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Optimization of Prodigiosin Production by A New Isolate of *Serratia* species

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Abstract

The bacterial compound Prodigiosin generated tremendous amount of interested due to its medical and industrial applications. A new prodigiosin-producing bacterium (strain AM8887) was isolated from the deep water of the Red Sea, Egypt. Classical cultural, morphological, biochemical tests and modern 16S rRNA gene sequence analysis identified this bacterium as a member of the genus *Serratia*. Specifically, the closest strain to the local isolate is the *Serratia plymuthica* AJ233433T; with a percentage similarity of 98.89%. A sequential combination of Plackett–Burman design and Central Composite Design optimized the bioprocess variables for prodigiosin production by this isolate. The optimum amounts of prodigiosin was produced when the isolate grown at 25°C on a rotary shaker (180 rpm) in a medium containing 6 g/l glycerol, 6 g/l sucrose, 1 g/l yeast extract, 15 g/l NaCl and 12 g/l fertilizer factory waste water at pH 6.5. Fertilizer factory wastewater enhanced prodigiosin production by 40%; raising the productivity from 1328 mg/l to 1805.28/l mg/l. In conclusion, the apparent up regulation of prodigiosin production by the exogenous addition of fertilizer industry waste water can be attributed to its chemical composition. The positive response of the local *Serratia* strain AM8887 to these chemicals warrants deeper investigation of what is called "wastes" some of them could contain huge hidden benefits.

Keywords: Prodigiosin (PG), Plackett–Burman design (PBD), Central Composite Design (CCD).

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Introduction

Plants and microbes are two major sources for natural pigments, although biopigments are produced by some animals, too. These biopigments serve as photosynthetic, sun protection, camouflaging warning signals and sexual selection (Gulani et al., 2012). Biopigments from plants suffer from light, heat or pH instability and low water solubility, contrary to pigment produced by microorganisms which can be produced in large amounts in cheap culture medium, independence from weather conditions (Venil and Lakshmanaperumalsamy, 2009b).

Serratia species inhabit many environmental niches including water, soil, plants, insects, vertebrates and human (Vora et al., 2014). Many useful medical and industrial compounds are produced by the species of this bacterial genus. These included the bacteriocin, chitinases, protease, lipase and nucleases (Mekhael and Yousif, 2009, Zarei et al., 2010). Prodigiosin (2-methyl-3-amyl-6-methoxyprodigiosene) is another important secondary metabolite of *S. marcescens*, *S. plymuthica* and *S. rubidaea*. This with low molecular weight a linear tripyrrole pigment produced in the late stage of bacterial growth and was reported to be associated with extracellular vesicles or intracellular granules (Gulani et al., 2012). Prodigiosin has no defined role in the physiology of the producing strains, but it was reported to have antifungal, antibacterial, immunosuppressive and anticancer activities (Priya et al., 2013).

These medicinal activities of prodigiosin motivated a number of scientific groups to produce it in large quantities, but the cost was even not encouraging, since 1mg costs up to $500 (Xu et al.). PG was reported to be significantly affected by various condition such as pH, temperature, carbon sources, nitrogen sources and phosphate source (Solé et al., 1994, Gibbons and Buchanan, 1975), optimizing such variables may lead to a significant increase in the productivity of the PG and may lead to significantly decrease the cost of the production.

Reduction of the overall production cost of prodigiosin by the local *Serratia* strain was set as the main goal. Employing modern statistical approaches such as Plackett-Burman design (PBD) followed by Response Surface Methodology (RSM) not only optimized the bioprocess variables for growth and prodigiosin production such as by *Serratia* AM8887 but identified fertilizer waste water as a positive enhancer.

Materials and Methods

Sample collection, isolation and cultivation condition

Water samples and deep sea core sediments were collected from a drilling stand in the Red Sea, Egypt. These samples were collected by a diver at 25 m depth latitude and longitude of (27.24-33.89) for the Red Sea sampling area. These samples were diluted in sterile water by serial dilution up to 10^{-7} and 100µl from the last three dilutions were plated out on LB agar plates (Sundaramoorthy et al., 2009). All the pigmented bacteria colonies were picked and purified further by the compound streak method and the purified colonies were kept at 4°C for future work. The best red pigment producer was selected, stocked in 20% glycerol and kept at -80°C.

Biochemical identification of the local isolate AM8887

Biochemical identification and characterization of *Serratia* isolate AM8887 was performed by MicroScan Walk-Away® system using Negative Breakpoint Combo 44 (NBPC 44) panel as per the manufacturer's instructions (Vivas et al., 2000). In addition, the capabilities of the purified strain to produce some common microbial enzymes such as amylase (Gaur et al., 2012), caseinase (Vashist et al., 2013), cellulose (Ariffin et al., 2006) and lipase (Kumar et al., 2012) were explored.

Antibiotic sensitivity test

MicroScan Walk-Away® using Negative Breakpoint Combo 44 (NBPC 44) panel determined the antibiogram of the isolated *Serratia* strain AM8887. The instrument tested the sensitivity of the local strain to the following 25 antimicrobials agents: ampicillin/sulbactam, ampicillin, amoxicillin/k-clavulanate, aztreonam, cefazolin, amikacin, cefepime, cefotaxime, ceftazidime, cefuroxime, ceftriaxone, cephalothin, ciprofloxacin, gentamicin, imipenem, ertapenem, levofloxacin, meropenem, Cefoxitin, tetracycline, tigecycline, trimeth/sulfa, Piperacillin, tazobactam and tobramycin. Moreover, it determined the minimum inhibitory concentration (MIC) and a qualitative degree of susceptibility like susceptible (S), intermediate susceptible (I) and resistant (R).

Molecular identification of the local bacterium, isolate AM8887

Total genomic DNA was extracted from strain AM8887 by the Insta-Gene Matrix Genomic kit (Bio-Rad, USA) and the 16S rRNA gene was amplified the 518F forward primer (5'- CCA gCAgCCgCggTA ATA Cg -3') and 800R reverse primer (5'-TACCAgggT ATC TAA TCC -3') (Cole et al., 2009). The PCR product was sequenced using BigDye™ version 3.1Terminator Cycle Sequencing Kit (PE Applied Biosystems, MA), followed by basic local alignment search tool (BLAST) and SeqMatch analysis. The quantity and quality of the sequence obtained was checked with Finch TV version 1.4.0. While, CAP3 software was used to assemble the gene. Then the 16S rRNA gene sequence was used to construct Phylogenetic tree by using Seaview software and automated BLAST searches to detect the closest type strain to the isolate AM8887.

Optimization of physico-chemical growth parameters

The bacterium was grown in LB broth (50 ml Flaks) and physico-chemical variables (temperature, pH and agitation speed) were optimized via a Central Composite Design (CCD) according the matrix displayed in Table 5 (Elrazak et al., 2013). However, the CCD matrix included five levels for each physico-chemical variable are shown in Table 1. Overall, the CCD provided an indication of the main effect of each factor in addition to the interaction between them.

Table 1: Physico-chemical variables and their levels

Variables	Levels				
	-2	-1	0	+1	+2
pH	5.81	6.5	7.5	8.5	9.18
Temperature (ºC)	23	25	30	35	38.4
Agitation speed (RPM)	0	50	125	200	251

Screening for best nutrient(s)

Placket-Burman Design (PBD) was used to screen ten relatively cheap and locally available materials for their effect on prodigiosin production and bacterial growth (Ward and Glassey, 2014). Each component was investigated at a high (+1, 6 g/L) and a low (-, 0 g/L) levels where the low level represented the absence of the investigated variable. In addition one dummy variable was included to calculate the standard error of the experiments. The experiments were done in Erlenmeyer flasks containing 50 ml of basal media (yeast extract, 1 g/l and NaCl, 15 g/l) in addition to component of each trial and incubated at optimized condition: 180 rpm and 25 °C for 48 hr. Responses were measured in terms of growth (OD_{620}) and prodigiosin concentration.

Optimizing the production medium for prodigiosin

CCD was used to detect the optimum level of each of the three selected variables (nutrients) and optimized the potential prodigiosin production medium (El Razak et al., 2014). Five different levels for each variable were tested as dictated by the CCD program (-2, -1, 0, +1 and +2), or 4g/L, 6, 9, 12 and 14g/L, respectively as shown in Table 2. The prediction of the optimum production medium composition for prodigiosin production generated by second-order polynomial model, as dictated by the program itself and calculation follow its equation. The significance of the experimental values was determined by the Analysis of variance (ANOVA) using Design Expert 8.0 statistical package (StatEase, Inc, Minneapolis, MN, USA).

Table 2: Variables and their levels

Variables (g/L)	Levels				
	-2	-1	0	+1	+2
Sucrose	4	6	9	12	
Fertilizer waste	14				
Glycerol	4	6	9	12	
	14				
	4	6	9	12	
	14				

Genetic Stability of *Serratia*

Genetic stability of the producing bacterial strain was monitored in consecutive runs lasted for two months (Walter et al., 1987). Where, one ml from a two days old culture (sucrose 6 g/l, glycerol 6 g/l and fertilizer waste 12 g/l) was transferred to freshly prepared medium and then each culture was used for subsequent inoculation for the two months duration. Genetic stability was tested at three levels: bacterial growth, prodigiosin concentration (desired product) and protein banding patterns (gene expression) of the local *Serratia* isolate number AM8887 (Ashwood-Smith, 1965).

Statistical analysis

All data obtained from screening and optimization experiments were analyzed by the statistical program Minitab 16. The experimental responses were subjected to the analysis of variance (ANOVA). The *P* value designates a stastistical confidance of a factor estimate. A *P* value of <0.1 was used as a cut-off point indicating the statistical significance of a factor at 90% confidence level. It detected the most significant factors with effects on bacterial growth and pigment production at 90 % level of confidence and $\alpha = 0.1$. Moreover, a *P*-value equal or less than α was considered significant; marked **Bold** in the data presented (Venil and Lakshmanaperumalsamy, 2009a).

Result

Morphological and biochemical identification of the bacterium AM8887

A prodigiosin-producing bacterium was isolated in pure form and was found to be Gram negative, small, rod-shaped and characterized by bright red, smooth and sticky colonies. Biochemically the *Serratia* AM8887 was found to be fermented sucrose, sorbitol, glucose, arabinose, raffinose, inositol, Enteric Septicemia of Catfish (ESC), utilized citrate, o-nitrophenyl-b (ONPG), D-galactopyranoside, colistin (CL_4), cephalothin (CF_8) and Nitrofuration (FD_{64}). The isolate was also found to be negative for lysine, arginine, ornithine, rhamnose and melibiose fermentation, H_2S, indole production, urease test, trptophandeaminase, maltose, oxidase, acetate, CET, nitrate reductase and tartarate. Moreover, this particular strain of *Serrtia* AM8887 possessed the capability to produce lipase, caseinase, but no amylase or cellulase activities.

Antibiogram of *Serratia* strain AM8887

The MIC values indicated the degree of severity of the antibiotic tested; the lower the MIC value the more toxic the antimicrobial agent. The MIC values depend on the bacterium under investigation. The antibiogram of the isolated *Serratia* strain AM8887 showing the MIC values and a qualitative susceptibility for each of the 25 antimicrobial agents tested. *Serratia* AM8887 showed sensitivity to amikacin, ampicillin/sulbactam, ceftazidime, aztreonam, cefepime, cefotaxime, ampicillin, ceftriaxone, ciprofloxacin, gentamicin, imipenem, piperacillin, tazobactam, ertapenem, tobramycin, tigecycline, trimeth/sulfa, levofloxacin, meropenem and tetracycline. While found to be resistant to cefazolin, cefuroxime, cephalothin, cefoxitin and amoxicillin/ k-clavulanate were reported to have an intermediate effect on *Serratia*.

Molecular identification

The 16S rRNA gene sequence of the isolate AM8887 was submitted to the Genbank under the accession number KU726587. This sequence was used to construct a phylogenetic

dendrogram. Careful analysis of this dendogram showed the phylogenetic position of the isolate AM8887 compared to closely related species of the genus *Serratia* sp. Strain AM8887 was found to be most closely related to *Serratia plymuthica* AJ233433T (98.89%) type strain with percentage similarity of 98.89% as shown in Figure 1.

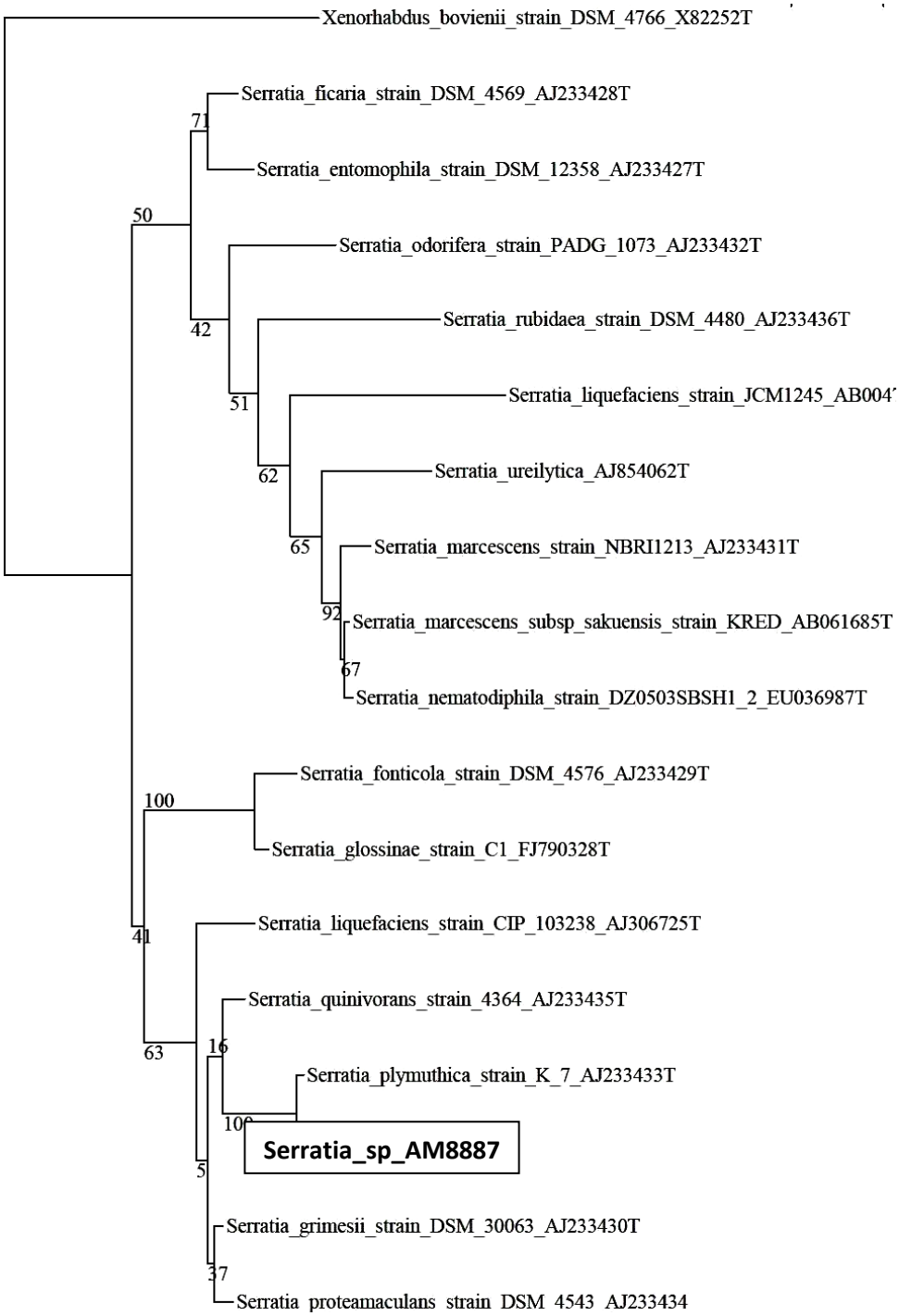


Figure 1: A phylogenetic dendrogram based on 16S rRNA gene sequence analysis showing the position of the local bacterial isolate AM8887 compared to closely related species of the genus *Serratia*. The sequence of *Xenorhabdus bovienii* strain DSM 4766^T used as external reference.

Physico-chemical parameters optimization

Agitation speed, temperature and pH significantly affected the bacterial growth and pigment production; where the matrix and responses were summarized in Table 3.

Table 3: Matrix and responses for physic-chemical optimization

Run	Variables			Responses	
	pH	Temperature °C	Agitation Speed (rpm)	Optical density OD_{620}	PG (mg/l)
1	6.5	25	50	0.7470	764
2	6.5	35	50	0.7510	136.59
3	8.5	25	50	0.7130	780.22
4	8.5	35	50	0.8870	118.07
5	6.5	25	200	1.6820	2208.69
6	6.5	35	200	0.3550	101.868
7	8.5	25	200	1.7900	240.779
8	8.5	35	200	1.5626	83.34
9	7.5	23	125	0.9530	1236.30
10	7.5	38.4	125	0.4780	90.29
11	5.8	30	125	1.3600	3445
12	9.18	30	125	0.5640	2694.88
13	7.5	30	0	1.3000	3009.75
14	7.5	30	251	1.6900	3222.74
15	7.5	30	125	0.4490	3226.16

16	7.5	30	125	0.5730	3102.35
17	7.5	30	125	0.4490	3426.16
18	7.5	30	125	0.5730	3102.35
19	7.5	30	125	0.4490	3426.16
20	7.5	30	125	0.5730	3102.35

Analysis of quadratic effects and interaction between factors and variance of the linear CCD matrix were summarized in S1. The experimental responses were subjected to the analysis of variance and parameter estimates and results were summarized in S1. The *P*-value designates a statistical confidence of a factor estimate. A *P*-value of < 0.1 was used as a cut-off point showing the statistical significance of a factor. The factors which have *P*-value < 0.1 were considered to be significant.

The optimum interaction between the different variable on the calculated growth response ($O.D_{620}$) and PG production can be divided into: 1) agitation and temperature, 2) pH and agitation and 3) pH and temperature. As for the growth response, the best following combinations of these variables were: 180-250 rpm and 22-28°C, pH values of 5.8 - 6.63 at static condition and 8.27- 9.10 at 180-250 rpm and pH range from 5.8 - 6.63 with an optimum temperature from 22 – 24.80°C, respectively. The optimum interaction between the different variable on PG production as summarized in the 3D surface plots in Figure 2.

Meanwhile, the overall optimum condition for PG production was found to be 25°C, pH 6.5 and 180 rpm for 48 hr.

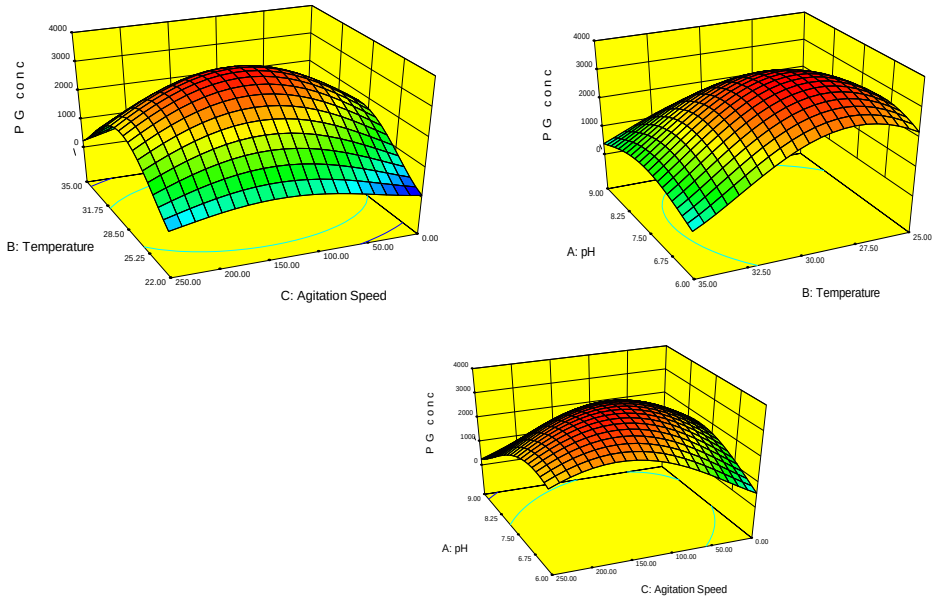


Figure 2: Three- dimensional surface plot showing the interaction between variables on maximum PG production

Optimizing media components

Screening of the ten media components showed that ammonium chloride, corn steep liquer, glycerol, urea, ground sesame, fructose and ammonia have a statistically significant effect on the growth. The matrix and responses are summarized in Table 4.

Table 4: 10 different cheap material studied by their levels on for bacterial growth and prodigiosin production via Plackett-Burman design for the isolate under investigation

Run	Variables											Responses	
	Ammonia	Ammonium chloride	Corn steep liquor	Whey	Glycerol	Urea	Fertilizer waste	Sucrose	Ground sesame	Fructose	Dummy	O.D620	PG (mg/l)
1	1	-1	1	1	-1	1	-1	-1	-1	1	1	0.499	2589
2	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	0.194	1328
3	-1	-1	-1	1	1	1	-1	1	1	-1	1	1.581	6795
4	-1	-1	1	1	1	-1	1	1	-1	1	-1	0.596	13855
5	1	-1	1	-1	-1	-1	1	1	1	-1	1	0.480	14961
6	-1	1	1	-1	1	-1	-1	-1	1	1	1	0.730	3209
7	1	1	1	-1	1	1	-1	1	-1	-1	-1	0.496	7701
8	1	-1	-1	-1	1	1	1	-1	1	1	-1	1.571	1549
9	1	1	-1	1	-1	-1	-1	1	1	1	-1	0.679	1527
10	1	1	-1	1	1	-1	1	-1	-1	-1	1	0.237	2501
11	-1	1	-1	-1	-1	1	1	1	-1	1	1	0.425	2324
12	-1	1	1	1	-1	1	1	-1	1	-1	-1	0.838	2457

The experimental responses were subjected to the analysis of variance and parameter estimates and results were summarized in S2.

The interaction among variables and the calculated responses could be mathematically modeled by using first order polynomial models were as follows:-

For optical density,

Y1 = 0.6938 - 0.126* ammonium chloride - 0.087*corn steep liquer + 0.0445* whey + 0.175* glycerol + 0.21* urea + 0.2860* ground sesame + 0.056* fructose – 0.0335* ammonia + 0.0157* sucrose + 0.014*fertilizer waste.

For pigment concentration,

Y2 = 5066 – 1780*ammonium chloride + 2396* corn steep liquer + 869* glycerol – 1164* urea – 891* fructose + 1023* ammonia + 2794* sucrose +1208*fertilizer waste

A normal probability plot for the standardized effect is presented in Figure 3, indicating whether a factor is significant or not. The factors with positive effect are present to the right of the line, representing statistical significance and those with negative effect to the left of the line. The further the factor lies from the line, the more significant and the greater the effect of this factor for the given responses.

a) b)

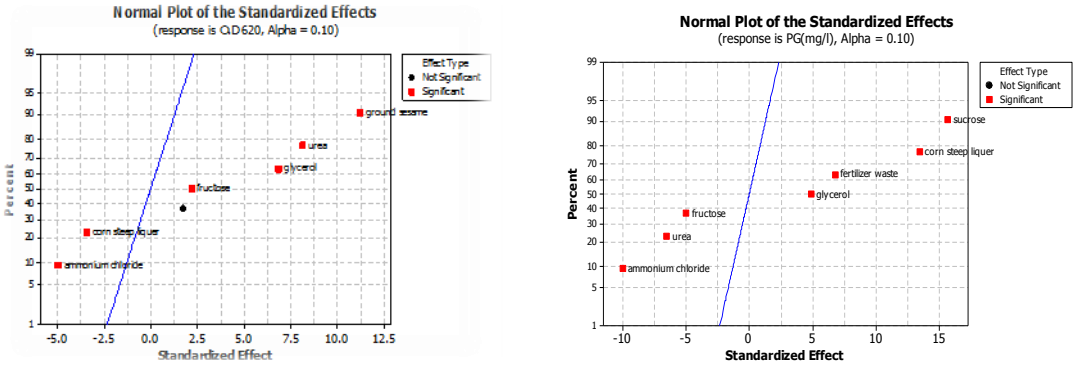


Figure 3: Normal plot of the standardized effect showing the significant effect of each variable on a) optical density, b) pigment concentration.

Ground sesame, urea, glycerol and fructose were found to have a positive statistically significant effect on bacterial growth with *P*-values <0.1. Sucrose showed no significant effect on bacterial growth, but Ammonium chloride and Corn steep liquor were found to have a negative

impact as shown in Figure 3a. Corn steep liquor, sucrose, fertilizer waste and glycerol were found to have a positive significant effect on prodigiosin production which has *P*-value <0.1 but fructose, urea and ammonium chloride were found to have negative significant effect as shown in Figure 3b.

Central composite design for the most significant cheap materials

Fertilizer wastewater, sucrose and glycerol showed the most positive significant effect on bacterial growth and PG production, beside 1g/L yeast extract and 15g/L sodium chloride. CCD matrix determined the optimum concentration ratios between the different medium components (Table 5). The optimum medium for PG production was found to contain (g/l): sucrose 6, glycerol 6, fertilizer wastewater 12, yeast extract 1 and NaCl 15.

Table 5: Matrix and Responses of CCD applied for significant cheap material.

Run	Variables			Responses	
	Sucrose g/l	Glycerol g/l	Fertilizer waste g/l	$O.D_{620}$	PG (mg/l)
1	9	9	9	0.505	989.5
2	6	6	6	0.660	1704
3	**6**	**6**	**12**	**0.758**	**1805.85**
4	4	9	9	0.575	1636.84
5	6	12	6	0.633	1592.85
6	9	4	9	0.566	1416.89
7	12	6	12	0.524	551
8	9	14	9	0.564	465.35
9	9	9	9	0.670	984.32
10	12	12	12	0.734	685.3
11	9	9	14	0.582	812.63
12	9	9	9	0.638	1317.34

13	9	9	9	0.505	989.48
14	12	12	6	0.506	523.23
15	9	9	4	0.674	1095.1
16	6	12	12	0.579	1833.63
17	14	9	9	0.571	662.14
18	12	6	6	0.450	435.25
19	9	9	9	0.670	984.3
20	9	9	9	0.638	1007.34

The experimental responses were subjected to the analysis of variance and parameter estimates and results were summarized in S3. The *P*-value designates a statistical confidence of a factor estimate.

From the ANOVA analysis, Sucrose was found to be the most significant factor affecting bacterial growth and PG production. The ANOVA table indicates that some of the interaction and quadratic effects of Sucrose, Glycerol and Fertilizer waste which have a significant impact on bacterial growth and PG production.

The interaction among the tested variables could be summarized using 3D surface plots as shown in Figure 4.

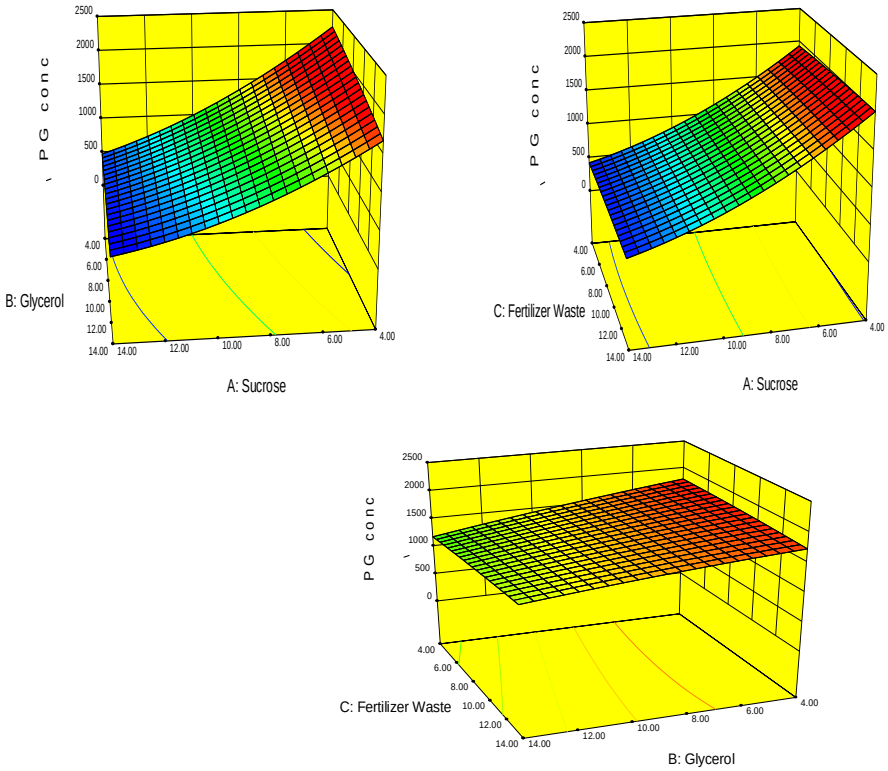


Figure 4: Three- dimensional surface plot showing the optimum interaction between variables on maximum PG production

Genetic Stability of *Serratia*

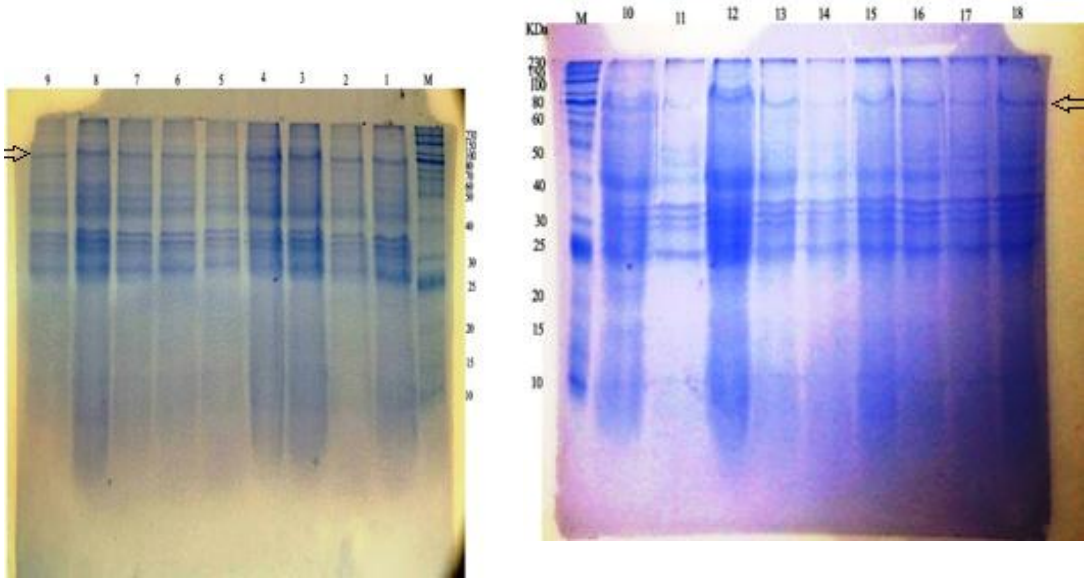
After twenty generation of bacterial transfers, no detectable abnormalities were observed in the bacterial growth (OD_{620}), prodigiosin concentration and protein banding patterns of *Serratia* strain AM8887 as shown in (Table 6 and Figure 5).

Table 6: No of generation and calculated responses for genetic stability of *Serratia*

No of generation	$O.D_{620}$	PG (mg/l)	No of generation	$O.D_{620}$	PG (mg/l)
1	1.129	6408.44	**11**	1.487	5537.93
2	0.993	4697.52	**12**	1.147	5639.8
3	1.062	3833.95	**13**	1.067	5500.89
4	1.183	4954.5	**14**	1.254	5454.58
5	1.004	5584.24	**15**	1.138	5547.2
6	0.987	5825.02	**16**	1.068	5500.89
7	0.948	5408.28	**17**	1.044	5482.37
8	1.1013	5852.79	**18**	1.055	5602.76
9	1.197	5760.2	**19**	0.992	5750.93
10	1.2	5528.67	**20**	1.152	5649.06

The OD values ranged from 0.949- 1.197 and PG concentration from 4954.5-6408.44 mg/L with a single odd value of 3833.95.

SDS-PAGE analysis of protein patterns of the different bacterial generations are grouped in the figure (5) below.



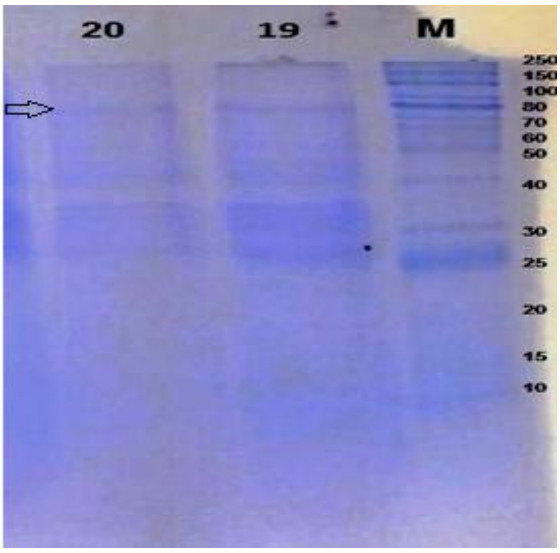


Figure 5: Electrophoretic patterns of SDS-polyacrylamide gel of twenty generation from lane (1-20) which represent the no of bacterial transfers. The arrowhead indicates the 100 kDa band which associated with prodigiosin formation

All protein banding patterns look similar with no differences. Moreover, The arrowhead indicates the 100 kDa band which is associated with prodigiosin formation and was retained in all the tested generations. It looks, as suggested by Kobayashi and Ichikawa (1989), associated with the inner cell membrane and/or the cytoplasm and not a secreted (protein) in the growth medium. The capability to produce the pigment and the appearance of the responsive band over the tested generations confirm the high genetic stability of the isolated *Serratia*.

Discussion

A *Serratia* strain AM8887, a prodigiosin-producing novel bacterium, was isolated from the deep water of the Red Sea, Egypt. Morphological, biochemical, antibiotic sensitivity and molecular tools were used to characterize and identify this local species. Moreover, the dendogram analysis of its 16S rRNA gene revealed its close belongs to the *Serratia plymuthica* AJ233433T; with percentage similarity of 98.89%. The low level of diversity does not qualify this isolate to be classified as a new strain which necessitates adoption of a new level of classification. It could be a new sub-strain or such new terminology to recognize these genetic differences. This isolate apparently produces high amounts of the prodigiosin-like pigment in growth medium. This red pigment belongs to the family of tripyrrole was found to exhibit antimycotic, antibacterial, immunomodulating, antimalarial and antitumor properties which raised the interest in obtaining it in high amounts (Priya et al., 2013). These pigments were found either present in intracellular granules or associated with extracellular vesicles (Gulani et al., 2012) (Vivas et al., 2000).

Fermentation technology devoted to optimize the potentials of the *Serratia* strain AM8887 for overexpression and synthesis of PG. The statistical experimental designs used for

optimization and enhancement included Plackett–Burman design and Central Composite Design (CCD). These strategies resulted in media which supported good growth of the bacteria and at the same time proved to enhance PG biosynthesis. The overall optimum physico-chemical condition for growth of the *Serratia* AM8887 strain and PG production inferred from these strategies were found to be 25°C, pH 6.5 and 180 rpm for 48 hr. The ANOVA table indicates that the main effect of agitation and temperature were found to be the most significant factor affecting bacterial growth, while pH was found to be insignificant. If a variable is significant with a negative effect, this means that the variable is effective on the response in low concentration. And if a variable is significant with a positive effect, this means that the variable is effective on the response in high concentration. The interaction between temperature and agitation speed in addition to quadratic effect of pH were found to be significant factor affecting bacterial growth while the main effect of temperature and quadratic effect of temperature were found to be the most significant factor affecting prodigiosin production. Additionally, the main effect of both pH and agitation were reported as insignificant.

Meanwhile, the ANOVA analysis suggested that the interaction and quadratic effects of Sucrose, Glycerol and Fertilizer waste have significant impact on bacterial growth and PG production. Specifically, ground sesame, urea, glycerol and fructose significantly affected bacterial growth (*P*-values <0.1); while, corn steep liquor, sucrose, fertilizer waste and glycerol were best for prodigiosin production. The maximum experimental response for PG production was 1805.85 mg/L.

It is important to note that the local *Serratia* AM8887 isolate physico-chemical condition of growth have great effect not only on the growth of the organism but on its potentials to produce PG. This isolate produces the pigment at 30 °C and the rate was reduced as the temperature increases; which is consistent with the mainstream trends in the literature. Almost all investigators reported that temperature ranges from 25-37 °C and pH7.0 is best for PG production. Samrot et al. (2011) have reported that a the highest PG productivity was obtained at 27°C, pH 7 for 72 hr, reduced when temperature reached 38°C and restored when returned to 27°C. Gulani et al. (2012) reported that pH and temperature affected the PG production. They also found that pH plays a significant role in secondary metabolite biosynthesis PG and the maximum production was attained at pH 7. Moreover, the temperature has influenced PG similar to Samrot et al. (2011), the maximum PG obtained at 25°C but its biosynthesis was blocked at 35°C. Similarly, Giri et al. (2004) found that *Serratia marcescens* produced PG at 28°C and 30°C in nutrient broth medium and once grown at 37°C no PG was produced.

Media components, beside the other growth conditions, were found crucial to PG productivity by different strains of *Serratia* (Kamble and Hiwarale, 2012, Sundaramoorthy et al., 2009, Tao et al., 2005 and (Venil and Lakshmanaperumalsamy, 2009a). No single medium was reported to be the best, but all are dependent on the *Serratia* strain and the experiment conditions used. However, one chemically-defined medium (without any other carbon source) reported consisted only of $(NH4)_2PO_4$ 6 g/L and trace salts 0.6 g/L yielded 1397.96 mg/L of PG at 30 °C (Venil and Lakshmanaperumalsamy, 2009a). (Kamble and Hiwarale, 2012) reported that the maximum PG observed after 72 hr was 1845.27 mg/L and 1335.38 mg/L in nutrient broth and peptone glycerol broth by *Serratia marcescens*. However, the maximum productivity of the same strain of *Serratia marcescens* grown at temperature 30 °C and pH 7.0 by (Sundaramoorthy et al., 2009) was 425±40 mg/L, almost 80% less efficient. Pilot-scale experiments (5L bioreactor) of (Tao et al., 2005) did not increase PG production higher than 583 mg/l, even so they used a two-step feeding strategy in which glucose was selected as the initial carbon source in the fermentation media and glycerol was fed as a PG inducer.

In our case, we found that addition of sucrose and glycerol enhanced the PG yield and this emphasized and highlighted the basic role of carbon source in increasing the PG production. The optimized medium for the local Serratia AM8887 isolate composed of sucrose, glycerol and fertilizer waste gave 1805.85 mg/L, similar to (Kamble and Hiwarale, 2012) when grown their strain in the rich medium; nutrient broth.

In conclusion, prodigiosin, a bacterial pigment created a much intrigue because of its enormous ability as medicinally important product. The isolated bacterial strain from Red Sea was found to be the closest to *Serratia plymuthica* AJ233433T based on the morphological, biochemical and 16s rRNA gene sequence. The combination of Plackett–Burman design with Central Composite Design (CCD) for optimizing the bioprocess variables for prodigiosin production by *Serratia* AM8887, proved to be successful and dependable tools to select the statistically significant factors and finding the optimal concentration of those factors in culture medium. The optimum condition for prodigiosin production was attained when incubated at 25 °C, with a pH of 6.5, at 48 hr of incubation, 180 rpm supplemented with glycerol, sucrose, yeast extract and fertilizer factory waste as best carbon, nitrogen and amino acid source for the selected bacterial strains. The utilization of less expensive and effectively available agro-industrial by-products (such as fertilizer factory waste water) in place of more expensive conventional complex medium ought to assist large-scale production of this useful bacterial secondary metabolite at a relatively low cost. Further studies are suggested at larger scale to maximize the growth and productivity of PG by this promising *Serratia* strain.

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Optimization of various physico-chemical parameters using Central Composite Design

S1: ANOVA table for the CCD experiment of environmental condition optimization; the *P* – Values of statistical terms were shown in bold

Variable	Responses					
	Optical density OD_{620}			PG (mg/l)		
Source	**Sum of squares**	**F value**	***P* value**	**Sum of squares**	**F Value**	***P* Value**
Model	3.64	5.06	**0.0092**	6.04	3.91	**0.0224**
A-Temp (°C)	0.35	4.33	**0.0641**	2.04	11.87	**0.0063**
B-pH	4.557E-004	5.697E-003	0.9413	0.12	0.70	0.4217
C Agitation (rpm)	0.64	7.98	**0.0180**	5.732E-003	0.033	0.8587
AB	0.20	2.52	0.1436	0.081	0.47	0.5089
AC	0.38	4.69	**0.0556**	6.561E-003	0.038	0.8489
BC	0.18	2.30	0.1602	0.12	0.72	0.4156
A^2	0.060	0.75	0.4053	3.40	19.82	**0.0012**
B^2	0.33	4.16	**0.0688**	0.31	1.80	0.2095
C^2	1.68	21.01	0.0010	0.31	1.79	0.2105

Optimizing media components

S2: Statistical analysis of Plackett-Burman experiment showing the effect, regression coefficient, T-value and *P*-value for each variable on the bacterial growth and prodigiosin production.

Variable	Optical density OD_{620}				PG (mg/l)			
	Effect	**Coefficient**	**T-value**	***P*-value**	**Effect**	**Coefficient**	**t-value**	***P*-value**
Ammonium Chloride	-0.2527	-0.1263	-4.94	**0.008**	-3559	-1780	-9.98	**0.001**
Corn steep liquer	-0.1747	-0.0873	-3.42	**0.027**	4792	2396	13.44	**0.000**
Whey	0.0890	0.0445	1.74	0.157	-225	-113	> 1	>1
Glycerol	0.3493	0.1747	6.84	**0.002**	1737	869	4.87	**0.008**
Urea	0.4157	0.2078	8.13	**0.001**	-2328	-1164	-6.53	**0.003**
Ground sesame	0.5720	0.2860	11.19	**0.000**	33	17	>1	>1
Fructose	0.1123	0.0562	2.20	**0.093**	-1782	-891	-5.00	**0.007**
Ammonia	-0.0670	-0.0335	-12.56	**0.05**	2047	1023	4.78	**0.041**
Sucrose	0.031	0.0159	5.87	0.107	5588	2794	15.68	**0.000**
Fertilizer Waste	0.021	0.014	4.52	0.110	-4496	-2248	-10.50	**0.009**

Central composite design for significant cheap material

S3: ANOVA table for the CCD experiment of media components; the *P* –Values of statistical terms were shown in bold

Variable	Responses					
	Optical density OD_{620}			PG (mg/l)		
Source	Sum of squares	F value	*P* value	Sum of squares	F Value	*P* Value
Model	0.027	1.81	0.1846	3.220E+006	4.30	**0.0163**
A-Sucrose (g/l))	5.792E-003	3.47	**0.0922**	2.981E+006	35.83	**0.0001**
B-Glycerol (g/l)	1.459E-004	0.087	0.7736	1.564E+005	1.88	0.2004
C-Fertilizer waste (g/l)	1.131E-003	0.68	0.4296	1547.90	0.019	0.8942
AB	0.011	6.73	**0.0267**	11677.74	0.14	0.7158
AC	3.825E-00	2.29	0.1611	525.04	6.310E-003	0.9383
BC	4.168E-008	2.495E-005	0.9961	4289.70	0.052	0.8250
A^2	2.367E-003	1.42	0.2613	58858.26	0.71	0.4200
B^2	3.110E-003	1.86	0.2023	1373.45	0.017	0.9003
C^2	2.979E-006	1.784E-003	0.9671	398.17	4.785E-003	0.9462
