Characterization of prodigiosin produced by *Serratia marcescens* strain isolated from irrigation water in Egypt

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Abstract: The spectroscopic analyses of the red pigment with UV–vis spectral analysis, Gas chromatography–mass spectrometry (GC/MS), Fourier-Transform Infrared Spectroscopy (FTIR Spectroscopy) and IH-Nuclear Magnetic Resonance Spectroscopy (NMR Spectroscopy), indicated that the red pigment produced by *Serratia marcescens* strain WSE is prodigiosin. The cluster genes (*pig* cluster) responsible for production of prodigiosin by *S. marcescens* strain WSE were amplified by PCR, sequenced and their nucleotide sequences were analyzed through bioinformatics. High degree of similarity was detected between the nucleotide sequence of the pig clusters and the nucleotide sequence of *S. marcescens* in the NCBI database (Acc. No. AJ833002, Acc. No. CP005927 and Acc. No. CP003959). Effect of precursor amino acids on prodigiosin production by *S. marcescens* isolate WSE were examined and the results revealed that maximum pigment production was obtained by growing *S. marcescens* in Nutrient Broth supplemented with 10 mg/ml L-tyrosine. This study suggests that L-Tyrosine plays an important role for maximum induction of pigment production by *S. marcescens* strain WSE. We describe a new method for estimation of prodigiosin concentrations intra and extracellular. We also report the organization of prodigiosin biosynthetic gene (*pig*) clusters in *S. marcescens* strain WSE based on predicted protein functions of the genes of the *pig* clusters.

[Faraag, A. H.; El-Batal, A. I. and El-Hendawy H. H. **Characterization of prodigiosin produced by *Serratia marcescens* strain isolated from irrigation water in Egypt.** *Nat Sci* 2017;15(5):55-68]. ISSN 1545-0740 (print); ISSN 2375-7167 (online). http://www.sciencepub.net/nature. 8. doi:10.7537/marsnsj150517.08.

Keywords: gene, prodigiosin, *Serratia marcescens*, BLAST, L-Tyrosine

1. Introduction: Prodigiosin, is a family of natural red pigments characterized by a common pyrrolyl pyrromethane skeleton and are produced by various bacteria although it was first characterized from *S. marcescens* **(Khanafari et al., 2006)**. This pigment is a promising drug owing to its reported characteristics of having antibacterial **(Darah et al., 2014)**, antifungal **(Kalbe et al., 1996; Someya et al., 2001)**, immunosuppressive and anti-proliferative activity **(Chang et al., 2011)**, immunomodulators **(Lee et al., 1995)**, antimetastatic **(Zhang et al., 2005).** It alsoinducesapoptosis in hematopoietic cancer cellswith no marked toxicity in nonmalignant cells **(Montaner et al., 2000)**, and has cytotoxic effects and induces apoptosis in HT-29 and T47D cancer cell lines **(Dalili et al., 2012)**, it also induces cell death and morphological changes indicative of apoptosis in gastric cancer cell line HGT-1 **(Díaz-Ruiz et al., 2001)** which increase the possibility of its therapeutic application as an antineoplastic drug **(Montaner et al., 2000; Dalili et al., 2012)**. It has been reported to have antiprotozoal effect **(Williams and Qadri 1980)**, antimalarial **(Castro 1967; Gerber 1975; Papireddy et al., 2011)**, and contain anticancer compounds **(Regourd et al., 2007; Chang et al., 2011).** Prodigiosin is produced by *S. marcescens* following a bifurcated biosynthesis pathway, in which mono and bi-pyrrole precursors are obtained separately and then coupled to form the linear tripyrrole red pigment **(Boger and Patel 1988)** during the stationary phase of bacterial growth **(Rokem and Weitzman 1987).**

Serratia pigment genes clusters contain 14 candidate genes common to Serratia and are arranged *pigA* through to *pigN*. Four genes (*pigB, pigD, pigG and pigK*) are unassigned function, with the remaining protein (*pigL*) proposed being involved in the posttranslational modification of some of the proteins in the cluster **(Cerdeño et al., 2001; Harris et al., 2004)**. However, **Williamson et al., (2006)** reported that the regulatory hierarchy of *pig* biosynthesis in *Serratia* strain ATCC 39006 employs at least 15 genetic loci. **Fineran et al., (2007)** reported that *pigX* controlled secondary metabolism by repressing the transcription of the target prodigiosin biosynthetic operon (*pigA-pigO*). This investigation was carried out to extract, purify and characterize the prodigiosin produced by *S. marcescens* strain WES and to study the effect of different amino acids on its production. Additionally, the organization of the prodigiosin biosynthetic gene (*pig*) clusters in *S. marcescens* strain WSE based on predicted protein functions of the genes of the *pig* clusters was investigated.

2. Materials and methods

Bacterial Isolate

S. marcescens strain WSE, was isolated from irrigation water collected from Al maryoteia canal, Giza Governorate, Egypt, (Marwa R. Abd El-Salam and Hoda H. El-Hendawy, unpublished). The bacterium was maintained on Nutrient agar slopes, subcultured monthly and kept at 4°C.

Extraction of prodigiosin

Prodigiosin extraction was carried out as described by **Pradeep et al., (2013).** A loop full of *S. marcescens* grown on NA plate for 24 h was inoculated into 250 ml flask containing 50 ml NB devoid of NaCl **(Giri et al., 2004)** and incubated in a shaking incubator (VS-8480 SRN, Korea) at 150 rpm and 28°C for 24 h. Then, 10 ml of the culture broth was centrifuged (Centurion Scientific K3 Series Centrifuges) at 6000 rpm and 4°C for 15 min. The amount of pigment was determined in each of the culture supernatant and in the cell pellet which was re-suspended in 10 ml of 95% ethanol, followed by incubation in the dark at room temperature for 30 min. This was followed by centrifugation at 6000 rpm and 4°C for 15 min. Prodigiosin was extracted using 95% ethanol, and the extract was further processed by probe sonication using an ultrasonic homogenizer (BioLogics Ultrasonic Homogenizer Model 150VT; BioLogics Inc, Manassas, VA, USA). Sonication time and pulsar rate were adjusted at 40% pulsed power and 30% pulsar rate for 3 min until the solution become colorless**.** The mixture was vortexed and the suspension was centrifuged at 6,000 rpm and 4°C for 10 min **(Slater et al., 2003),** then filter sterilized through 0.45-µm Whatman® Optitran® reinforced nitrocellulose membrane filter. The extracted pigment were transferred into round-bottom flasks (Schott Duran, Germany), then evaporated by vacuum rotary evaporator (Heidolph WB 2000, Germany) at 50 °C and dry weight was determined according to **Garg et al., (2013),** the amount of concentrated pigment obtained as a dry weight was calculated.

Pigment Estimation

The amount of pigment produced in bacterial cells and in the supernatant were determined by the addition of 100 µl of 0.1N HCl to 10 ml of the supernatant for extracellular pigment estimation. However, for the intracellular estimation, also 100 µl of 0.1N HCl was added to 10 ml of ethanolic extract. The total pigment was estimated according to the Spekwin32 spectroscopy Software for optical spectroscopy. Prodigiosin concentration was calculated by measuring the absorbance at 535 nm **(Chen et al., 2006)** using the extinction coefficient 51.3×10^3 l/(mol*cm) **(Williams *et al.*, 1961 and 1971)**, using Spekwin 32, software version 1.71.6, the concentration of the total pigment yield (mol/l) was calculated **(Menges 2011).** The calculation is carried out via Lambert-Beer Law **(Swinehart 1962)**. Upon entry of molar absorption coefficient, wavelength and absorption path length (Figure 1), concentration can be calculated, with the button [Calculate]. Finally, the concentration of pigment was converted from (mol/l) into (mg/l) using molecular weight of 323.4 Da as in the following equation:

Equation1:

Molar concentration (moles per liter) X molecular weight (gram per mole) X 1000 = milligram per liter

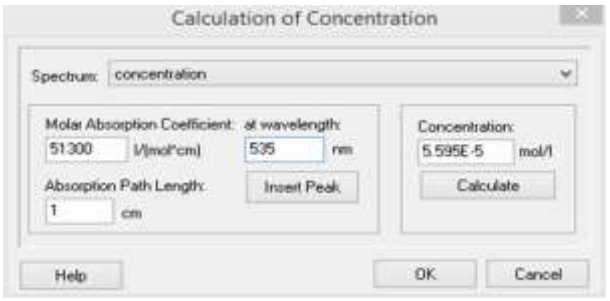

Figure 1. Prodigiosin concentration calculation using Spekwin 32, software.

Purification of the pigment

The extracted prodigiosin was applied to thin layer chromatography (TLC) plate for further purification using the mixture of chloroform and methanol (9:1) as the solvent system **(Casullo de Araújo et al., 2010),** then prodigiosin was separated by silica gel column chromatography (2.5×30 cm; Kieselgel 60; Merck, Darmstadt, Germany), mesh size: 60–80, which was used as the stationary phase for separation of the noncolored impurity from the pigment **(Montaner and Pérez-Tomás 2002)**. The dried concentrated sample was dissolved in 5 ml of n-hexane and loaded onto the silica gel column and solvent system comprising n-hexane and ethyl acetate (2:0; v/v to 0:2; v/v) at a flow rate of 1 ml/min. The eluted red colored fractions were collected and assayed for the presence of prodigiosin as previously described. The fractions containing prodigiosin were pooled and concentrated by evaporation at 50°C using rotary evaporator.

Characterization of the Pigment

Preliminary identification of prodigiosin

Preliminary identification of prodigiosin was carried out as follows: the clear solution was divided into two portions. One part was acidified with a drop of concentrated HCl; the other part was alkalinized with a drop of concentrated ammonia solution **(Gerber and Lechevalier 1976)**.

UV–vis spectral analysis

The pigment was analyzed for maximum UV–vis absorbance at pH values of 2, 7 and 9 between 200 and 800 nm, using ethanol as blank. The pH value of the purified and ethanol dissolved pigment was pH 7.0, the pH value was adjusted to 2.0 using 0.1 N HCI, and pH value 9.0 was adjusted using 0.01 N NaOH.

Thin-Layer Chromatography (TLC)

Thin layer chromatography (TLC) was performed on silica gel TLC-cards (Alugramm® SIL G, layer thickness 0.20 mm, Fluka) for further purification using the mixture of chloroform and methanol (9:1) as a solvent system **(Song et al., 2006; Casullo de Araújo et al., 2010).**

HPLC analysis

HPLC analysis, Chromatographic separation was carried out using a C18 column for reverse phase chromatography (Eclipse plus C18, 4.6 x 100 mm), with a flow rate of 0.8 ml/min and an injection volume of 10 µl. Mobile phase: acetonitrile/HPLC water (60:40, v/v).

Mass Spectroscopy

The molecular weight of the sample was determined using gas chromatography–mass spectrometry (GC/MS), mass spectrophotometer, Schimadzu GCMS-Qp 2010 Plus at the Center for Microanalysis, Cairo University, Cairo, Egypt.

Fourier-Transform Infrared Spectroscopy (FTIR Spectroscopy)

The purified pigment sample was subjected to FTIR spectroscopic analysis; FTIR spectroscopy was carried out by mixing the dried pigment with finely ground KBr (1:100). The parameters used in the FTIR analysis were: spectral range: between 4,000 and 400 cm^{-1}. Upon pressing under 2,000 kPa, pellet disc obtained was analyzed using JASCO FTIR-3600 infra-red spectrometer by employing KBr Pellet technique, equipped with KBr beam splitter with DTGS (Deuterated triglycine sulfate detector (7800-350 cm-1) at the National Center for Radiation Research and Technology (NCRRT), Atomic Energy Authority, Egypt.

IH-Nuclear Magnetic Resonance Spectroscopy (NMR Spectroscopy)

Proton (1H) NMR spectra of the purified pigment was analyzed using FT-NMR Braker Ac 200 spectrometer, at the Center for Microanalysis, Cairo University, Cairo, Egypt. The purified product was dissolved in D-chloroform (CDCl3) and the solution then was analyzed by NMR to identify the structure of the purified product. The parameters used in the NMR analysis were: Temperature 30^{o}C, 303.1 K Mercury-300BB, Relax. Delay 1.000 Sec, Pulse 12.0 degrees, Acq. Time 4.004 Sec, Width 6600.7 Hz.

Analysis of prodigiosin biosynthesis gene cluster of *S. marcescens*

Primer design

Primers for analysis of prodigiosin biosynthesis gene cluster of *S. marcescens* were designed using the program OLIGO 7.57 primer analysis software (Molecular Biology Insights, Inc) according to its primer and probe search protocol (Table 1). The nucleotide sequences used for primers design in this study have been deposited in the GenBank database under accession number **AJ833002 (Harris et al., 2004).**

Table 1. Primers and conditions for PCR.

Target gene	Primer	Sequence	Tm (^{o}C)	Optimum Ta (^{o}C)
pigA	248F21	5' TCGGCATGTCCTTCTCGCTCT 3'	62.7	60.1
	560R22	5' CCTGGCATCCCTTTCTCGAGCA 3'	62.0	
pigC	727F19	5' CTGCAAACGCCCGAAGCCA 3'	62.7	61.0
	1114R23	5' ACGTAGCCTTGCAAATACCCCAT 3'	63.1	
pigE	385F22	5' GGCAACTCGCTGACCACCTACG 3'	63.9	62.4
	701R17	5' TTGCAGTCTGGCGGGGT 3'	63.5	
pigF	704F21	5' GCGGCTATGACCTGATCCACC 3'	62.5	61.1
	944R21	5' CGTTGCTGAATCCCGCCAGAC 3'	63.1	
pigH	1103F21	5' TCACCGACGCCATCAACCACA 3'	64.5	61.2
	1410R21	5' GTGCCCAGTTCGAGATCCACC 3'	62.6	
pigI	536F22	5' CCAACCATGCCAGTTTCGCCTT 3'	64.9	62.5
	848R21	5' TTTCCGTCGGGCCATACCAGT 3'	65.2	
pigJ	665F25	5' CCGTACAATGCTTCCACCATCAACC 3'	63.6	62.6
	1026R23	5' CCTGCGCGGTGATGGGCTGATAG 3'	66.9	
pigL	360F23	5' ACAGCGATTGGGACACATACAGG 3'	63.6	60.9
	575R21	5' TCGCTCTGAACGACATGCTCT 3'	62.2	
pigN	164F20	5' GCCACAAAGCCCTGATCCTT 3'	61.3	60.5
	675R22	5' ACGTTTATAGTTGCGCCAGACC 3'	62.1	
Optimum Tm and Ta for *pig* genes of PCR			**61**	**59**

Forward (F) and reverse (R) primers are indicated. (Tm), melting temperature and (Ta), the annealing temperature used during PCR

Genomic DNA Isolation

The genomic DNA of *S. marcescens* isolate WSE was extracted by Wizard® Genomic DNA Purification Kit. According to the manufacturer's recommended procedure. PCR was performed in a thermal cycler (Biometra® cycler personal). PCR amplification of the *pig genes* were carried out using the designed primers (Table 1) in a thermal cycler (Biometra® cycler personal). PCR reactions mixtures contained 1 µl of a10 µM working solution of each primer, 1 µl of genomic DNA, 12.5 µl of a DreamTaq Green DNA Polymerase (2x) (© 2012 Thermo Fisher Scientific Inc.) and 9.5 µl of Water, nuclease-free following the manufacturer's guidelines. However, PCR conditions used for the amplification of the *pig* genes were: 95°C for 5 min., followed by 35 cycles of 95°C for 1.5 min, 59°C for 1 min and 72°C for 1.5 min, with a final 10 min extension at 72°C. Then DNA molecules were separated in 0.8% agarose-TBE (90 mM Tris-borate, pH 8.0, 2 mM EDTA. The approximate size of PCR products was estimated by comparison to DNA marker, Gene Ruler™ 1kb Plus DNA Ladder (Catalog number: SM1331), which was electrophoresed alongside the samples. DNA bands were visualized by using a UV transilluminator (LMS-26, 302 runs, Wolf laboratories. UK), and photographed. The PCR products were purified by using Thermo Scientific Gene JET PCR purification kit (© 2013 Thermo Fisher Scientific, Inc). The purified PCR products was sequenced in one direction using the previously forward designed primers (Table 1), in an automated sequencer ABI prism 3730XL (Applied Biosystems, Foster City, CA, USA) at Macrogen Inc., Korea. Sequences were analyzed by using Geneious Pro 8.1.1 software. To determine sequence similarity of the *pig* genes, these nucleotide sequences were compared against sequences found in nucleotide database using the National Center for Biotechnology Information (NCBI) using BLASTN tool available at Geneious Pro 8.1.1 software.

Influence of inductor (different amino acids)

The Effect of amino acids on pigment production was investigated by adding alanine, proline, serine and L-tyrosine to a final concentration of 0.5% (250 mg/l) level in the nutrient broth medium. Pigment production was determined after 24 h of incubation at 23^{o}C and pH 8.5 in a 250 ml -volume Erlenmeyer flask then placed in a shaking incubator (VS-8480 SRN, Korea) at 150 rpm. After incubation in an optimum condition, the prodigiosin was quantified.

Effect of different concentrations of L-Tyrosine

Effect of different concentrations of L-Tyrosine on pigment production was determined by the addition of 0.25%, 0.5% and 1.0% (w/v) L-Tyrosine, to nutrient broth. Pigment production was determined after 24 h of incubation as previously described.

3. Results

Confirmation of prodigiosin production

Different colors were obtained when the pH value of the extracted red pigment was adjusted at 2, 7, and 9, these colors were pink, red and yellow, respectively (Figure 1). This result confirmed that the extracted red pigment is prodigiosin.

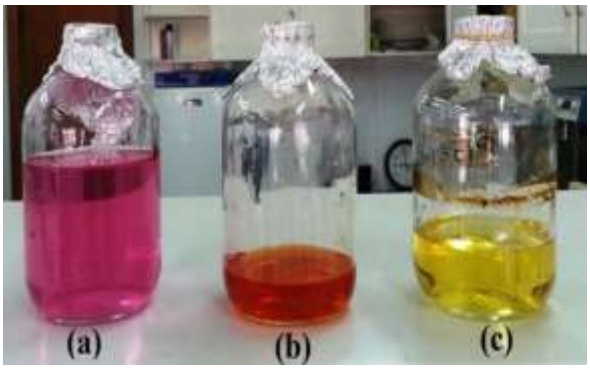

Figure 1. Different coloration of the red pigment at (a) pH 2 (b) pH 7 and c) pH 9

UV–vis spectral analysis

Absorption spectra of the red pigment in ethanol showed a maximum absorbance at 535 nm evidenced by the presence of a peak at this wave length. The maximum absorbance of the pigment at the pH values 2, 7 and 9 was found to be 535 nm, 458 nm and 469 nm, respectively (Figure 2).

Purification of the pigment

The pigment produced by the bacterium, was extracted from the medium and purified by column chromatography. The results obtained are presented below.

Column chromatography

The concentrated pigment was purified by column chromatography using a silica gel column and a solvent system comprising n-hexane and ethyl acetate (2:1; v/v). The red colored eluted fractions were pooled and concentrated by evaporation at 50°C by using rotary evaporator. The absorption pattern of the purified pigment dissolved in 95% ethanol is shown in Figure 3. A reduction of absorbance in the range of 430 to 490 nm wavelength (Figure 3) indicates the removal of impurities. The purified pigment was then subjected for further characterization.

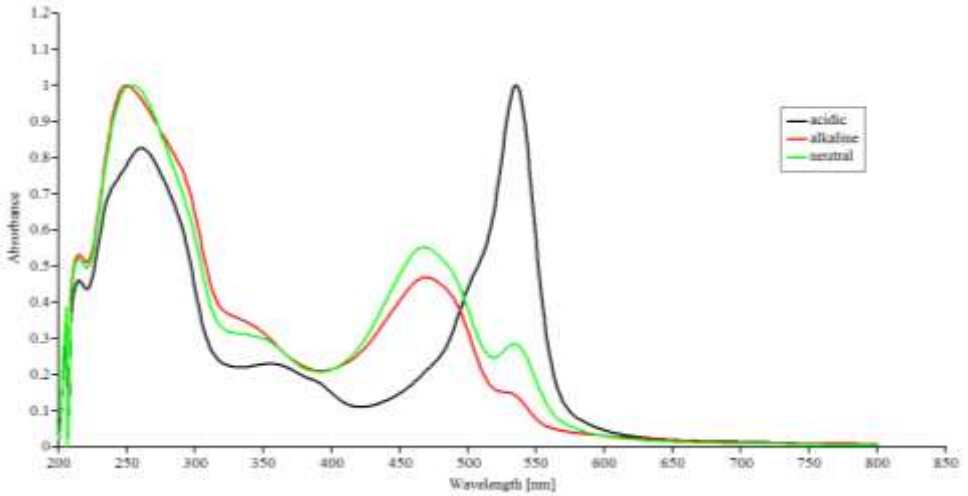

Figure 2. Maximum absorption of the red pigment dissolved in ethanol at pH values of 2, 7 and 9.

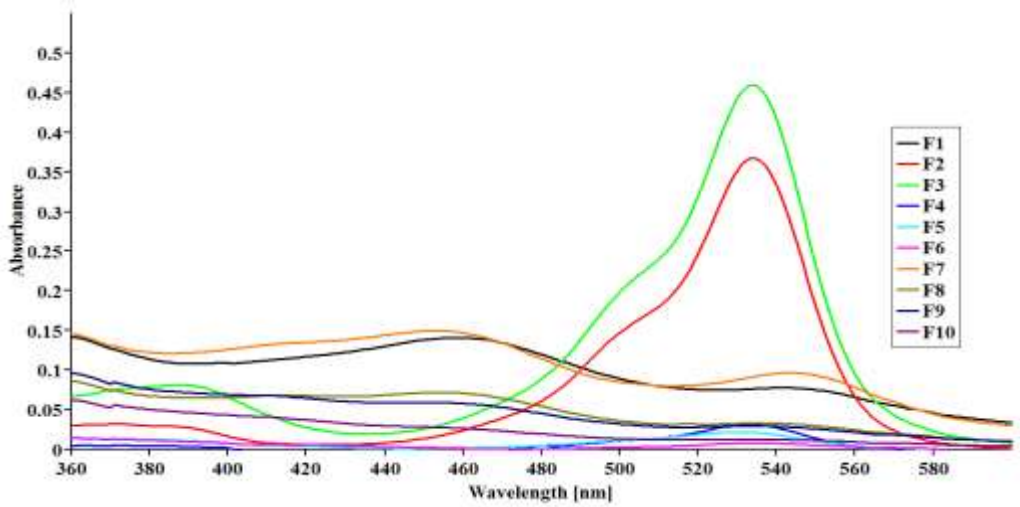

Figure 3. Absorption spectra of 10 fractions from column chromatography.

Characterization and identification of the pigment
Thin-layer chromatography TLC

10 µl containing 5 µg of the purified pigment were spotted on TLC plates and separated using chloroform: methanol (9:1) solvent system. A single band with an Rf value of 0.9 was obtained as showed in Figure 4.

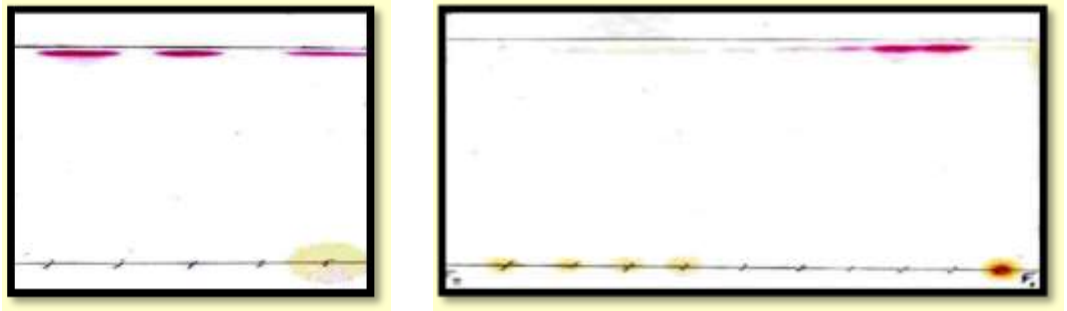

Figure 4. Thin layer chromatogram of the fractions obtained from the column chromatography and containing prodigiosin produced by *S. marcescens* strain WSE

Gas Chromatography-Mass Spectroscopy (GC-MS).

Characterization of the pigment was carried out by mass spectrometry-GCMS, the obtained results revealed that the molecular weight of the red fraction corresponded to 323 D m/z, thus confirming that the pigment extracted from *S. marcescens* strain WSE is prodigiosin pigment (Figure 5).

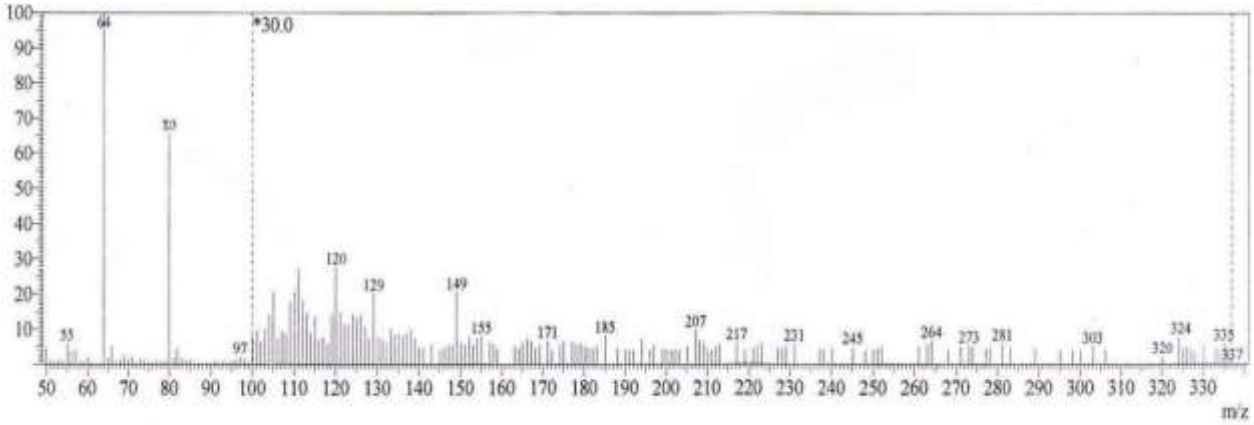

Figure 5. Mass spectrometry (GC-MS) of the purified red fraction (prodigiosin) produced by *S. marcescens* strain WSE.

HPLC analysis

Figure 6 indicated that prodigiosin was separated by HPLC, with 94.84% purity and a single peak at retention time of 2.062 min.

Fourier Transform Infrared Spectrum of prodigiosin Pigment

FT-IR absorption in KBr was at νmax 3423, 2955.97, 2920, 2851, 2362, 2343, 2025, 1631, 1600, 1466, 1384, 1351, 1217, 1119, 1067, 1020, 908, 850, 762, 622 and 583cm^{-1}, Respectively (Figure 7).

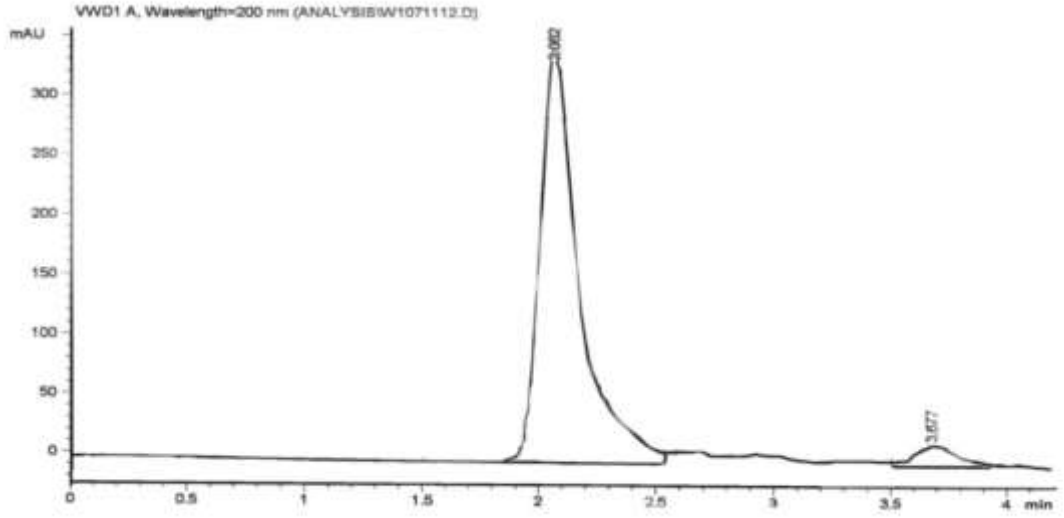

Figure 6. HPLC profile of purified prodigiosin from *S. marcescens* strain WSE.

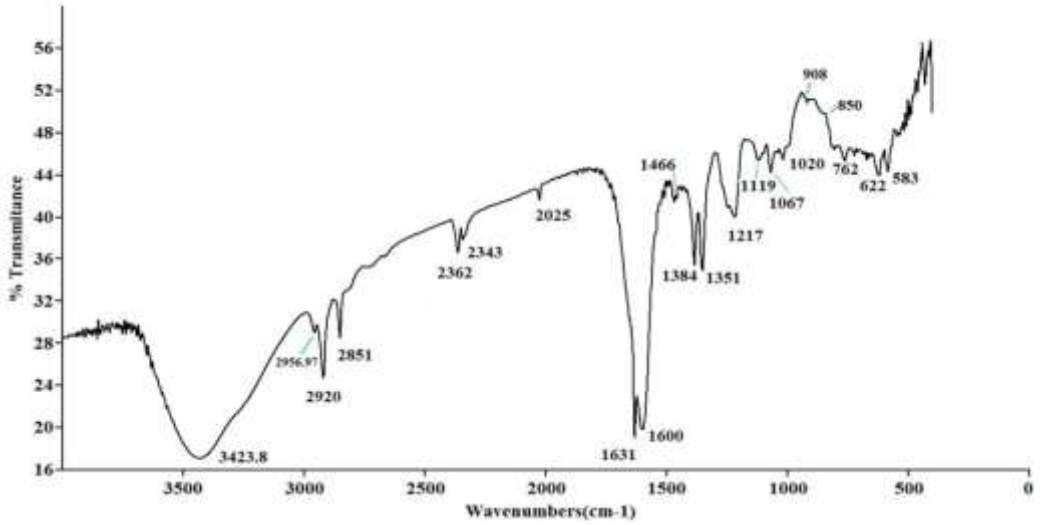

Figure 7. FT-IR analysis of the red pigment

1H-NMR Spectral Studies

The NMR spectral analysis showed that a distinct spectrum, which indicates the position of each proton in the sample molecule. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.27, 6.79, 6.71, 6.12, 6.07, 5.01, 4.02, 3.06, 3.04, 3.02, 3.01, 2.54, 2.35, 2.26, 2.24, 2.23, 2.22, 2.21, 1.26, 0.89 (Figure 8).

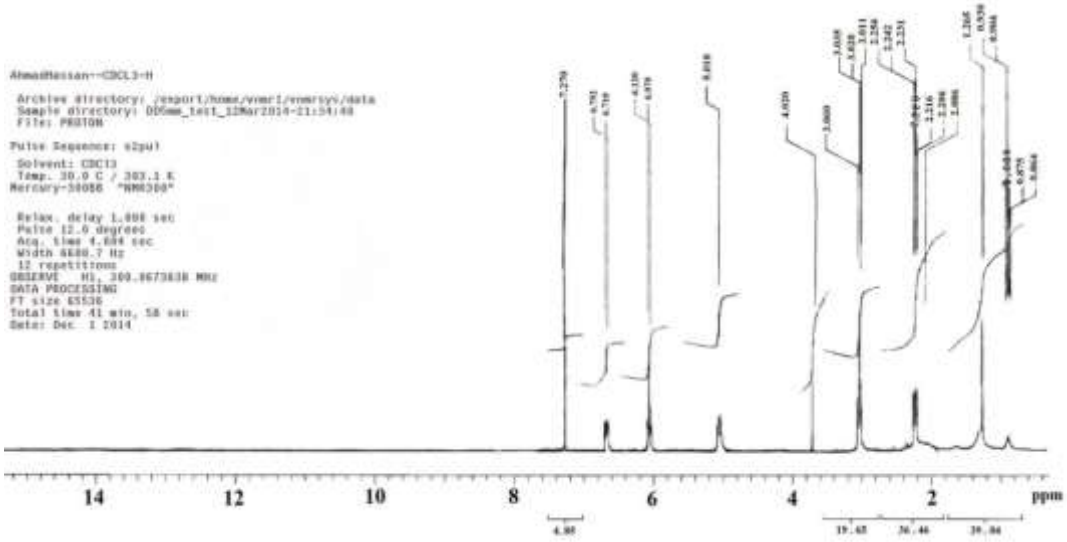

Figure 8.^{1}H NMR analysis of the purified pigment.

Analysis of prodigiosin genes cluster of *S. marcescens* strain WSE

The amplified prodigiosin genes were separated by agarose gel electrophoresis (Figure 9). The purified and sequenced by the Korean Macrogen Sequencing Services. The obtained results revealed that *pigA* fragment was 334 bp long; and that of *pigC, pigE, pigE, pigF, pigH, pigI, pigJ, pigL and pigN* fragments were 410 bp, 333 bp, 261 bp, 328 bp, 333 bp, 372 bp, 236 bp and 533 bp long, respectively. The Blast Hit of pig genes that have been included in Figure 10, are *pigA, pigC, pigE, pigF, pigH, pigI, pigJ, pigL* and *pigN as* indicated by red color. Of these, *pigL* has sequence identity with the pig sequences of *S. marcescens* based on BLASTN search results. There is a small variation in the level of identity between genes in the pig clusters and the PCR product. Table 2 shows the length and percentage of identity of *pig* genes based on BLAST searches of Geneious pro 8.0.1. Software. For example, the percentage of identity of Serratia *pigA, pigC, pigE, pigF, pigH, pigI, pigJ, pigL and pigN*, are 98.7%, 100%, 98.2%, 97.7%, 98.8%, 97.3%, 99.5%, 98.3% and 99.4%, respectively.

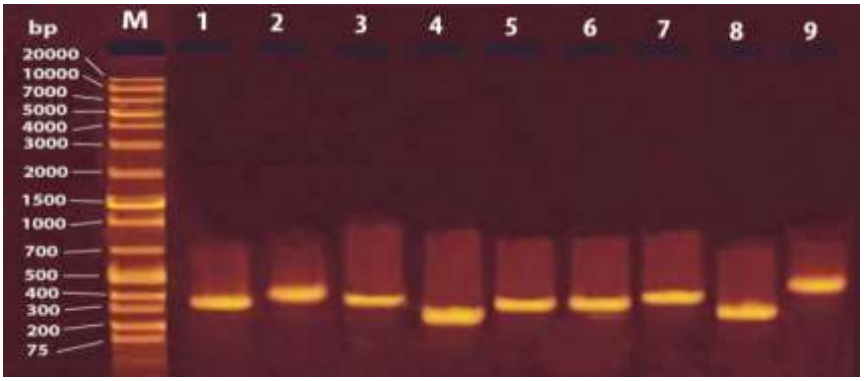

Figure 9. Agarose gel electrophoresis of the prodigiosin genes amplified by PCR. Lane M: 100 bp molecular weight DNA marker; lanes 1-9, *pigA, pigC, pigE, pigF, pigH, pigI, pigJ, pigL and pigN*, respectively.

Table 1. NCBI BLASTN homology search for highly similar sequences of *pig genes*

Target gene	Length (bp)	Accession number	Percent of Identity
pigA	**334**	AJ833002, CP005927, CP003959	98.7%, 97.8%,97.8%
pigC	**410**	AJ833002, CP005927, CP003959, HQ833702	100%, 99.0%,99.0%, 99.0%
pigE	**333**	AJ833002, CP005927, CP003959	98.2%, 97.3%,97.0%
pigF	**261**	AJ833002, CP005927, CP003959, AJ833001	97.7%, 96.9%,96.2%,80.2%
pigH	**328**	AJ833002, CP005927, CP003959, CP000116, EF063590	98.8%, 97.9%,97.9%,77.9%,76.5%
pigI	**333**	AJ833002, CP005927, CP003959	97.3%, 95.5%,95.2%
pigJ	**372**	AJ833002, CP005927, CP003959	99.5%, 97.7%,97.4%
pigL	**236**	AJ833002, CP005927, CP003959	98.3%, 96.2%,95.7%
pigN	**533**	AJ833002, CP005927, CP003959	99.4%, 97.4%,97.4%

Figure 10. The BLAST Hit of sequenced *pig* genes (red color)

Effect of precursor (amino acids) on prodigiosin production

Figure 11 and Table 3 indicate the different levels of prodigiosin produced by *S. marcescens* strain WSE in presence of different amino acids. The highest amount of the pigment was induced by L-tyrosine, followed by NB, alanine, proline and serine. Nutrient broth supplemented with L-tyrosine induced the highest amount of prodigiosin both intracellular and extracellular relative to the tested amino acids in comparison with alanine. However, the addition of serine suppressed pigment production.

Table 3. Effect of different amino acids on prodigiosin production by *S. marcescens* strain WSE. using Nutrient broth at 23°C at 24 h.

Amino Acids (5 mg/ml)	Concentration of prodigiosin (OD 535) mg/l	
	Intracellular	**Extracellular**
NB	7.58 b	2.78 b
Alanine	6.39 c	2.59 b
Proline	4.15 d	2.20 c
Serine	0.55 e	1.48 d
L-Tyrosine	21.44 a	8.87 a
LSD at 5%	1.19	**0.39**

Different letters indicate significant differences between treatments (Duncan test, $P \leq 0.05$). Means, in each column, followed by similar letter are not significantly different. The results are means of three experiments.

Effect of L-Tyrosine concentrations on pigment production

The effect of different concentrations of L-Tyrosine was investigated as an inducer for prodigiosin production. Experiments were conducted to determine prodigiosin production in presence of 2.5, 5 and 10 mg/ml of L-Tyrosine. The results showed that addition of L-Tyrosine could enhance the yield of prodigiosin by two times more than the production of pigment as the previous experiment. Figure 12 showed that 0.5% is the most suitable concentration for pigment production (21.48 mg/l).

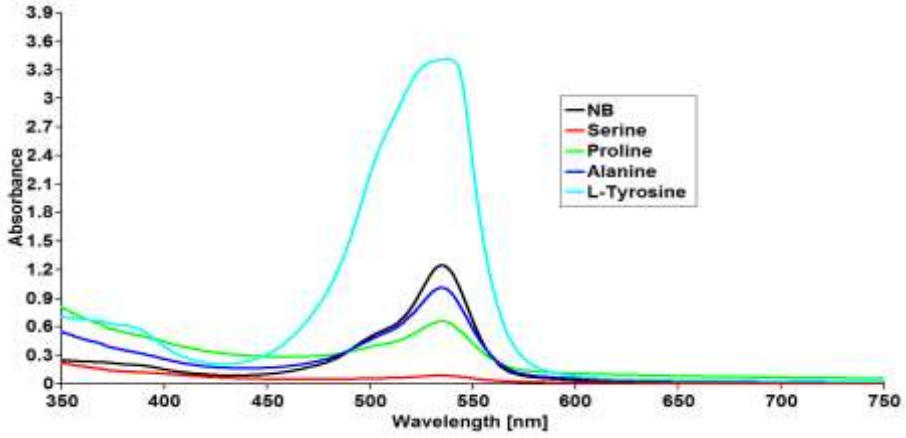

Figure 11. Spectral analysis of the effect of different amino acids on prodigiosin production by *S. marcescens* strain WSE.

Table 4. Effect of L-Tyrosine concentration on prodigiosin production by *S. marcescens* using Nutrient broth at 28°C at 24 h

L-Tyrosine concentration	**Concentration of prodigiosin (OD 535) mg/l**	
	Intracellular	**Extracellular**
2.5 mg/ml	21.37 a	4.03 c
5mg/ml	21.46 a	7.82 a
10mg/ml	20.67 b	4.63 b
LSD at 5%	0.70	0.59

Different letters indicate significant differences between treatments (Duncan test, $P \leq 0.05$). Means, in each column, followed by similar letter are not significantly different. The results are means of three experiments.

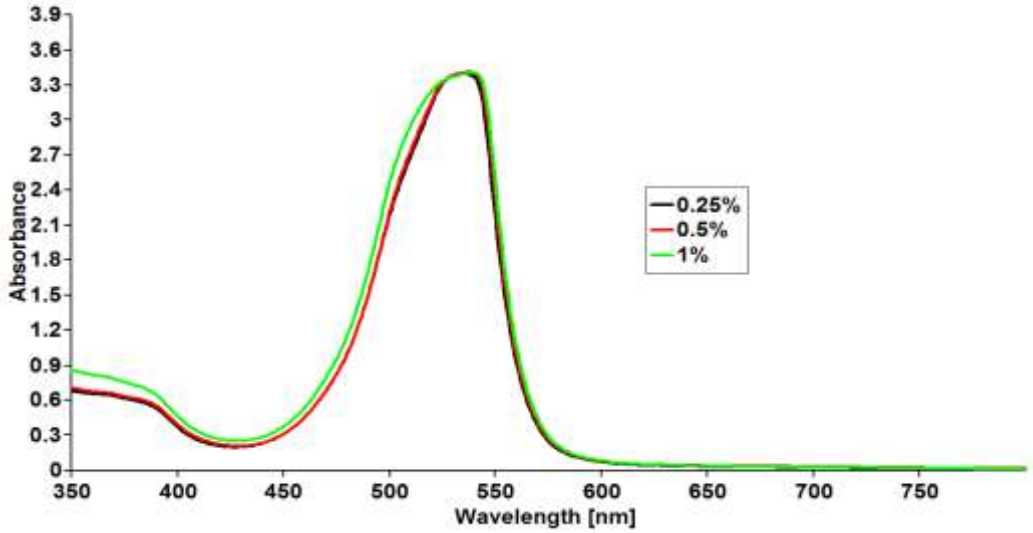

Figure 12. Effect of L-Tyrosine concentration on prodigiosin production by *S. marcescens* strain WSE.

4. Discussion

In the present study, the red pigment produced by *S. marcescens* strain WSE was extracted with ethanol then purified followed by microfiltration (0.45 µm) **(Wang et al., 2013)**, and dried by evaporation of the solvent at 50°C using rotary evaporator. The resulting extracted pigment was identified by measuring the absorption spectra over the range of 200–800 nm in 95% ethanol **(Casullo de Araújo et al., 2010; Patil et al., 2011),** which was found to be dependent on pH value as different colors and different absorption spectra was obtained at different pH values. For example, pH 2.0, the pigment was red and showed a maximum absorption at 535 nm, which is identical to that of prodigiosin hydrochloride. Under neutral condition (pH 7.0), its absorption intensity decreased and the color of the pigment changed to pink. However, in alkaline condition (pH 9-10.0) the color was orange or yellow and its absorption spectrum shifted to 470 runs **(Gerber and Lechevalier, 1976; Ding and Williams, 1983).** It has been suggested that the nitrogen of the three conjugated pyrrole rings are protonated by NaOH **(Rizzo et al., 1999)**. Prodigiosin has long been known to respond to pH change since the addition of acid causes a bright red colour and the addition of alkali produces an orange shade **(Lewis and Corpe 1964)**. The coloration of prodigiosin solution depended on the ratio of the red (absorption maximum, 535 nm) and yellow (460–470 nm) pigment forms, which, in turn, depended on the final pH of the solution. The neutral and slightly alkaline solutions were characterized by the presence of both absorption peaks; a change in the final pH resulted in a corresponding change in the absorption spectrum **(Andreyeva and Ogorodnikova 2015).**

A single band with an Rf value of 0.9 was obtained after thin-layer chromatography with chloroform: methanol (9:1; v/v) solvent system. Song et al. (2006) reported single red prodigiosin band with Rf value 0.43. However, a single red prodigiosin band with the same Rf value 0.9 was reported **(Patil et al., 2011; Sumathi et al., 2014)**. A single red colored band was obtained after column chromatography using a silica gel column and the same was eluted with n-hexane and ethyl acetate (2: 1; v/v) and concentrated by evaporation **(Williams et al., 1956; Williams et al., 1961).**

The FTIR spectra of the red pigment showed that it has several degrees of similarity to the spectra of prodigiosin **(Sumathi et al., 2014).** The obtained spectrum showed a broad envelope around 3600-3300 cm^{-1}centered at 3423.8 cm^{-1} which attributed to the –N–H stretch. A strong and broad absorption for NH was evident at *vmax* 3,423 cm^{-1}. This result indicated that the pattern of this pigment is similar to that of prodigiosin **(Patil et al., 2011).** The peaks at 2920 (aromatic CH) are due to asymmetrical stretching of methylene groups and 2851 cm^{-1} are characteristics of symmetrical stretching of methylene groups in prodigiosin structure. FTIR absorption in KBr for the red pigment was dominated by very strong bands at 1600 cm^{-1} (aromatic C=C). Prodigiosin exhibits very strong bands at 1630 due to the presence of –NH. The peaks at 1119 and 1067 cm^{-1} were attributed to C-O stretching. The visible peak at 1384 cm^{-1} was due to the presence of C–O group in prodigiosin. The peaks around 1217 cm^{-1} and 762 cm^{-1} are attributed to carbon-carbon double bond prodigiosin **(Song et al., 2006; Sumathi et al., 2014)**. CH and CH2 bending occurred at 908 cm^{-1}, C-H bending and ring puckering caused a spectra at 850 and 762 cm^{-1} **(Garg et al.,**

2013). The FTIR spectra of the red pigment showed that it has several degrees of similarity to the spectra of prodigiosin **(Rustom et al., 1990; Song et al., 2000).** The mass spectrometry of the purified pigment produced by *S. marcescens* strain WSE was 324.2 Da, which corresponds to that of the molecular mass of prodigiosin as reported by **Giri et al., (2004); Kim et al., (2008); Lins et al., (2014); Siva et al. (2012); Song et al., (2006) and Sundaramoorthy et al., (2009)**. In the NMR spectrum, a chemical shift of the methoxy group in the molecule exhibited δ 4.02 ppm as a single peak. In addition, two peaks at δ 7.27 ppm and δ 5.01 ppm corresponding to the chemical shift (in CDCl3) of NH^{2+} protons in the pyrrole ring was δ 7.27 ppm and the chemical shift (in CDCl3) of NH^{2+} protons in the pyrrole ring was δ 5.01 ppm. It is therefore concluded that the identity is prodigiosin-like pigment **(Sumathi *et al.*, 2014)**. The NMR spectrum peaks of the product indicate the consistency of chemical shifts of methylene groups of prodigiosin like pigments at δ 2.54 ppm, δ 2.35 ppm, δ 2.26 ppm, δ 2.24 ppm, δ 2.23 ppm, δ 2.22 ppm, δ 2.21 ppm, δ 1.26 ppm, the peaks corresponding to chemical shifts at δ 2.086 ppm and δ 0.89 ppm assigned to methyl group. The peaks δ 6.792 ppm (C2-H), δ 6.710 ppm (C6-H) and δ 6.070 ppm (C6-H) of NMR of the purified product is corresponding to the chemical shift (in CDCl3) of C-H protons in the pyrrole ring **(Wei and Chen 2005)**.

The *S. marcescens pig* clusters contain 14 candidate genes common to both strains and are arranged *pigA* through to *pigN*, five are assigned genes products to the biosynthesis of MBC (red) and four to the MAP (blue) pathway. Four are unassigned, with the remaining protein (*pigL*) proposed to be involved in the post-translational modification of some of the proteins in the cluster. Of these, *pigL* has sequence identity with phosphopantetheinyl transferases (PPTases) **(TSAO et al., 1985; Lambalot et al., 1996; Reuter et al., 1999; Cerdeño et al., 2001; Harris et al., 2004).** In the present study, sequence Blastn result for the *pigA* fragment of *S. marcescens* isolate WSE showed 98.7%, 97.8% and 97.8% similarity to *S. marcescens* prodigiosin biosynthesis gene cluster (Acc. No. AJ833002), *S. marcescens* WW4, complete genome (Acc. No. CP005927) and *Serratia* sp. FS14, complete genome (Acc. No. CP003959) of *S. marcescens* in NCBI EMBL-Bank database. Sequences of *pigC, pigE, pigF, pigH, pigI, pigJ, pigL* and *pigN* in this study were similar to the same sequences in the database having a maximum identity of (100%, 99.0%,99.0%), (98.2%, 97.3%,97.0%),(97.7%, 96.9%,96.2%), (98.8%, 97.9%,97.9%),(97.3%, 95.5%,95.2%),(99.5%, 97.7%,97.4%),(98.3%, 96.2%,95.7%) and (99.4%, 97.4%,97.4%), respectively, similar to *S. marcescens* prodigiosin biosynthesis gene cluster (Acc. No.AJ833002), *S. marcescens* WW4, complete genome (Acc. No.CP005927) and *Serratia* sp. *FS14*, complete genome (Acc. No. CP003959) of *S. marcescens*.

It should be specially noted that *pigC, pigF and pigH* also are identical 99.0%, 80.2% and (77.9%, 76.5%) to *S. marcescens* strain jx1 prodigiosin synthase *(pigC)* gene, complete cds (HQ833702), *Serratia* sp. ATCC 39006 prodigiosin biosynthesis gene cluster (AJ833001) and (*Thiobacillus denitrificans* ATCC 25259, complete genome (CP000116) and *Janthinobacterium lividum* strain BR01 clone pBR red genomic sequence (EF063590) as mentioned above. These results suggest that the fragment is most likely the pig genes fragment of *S. marcescens* isolate. The analysis of the nucleotide sequences of *S. marcescens* isolate exhibited a high degree of sequence similarity with the genes involved in prodigiosin biosynthesis in some bacterial species**.** The prodigiosin biosynthesis gene cluster (*pig* cluster) from *S. marcescens* has been sequenced. Sequence analysis of the nine pig genes indicated that the PCR products have high similarity to *S. marcescens* prodigiosin biosynthesis gene cluster (Accession number: AJ833002) **(Harris et al., 2004)**.

There is an evidence that the amino acid precursor may play a crucial role in synthesis of prodigiosin by *S. marcescens* **(Cerdeño et al., 2001; Thomas et al., 2002; Williamson et al., 2005).** In the present study, L-tyrosine enhanced the maximum pigment production in relative to other amino acids. Many amino acids, which contain pyrrole-like structures have inductive effect for prodigiosin production **(Wei et al., 2005). Siva et al., (2012)** reported that proline acts as a good precursor for pigment production when used individually or in combination; however, serine resulted in reduction in pigment production. Proline did not cause biosynthesis of prodigiosin in non-proliferating cells unless it was catabolized **(Scott et al., 1976).** To understand the rate of L-tyrosine as an inducer for prodigiosin production, experiments were carried out using different concentrations of L-tyrosine. The results indicated that addition of 2.5, 5 and 10 mg/ml of L-tyrosine could enhance the yield of prodigiosin in comparison with other precursor amino acids, but higher L-tyrosine concentration had a very little significant effect.

Conclusion

This study suggests that L-Tyrosine play an important role for maximum induction of pigment production by *S. marcescens* isolate WSE. The prodigiosin biosynthesis gene cluster (pig cluster) from *S. marcescens* has been sequenced. Sequence analysis of the nine pig genes predicted that the PCR

products showed high similarity to *S. marcescens* prodigiosin biosynthesis gene cluster with an Accession number: (AJ833002).

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