018).

### 2.1: **Isolation of red pigment producing isolate**

The isolation of the red pigment producing was done on the nutrient agar plates supplemented with 1% skimmed milk. The red pigment producing colony was separated and subculture on

the sterile nutrient agar supplemented with 1% skimmed milk. (Abdhul, Sakthinarayanan, and Murugan 2018)

**2.3: Molecular identification of the red pigment producing isolate**

The identification of the red pigment producing isolate was done by using 16S rRNA method. The CTAB lysis buffer was added to the cell suspension and genomic DNA was extracted from the cells by using chloroform and isoamyl alcohol (24:1). The DNA was spooned from the suspension using chilled absolute ethanol. The amplification was done on the 1% agarose gel supplemented with the 0.5 µg µL-1 ethidium bromide. Chromatograms were generated and the raw data was analysed using the Multalin software and BLAST. Sequenced samples showing maximum homology to the cultures deposited in the database were selected and its genus and species name was assigned to the isolated cultures(Mignard and Flandrois 2006). The sequence was submitted to the genebank.

**2.4: Optimisation of the pigment production**

Optimization of the prodigiosin pigment production in *Serratia marcescenes*: The optimization study was conducted for the prodigiosin pigment production using the Taguchi method with the 6-factorial design. Concentration of glycerol, dextrose, lactose, sucrose, maltose and histidine were used as the different factors on nutrient agar and tryptic soya agar. The factors were 2% and 4 % glycerol and 1% and 2% of different sugars were used for the study. (Desai and Desai 2018)The histidine concentration was studied at 0.1 % and 0.2 % level.

**2.5: Extraction and identification of prodigiosin**

The extraction of the prodigiosin was carried out using different solvents such as methanol, chloroform, acetone, hexane and petroleum ether. (Lapenda et al. 2015). The extracted pigment was dried at room temperature and resuspended in methanol to get 1 mg/ml stock concentration. The absorption maxima of the extracted pigment (1mg/ml conc.) were estimated. (Zaghdoudi et al. 2017)

**2.6: Study of the toxicity of the pigment**

The toxic effect of the pigment was studied on the human keratinocytes cell line procured from NCCS Pune. The dried pigment was suspended in DMSO at the conc. ranging from 100-1000µg/ml. The keratinocytes cell lines were grown in DMEM+ 10% FBS + antibiotics (Penstrep) for 24 hrs in $CO_2$ incubator. The cells were treated with the test compound and the survival of the cells was monitored by using the MTT (5mg/ml). The MTT added to the cells and incubated for 3 hrs at $37^0$C. The supernatant was collected and formazan crystal were dissolved in DMSO. The absorbance of each cell was monitored at 540nm using plate reader. The percent survival was calculated based on the positive and negative control(Madaan et al. 2017).

**2.7: Temporary hair colour formulation**

The temporary hair colour formulation was prepared in the form of the hair gel cream. The gel cream was formulated using the oil in water formulation. The gel was formulating using gum arabic and carbopol 940. The phase A consist of the gum arabic, propylene glycol, sodium

gluconate and carbopol 940. Phase B consist of the light liquid paraffin, coconut oil, glyceryl monostearate, Cetostearyl alcohol and Cyclopentasiloxane.1 % dried pigment was added to the formulation. Phase B was added to the phase A at $85^0$C. pH adjusted to 5.5 to 6. Phenoxyethanol: ethyl hexyl glycerol (90:10) was used as preservative for the formulation. (www.galaxysurfactants.com, 2015.)

The compatibility of the pigment was checked with the ingredients of formulation using centrifugation at 13500 rpm for 15 min, turbidity or any precipitation for period of 8 days.

**Preparation of hair traces**

A lock of bleached hair samples of Asian origin was collected for the study. 5 gm weighted lock of bleached hair was prepared with approximately 10 cm long each.

**2.8: Performance of the temporary hair colour on the hair traces**

The performance of hair gel cream was tested on the bleached hair strands. The 1 gm test hair colouring gel cream formulation was applied on the bleached hair traces for 5 min and dried using hair dryer. The colour developed on the hair traces was observed.

The performance of the gel cream formulation was tested using the 3 parameters such as only single application, double application and multiple applications. The evaluation was done by using the photography and rating criteria as 1 for no colouration of hair, 2 for medium colouration on hair strands and 3 vibrant warm colourations on hair strands. (Mala 2018)

**2.9: Stability of the formulation (**Sanap et al, 2016)

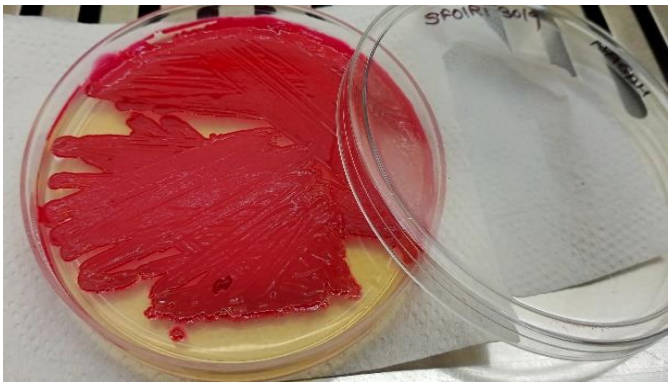
The stability of the formulation was done by using routine method. The formulation was subjected to the different conditions of temperature. The test temperature conditions were $25^OC\pm2^OC$ at 65%±5% relative humidity, $45^OC\pm2^OC$ at 75%±5% relative humidity and $5^OC\pm2^OC$. The rheological parameters, physicochemical parameters and microbiological parameters were assessed for the determination of the stability of formulation over a period of 6 months.

## 3: Results and discussion

**3.1: Isolation of the pigment producing isolate:**

The water sample collected from the industrial water treatment plant showed the presence of the red pigment producing colony. The red pigment producing colony was identified as the *Serratia spp* based on the cultural and biochemical characteristics. The *Serratia spp.* are mostly found in the extreme environmental conditions.

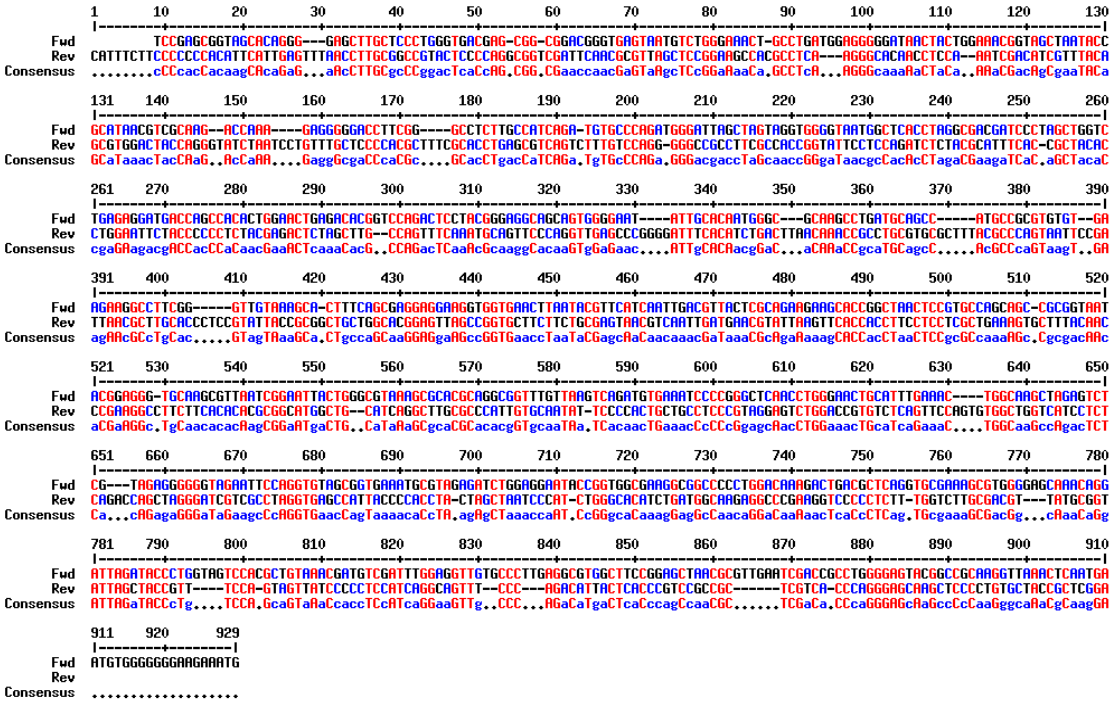
Image-1: Red pigment producing *Serratia marcescen*e isolated from the industrial water system.



### 3.2: Biochemical identification of the red pigment producing isolate:

The 16S rRNA technique was performed to identification of the red pigment producing isolate. The 929bp DNA sequence was obtained and sequence was analysed on the BLAST. The isolate was identified as *Serratia marcescens* and the accession number was MW265916.1

Image-2: Multiple sequence alignment for SF01R1



### 3.3: Optimization of the pigment production

The intense red pigment was produced in presence of the 4% dextrose sugar with 4% glycerol, 0.1% histidine in nutrient agar media at pH 8. Whereas in case of the mannitol, sucrose and lactose the intensity of the red pigment production was minimal compared to the dextrose. The effect of the intensity of the pigment production with respect to presence of the sugar was dextrose > Lactose > Mannitol > Sucrose. The response of the pigment production was comparable in nutrient agar and tryptic soya agar. Nutrient agar showed positive impact on the production of the intense red pigment by pigment producing isolate SF01R1. The synthesis of

the prodigiosin pigment was higher in case of the nutrient agar compared to tryptic soya agar due to the nitrogen source. (Bhagwat and Padalia 2020)**.** The optimised condition for the Prodigiosin pigment production was nutrient agar containing 0.1% histidine , 4% glycerol and 1% glucose with minimal cost. Nakamura et al 2009 reported that the nutrient agar media containing carbon source and amino acid as precursor stimulates that pigment production in bacterial cells resulting in the overproduction of the pigment. (da Silva, Mack, and Contiero 2009). (Nagar et al. 2009)The details of the optimisation study given in the table no:1

Table no-1: Optimisation study of the pigment production in red pigment producing isolate SF01R1.

| Media | pH | Media supplement | | Dextrose | | Sucrose | | Lactose | | Mannitol | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Histidine % | Glycerol % | 2% | 4% | 2% | 4% | 2% | 4% | 2% | 4% |
| Tryptic soya agar | 8 | 0.1 | 2 | + | ++ | + | - | + | ++ | + | - |
| | | | 4 | ++ | ++ | + | - | + | ++ | + | - |
| | | 0.2 | 2 | ++ | ++ | + | - | ++ | ++ | + | - |
| | | | 4 | ++ | ++ | + | - | ++ | ++ | ++ | - |
| | 7 | 0.1 | 2 | + | + | + | + | + | - | + | - |
| | | | 4 | + | + | + | + | + | - | + | - |
| | | 0.2 | 2 | + | + | + | + | + | - | + | - |
| | | | 4 | + | + | + | + | + | - | ++ | - |
| Nutrient agar | 8 | 0.1 | 2 | + | ++ | + | - | + | - | + | - |
| | | | 4 | ++ | ++ | + | - | + | - | + | - |
| | | 0.2 | 2 | ++ | ++ | + | - | ++ | - | + | - |
| | | | 4 | +++ | ++ | + | - | ++ | - | ++ | - |
| | 7 | 0.1 | 2 | + | + | + | + | + | - | + | - |
| | | | 4 | + | + | + | + | + | - | + | - |
| | | 0.2 | 2 | + | + | + | + | + | - | + | - |
| | | | 4 | + | + | + | + | + | - | ++ | - |

### 3.4: Extraction and identification of prodigiosin:

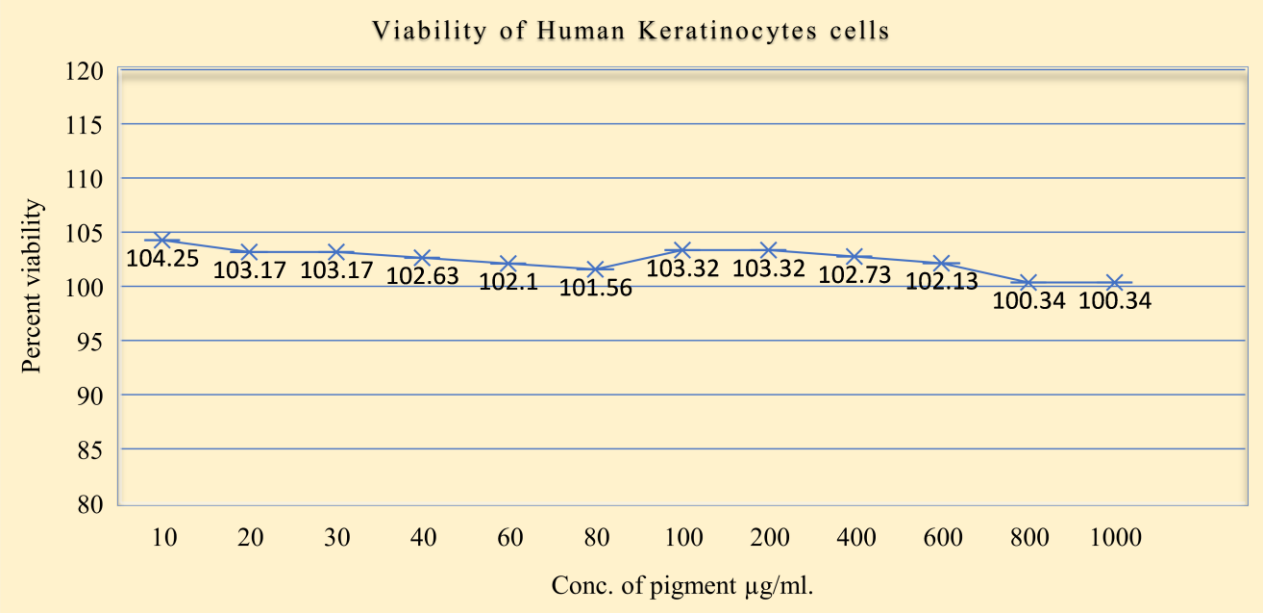
Methanol and chloroform (1:1) enables the complete extraction of the pigment from the cells and the absorbance maxima was observed as 515 nm in methanol solvent which matched with the reported standard prodigiosin pigment. (Phatake and Dharmadhikari 2016).

### 3.5: Study of the toxicity of the pigment

The toxic effect of the pigment on the human keratinocyte cell lines was assessed by using the MTT assay. The prodigiosin pigment extracted from the red pigment producing isolates showed 100% viability of the human keratinocytes cells at the concentration ranging from the 10 µg/ml to 1000 µg/ml. The viability of the human keratinocytes was not reduced in presence of the prodigiosin pigment. The prodigiosin was found to be non toxic against the human

keratinocytes whereas as reported by Sumathi 2007 the prodigisin induces apoptosis in the carcinoma cell lines. (Babitha, Soccol, and Pandey 2007)

Graph no.-1: Graphical representation for the cytotoxic study of the prodigiosin pigment on the human keratinocytes.




### 3.6: Formulation of the temporary hair colouring gel cream

The hair gel cream was formulated using the extracted dried prodigiosin pigment at 0.1% concentration. The formulation was developed based on the colour developed, stability or any other reaction with the formulation ingredients. The formulation was tested for the colour fastness on the bleached hair traces. The compatibility of the formulation was tested for the centrifugation at 13500 rpm for 5 min and there was no any phase separation in the formulation is intact. The formulation was kept at 35 deg. C for the period of 8 days and there was no any precipitation or flocculation observed in the formulation. The no any change in the colour of the formulation showed that the formulation is intact.

**Image No.-3: Temporary hair coloring gel cream with 0.1% prodigiosin pigment extracted from the *Serratia marcescens***

**Table no-1: Compatibility of the prodigiosin pigment with the ingredients of the hair coloring gel formulation**

| Parameters | Observation |
|---|---|
| Phase separation at 13500rpm for 5 min | NO |
| Precipitation | NO |
| Colour of formulation | Bright red colour formulation |
| Flocculation | NO |

### 3.7: Performance of the hair colouring gel on hair traces:

The formulation was evaluated on the bleached hair traces for the colour fastness. The hair gel cream was applied on the bleached hair traces with single application, double application and multiple applications. The colouration of the hair strands was with the single application the light pinkish red colour observed on the hair strand. Whereas in the double application the colour became darker rates as 2. In case of the multiple applications the colour on the hair strand rated as 3 as they became vibrant and warm. The photographs of the study are given below in image 4 and 5

Image -4: Bleached Hair Vs Coloured hair

Image-5: No. of applications of colouring gel formulation

Multiple Double Single

Table no.2: The rating criteria for the colourfastness of the hair colouring gel cream on the hair traces

| No. of application | Rating based on the colourfastness | Description |
|---|---|---|
| Single | 2 | Orange red colour on hair traces |
| Double | 2 | Red colour on hair traces |
| Multiple (3/4 times) | 3 | Vibrant and warm red colour on hair traces |

### 3.8: Stability of the formulation

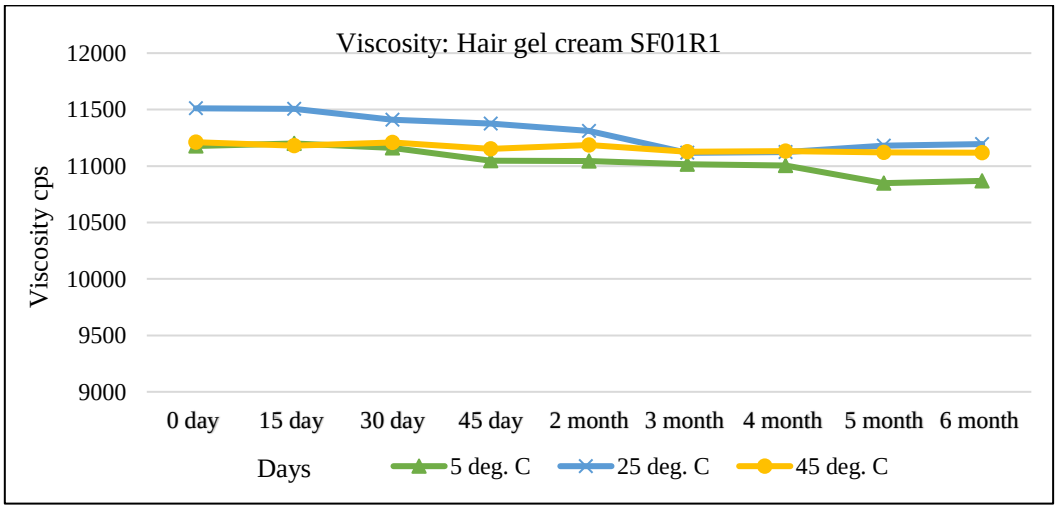
The stability of the hair colouring gel cream was checked for 6 months at the interval of 0-day,15-days,30-days, 45-days, 2-months,3-months, 4-months,5-months and 6-months. The stability testing conditions of temperature and relative humidity of $25^{O}C\pm2^{O}C$ at 65%±5% relative humidity, $45^{O}C\pm2^{O}C$ at 75%±5% relative humidity and $5^{O}C\pm2^{O}C$. The physicochemical parameters such as pH and viscosity were monitored using pH meter Elico make and Brookfield viscometer.

Organoleptic test: The organoleptic test for the hair colouring shampoo gel cream was performed over the period of the 6 months at specified time interval. There was no any change in colour of the formulation, it was found to be stable. There was no any malodour in the formulation and appearance of the formulation was also stable. The formulation was found to be intact during the period of 6 months at room temperature.

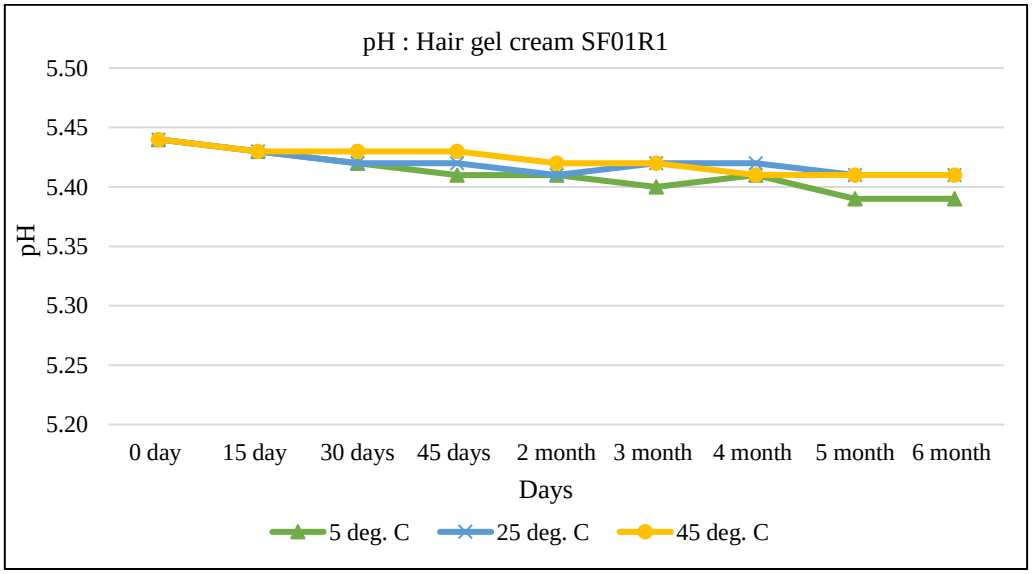
Microbiological test: The hair colouring gel formulation was found to free of microbial contamination over the period of 6 months for total bacterial count and yeast mould count analysed using microbial limit test.

Physicochemical test: The physicochemical test was performed to check the integrity of the formulation. There was no any phase separation in the formulation after centrifugation at the 13500 rpm for 5 min. The Viscosity and pH of the formulation was monitored quantitatively. The initial pH of the formulation was 6.45 was shifted to the 6.39 for 6 months at $5^0$C. temperature. The pH of the formulation was shifted to the 6.38 at 6 months for temperature 25deg.C. For the temperature $45^0$C the pH of the formulation remains stable at 6.41pH the results indicates that the pH of the hair colouring gel cream was stable for the period of the 6 months. The viscosity of the hair colouring gel cream was 34829 cpc at 0 day. There was no any change in viscosity of the formulation for the temperature $25^0$C and $45^0$C. Refer graph no. 1 and 2.

Graph No-1: Graphical representation for the viscosity of the hair colour prepared with the SF01R1




Graph No-2: Graphical representation for the pH of the hair colour prepared with the SF01R1



## 4: Conclusion:

The prodigiosin pigment was isolated from the stressed environment was having the capacity to produce prodigiosin pigment. The red pigment producing strain was identifies as *Serratia marcescenes*. The extracted pigment showed the lambda max as 530 nm (MeOH) and the Rf value 0.5 revealed the molecule identity as prodigiosin molecules. The enhancement of the prodigiosin pigment production was observed for the glycerol as substrate and histidine as the precursor. The toxic effect of the prodigiosin was evaluated on the human keratinocytes and found to be non-toxic at 1000 µg/ml. The viability of the keratinocytes was observed to be 100% for the conc. ranging from the 10 µg/m to 1000 µg/m. The prodigiosin pigment is not having toxic effect on the keratinocytes indicates that the pigment is non toxic and hazardous for human application. The hair coloring gel cream was formulated using the 1000 µg/ml showed the compatibility with the formulation ingredients. The formulation was evaluated on the bleached hair traces and found to be satisfactorily. The bleached hairs showed vibrant red colour on the hair. The hair coloring gel formulation was remained stable for the period of the 6 months at 3 temperatures ranges 5 $^{O}$C, 25 $^{O}$C and 45$^{O}$C. The formulation was intact and no precipitation observed in the formulation indicates that there is further scope to study the microbial pigment derived formulation for hair care products. However intense research and significant improvement are required in the field to commercialize the concept of the application of microbial pigment in the hair coloring products.

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