

Optimization of Bioreactor Cultivation Parameters by Taguchi Orthogonal Array Design for Enhanced Prodigiosin Production

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ABSTRACT: *One of the major steps toward the industrialization of the microbial product(s) is to optimize the cultivation conditions at the large-scale bioreactor and successfully control the microbial behavior within a large scale production environment. Statistical Design of Experiment was proven to optimize a vast number of microbial processes to achieve robustness and explore possible interactions among the variables. In this research, Taguchi Orthogonal Array was applied to optimize the cultivation condition of a newly isolated Prodigiosin-producing marine bacterial strain, Serratia AM8887, at the bioreactor level. The two-steps fermentation process was applied; as the productivity was scaled up from shake flask level to a benchtop bioreactor (5L) and subsequently to an in-situ sterilization bioreactor system (20L); yielding a 7g/L of the pigment; compared to 100mg/L before optimization confirming that; applying Taguchi experimental design is a reliable and good positive option for the optimization of biotechnological processes. The produced pigment was purified and the chemical structure was revealed using Spectrophotometric, Mass Spectrum (MS), Fourier Transform InfraRed (FT-IR), and proton Nuclear Magnetic Resonance (¹H-NMR) spectroscopy analysis. The biological activity including antibacterial (against multidrug resistance), antioxidants (against 1,1-diphenyl-2-picrylhydrazyl) and cytotoxicity to cancer cell lines (against breast cancer MCF-7 and liver cancer HepG-2) of the pigment were explored showing very characteristic features that could be helpful in food, pharmaceuticals, and/or textile industries.*

KEYWORDS: *Bioreactor; Taguchi; Prodigiosin; Antimicrobial; Antioxidant; Anticancer.*

INTRODUCTION

Prodigiosin (PG) attracted much research recently due to its clinical importance. It is a secondary metabolite of some bacteria such as *Serratia* sp., *Pseudomonas* sp., and *Vibrio* sp. [1,2]. PG was reported to have antifungal,

antibacterial, antimalarial, antiprotozoal, and anticancer activities [3]. More importantly, PG was reported to induce apoptosis in different human hematopoietic tumor cell lines with no impact on nonmalignant cell lines [4].

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1021-9986/2020/2/319-330 12/\$/6.02

Producing enough amounts of any microbial metabolite constitute a great challenge to industrial microbiology. This is because conventional optimization strategies are not only time consuming, require more experimental data sets but it cannot provide information about the mutual interactions of the parameters. The modern procedures depending on Design of Experiments (DOE) and statistical tools are more helpful in gaining new insights into the optimization conditions in a relatively few trials [5]. Among different statistical tools, Taguchi experimental design offers distinct advantages. It is a fast and considerable way of optimization conferring remarkable outcome in simultaneous study of many factors. It also makes imprint in quality products supplemented with better process execution, and rendering high yield and better stability [6]. Taguchi experimental design involves a study of a given system by a set of factors over a specific region of interest (levels) by identifying the influence of individual factors, establishing the relationship between variables and also the performance at the optimum levels. By studying the main effects of each of the factors, the levels of factors to produce the best results can be predicted [6]

The main target of this study was to optimize and scale up the production of PG from a local isolate of bacterial strain, *Serratia* AM8887, which has been documented to produce significant amounts of PG [7]. The production was optimized at Bioreactor scale (20L) in terms of pH, temperature and dissolved oxygen levels via applying a Taguchi matrix. Upon optimization, the pigment was extracted and screened for its biological activity. To our Knowledge, this is the first time to report apply Taguchi algorithm to optimize PG production at pilot scale level.

EXPERIMENTAL SECTION

Strain and Cultivation Conditions:

The selected bacterial strain, was previously isolated from Red Sea Sediments, was identified as *Serratia* AM8887 (GenBank accession number **KU726587**) based on the morphological and 16S rRNA gene sequencing and analyzing. For PG production, the isolate was inoculated to the previously generated production medium which consisted of; Sucrose 6 g/l, Glycerol 6 g/L, Fertilizer Waste 12 g/l and NaCl 15 g/l [7]. The organism was cultivated at an agitation speed of 180 rpm and 25°C

with a pH of 6.5 for 48hrs in a 250 mL Erlenmeyer flask containing 100 mL of the generated production medium.

Optimization of Physico-Chemical parameters controlling PG production in bioreactor

Batch runs were performed, according to the developed Taguchi matrix, using Eppendorf - New Brunswick 5L Rushton turbine Stirred Tank Bioreactor (STR) with a working volume of 3L. After sterilization, the bioreactor was set up as described in the manufacturer's instructions. Taguchi matrix was applied to determine the optimum levels of pH, Temperature and dissolved oxygen (DO) which control bacterial growth and PG production. DO values were maintained constant by applying cascade control. The design included nine batch fermentations; each was prepared in 2.7L of the PG production medium and incubated according to the unique condition of each trial as shown in Table 1.

Seed culture was prepared as 300mL medium with the same nutrients concentrations of the production medium and incubated in orbital shaker incubator at 25°C and 180rpm for 48hrs then transferred to the bioreactor.

The bioreactor is equipped with pH probe, oxygen probe, foam sensor and stirrer of two-six bladed Rushton turbines. For controlled pH cultivations, the pH was maintained by addition of 2M NaOH and 2M H₃PO₄ solution.

Taguchi experimental design, a standard orthogonal array L₉ (3³), was used to examine three factors in three levels. The L and the subscript (9) represent the Latin square and the number of experimental runs respectively. The levels of the factors studied and the layout of the L₉ Taguchi's orthogonal array are shown in Tables 1 and 2 respectively. The experimental results were analyzed to calculate the main effects of the factors and subsequently; the analysis of variance technique was then applied to determine which factors were statistically significant. The optimal conditions were determined by combining the levels of factors that had the highest main effect value. All calculations were performed using Design Expert 8.0 statistical package (StatEase, Inc, Minneapolis, MN, USA) [8].

The exact amount of PG was estimated using the recently developed method described previously [9], with authentic prodigiosin hydrochloride (Sigma, Aldrich UK) as a standard to generate the standard curve.

Table 1: Factors and their levels employed in the Taguchi's experimental design for bacterial growth and prodigiosin production (PG) at Bioreactor Level.

Factors	Levels		
	1	2	3
pH	6	7	8
Temperature (°C)	10	20	30
Dissolved oxygen (%)	10	30	50

Table 2: L₉ orthogonal array of Taguchi experimental design and corresponding responses.

Run	Variables			Responses	
	pH	Temperature (°C)	Dissolved oxygen (%)	O.D ₆₂₀	PG Conc. (mg/L)
1	6	10	10	2.49 (±0.05)	347 (±30)
2	6	20	30	2.53 (±0.10)	421 (±36)
3	6	30	50	1.96 (±0.08)	449 (±14)
4	7	10	30	1.59 (±0.12)	57 (±8.8)
5	7	20	50	1.40 (±0.13)	252 (±35)
6	7	30	10	1.89 (±0.30)	208 (±37)
7	8	10	50	1.49 (±0.18)	720 (±10)
8	8	20	10	1.98 (±0.09)	2338 (±77)
9	8	30	30	1.14 (±0.15)	81 (±12)

Upon determining the optimum cultivation conditions; the cells were transferred to a 20L CelliGen[®] 510 stainless steel Bioreactor for a pilot scale production of the optimized PG. The *In-Situ Sterilization* Fermenter was set up as described in the manufacturer's instructions. All the conditions were monitored in addition to sample withdrawing at time intervals for off line readings and estimation of growth and pigment production over the run time.

Batch run in a 20L In-Situ Sterilization bioreactor system

The optimum bioreactor cultivation condition was predicted to be; Temperature 20°C, pH= 8.5 and dissolved oxygen= 5% leading to approximately 3 g/L representing 30 times increase in the productivity compared to the amount obtained in shake flasks prior to optimization.

The contribution of three factors in Taguchi experimental design showed that pH played a leading role than the other factors (pH 34.99 %, dissolved oxygen 24.27 % and temperature 22.62 %).

In the second step; the optimum levels achieved were applied to *In-Situ Sterilization* (20L) with precise control and online monitoring of the microbial behavior. The achieved yield upon the scaling up process was approximately 7g/L of PG after 19hrs from inoculating the bioreactor, end of log phase, representing 70 times more yield compared to the amount produced prior to optimization. The attained results confirmed that; Taguchi experimental design is a good positive option for the optimization of biotechnological processes.

Pigment extraction and purification

The Pigment produced by the bacteria was purified according to the method described previously [10.11]. Upon extraction; the crude pigment was dissolved in ethanol then the solution passed through silica balanced gel (230- 400) mesh particle size and then the adsorbed pigment was eluted with 10M ethyl acetate to get pure pigment and then concentrated by rotatory evaporator at 50°C, which was then analyzed by scanning in UNICAM UV-Visible spectrophotometer to detect

the maximum absorbance of prodigiosin with scanning range from (400-600) [12].

Chemical characterization of the produced prodigiosin

The chemical structure and molecular mass of the produced Prodigiosin by the isolate *Serratia* AM8887 were estimated by the following methods;

a) Nuclear Magnetic Resonance (NMR) spectroscopy

The purified pigment was suspended in deuteriochloroform (CDCl₃). By using model (JEOL NMR ECA-500) at 500 MHZ, the ¹H NMR spectra of sample was obtained. The chemical shift scale was in parts per million (ppm) [2].

b) Fourier Transform InfraRed (FT-IR) spectroscopy

The FT-IR spectrum of the prodigiosin was recorded with a spectrometer (Perkin Elmer, USA).in the range of 4000 - 400 cm⁻¹[13].

c) Mass spectrum of prodigiosin

The molecular weight of the pigment was determined by using mass spectrometry by using model DSQ [14].

Evaluation of in vitro antimicrobial activity of Prodigiosin

The antimicrobial activities of Prodigiosin (crude and pure) were studied on LB agar by the disc diffusion technique against clinical isolates of *Bacillus subtilis*, *Staphylococcus epidermidis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *klebsiella pneumoniae*, *Candida albicans*, *Erwinia carotovora*, Methicillin Resistant *Staphylococcus aureus* (MRSA), *Proteus vulgaris*, *Streptococcus pyogenes*, *staphylococcus aureus* and *Enterobacter cloacae*. All the isolates were obtained from Mansoura University General Hospital, Egypt.

Sterile filter paper discs (6mm) were individually immersed in 500µl of ethanol extract of prodigiosin and ethanol was taken as control. All the discs were dried and placed on the surface of the test bacterial and fungal lawn. Following of incubation at 37°C; the plates were examined for the zones of inhibition [15].

Evaluation of antioxidant activity of prodigiosin

The effect of prodigiosin on 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Fluka, Switzerland) radical

was estimated using the method described previously [16]. Five dilutions of the pigment were prepared in methanol. 1 mL of the prepared concentrations of the tested pigment was added to 1 mL of DPPH[·] (0.135mM). The absorbance was measured at 517 nm after 30 minutes of incubation at exclusion of light. A solution free of the pigment was used as a blank and contained methanol instead of the sample. The percentage of remaining DPPH[·] of each tested concentration at the steady state was calculated as follows:
% DPPH[·] remaining = [DPPH[·]]_T/ [DPPH[·]]_{T=0} X 100

These values were plotted against mg of prodigiosin to show the amount of antioxidant necessary to decrease the initial DPPH[·] concentration by 50% (IC₅₀). Catechine, Ascorbic acid and gallic acid were used as references to compare with.

Evaluation of cytotoxicity effects of purified prodigiosin against MCF-7 cell line and HepG-2 cell line

These experiments were performed to test the potentiality of the obtained PG to inhibit two different cancer cells including; Breast and Liver cancer cells.

Mammalian cell line

MCF-7 cells, HepG-2 cell line (human breast cancer cell line and human liver cancer cell line were obtained from VACSERA tissue culture unit, Cairo, Egypt).

Cell line propagation

The cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum, 1% L-glutamine, HEPES buffer and 50µg/ml gentamycin. All cells were maintained at 37°C in humidified atmosphere with 5% CO₂ and were sub cultured two times a week. Cell toxicity was monitored by determining the effect of the test samples on cell morphology and cell viability.

Cytotoxicity evaluation using viability assay

For cytotoxicity assay, the cells were seeded in 96-well plate at a cell concentration of 1×10⁴ cell per well in 100µl of growth medium. Fresh medium containing different concentration of the test sample was added after 24hrs of seeding. Serial two-fold dilutions of the tested chemical compound (PG) were added to confluent cell monolayer's dispensed into 96-well, flat-bottomed micro titer plates (Falcon, NJ, USA) using a multichannel pipette. The micro

titer plates were incubated at 37°C in a humidified incubator with 5% CO₂ for a period of 48hrs. Three wells were used for each concentration of the test sample. Control cells were incubated without test sample and with or without DMSO. The little percentage of DMSO present in the wells (maximal 0.1%) was found not to affect the experiment. After incubation of the cell for 24hrs at 37°C, various concentration of the tested pigment (50, 25, 12.5, 6.25, 3.125, 1.56µg) were added, and the incubation was continued for 48hrs and viable cells yield was determined by a colorimetric method using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay.

In brief, after the ends of the incubation period, media were aspirated and the crystal violet solution (1%) was added to each well for at least 30 minutes. The stain was removed and the plates were rinsed using tap water until all excess stain is removed. Glacial acetic acid (30%) was then added to all wells and mixed thoroughly, and then the absorbance of the plates was measured after gently shaken on micro plate reader (TECAN, Inc.), using a test wavelength of 490 nm. All results were corrected for background detected in wells without added stain. Treated samples were compared with the cell control in the absence of the tested compound. All experiments were carried out in triplicate. The cell cytotoxic effect of each tested compound was calculated [17, 18].

Statistics

All the statistical tests of the developed method including the multi-way analysis of variance (ANOVA) were performed using Minitab[®] 17 software, Minitab Inc., USA and Design Expert 8.0 statistical package (StatEase, Inc, Minneapolis, MN, USA). All the data are presented as the mean ± the Standard Deviation (SD) and p values < 0.1 were considered to be statistically significant

RESULTS AND DISCUSSION

Statistical Optimization Using Taguchi Orthogonal Array Design

Serratia AM8887 was previously isolated from deep sea core sediments of the Red Sea and was reported to be a potential high PG producer with approximately 100 mg/L when growing on a combination of wastes [7].

As a step forward in the direction of PG production scaling up; the productivity of the isolate *Serratia* AM8887 was optimized at bioreactor level via two steps

sequential fermentation process. In the first step; the optimum levels of pH, temperature and dissolved oxygen (DO) were addressed in a bench top 3L bioreactor with accurate control and online monitoring of the selected variables to be optimized.

In this research, the influence of 3 factors on the growth of *Serratia* sp AM8887 and its PG production were tested in Taguchi experimental design in 9 runs as shown in Table 2. Nine different trials, each with its unique combination, were performed to estimate the optimum conditions for PG production at Bioreactor level. The values of the targets to be optimized (growth and pigment concentration) were calculated and analyzed (Table 2).

The efficiency of PG production by *Serratia* sp ranging from 57.88 to 2338 mg/L corresponding to the combined effect of the three factors in their specific ranges. The experimental results suggest that these factors at optimum level strongly support the production of Prodigiosin. In trial number 4; the lowest production of PG was observed (57.88 mg/L), while in trial number 8; the highest amount of PG (2338 mg/L) was observed.

The outcomes of the fermentation runs were analyzed by statistical software package Design Expert 8.0. The signal-to-noise ratio (S/N), which is the logarithmic function of desired output, served as objective function for optimization. For each run, S/N ratio corresponding to larger-the-better objective function as shown in Table 3.

A main effects plot was used to examine differences between level means for one or more factors. There is a main effect when different levels of a factor affect the response differently. A main effects plot for S/N Ratios and means graphs the response for each factor level connected by a line (Fig. 1).

The Taguchi optimized fermentation process parameters are shown in Fig. 1 (a, b). The best process parameters for bacterial growth was pH 6, temperature 20°C and dissolved oxygen 10 % as shown in Fig. 1a while the best process parameters for PG production was pH 8, temperature 20°C and dissolved oxygen 10% as shown in Fig. 1b.

The factors affecting the bacterial growth can be ranked as pH then dissolved oxygen and finally temperature while the factors affecting the PG production can be ranked as dissolved oxygen then pH and finally temperature as shown in Table 4.

Table 3: S/N Ratios and Means for each response of L_9 Taguchi orthogonal array design.

Run/expt.no	O.D ₆₂₀		PG Conc. (mg/L)	
	S/N ratio	Mean	S/N ratio	Mean
1	7.92399	2.4900	50.8141	347.30
2	8.08641	2.5370	52.4931	421.36
3	5.84512	1.9600	53.0476	449.14
4	4.04977	1.5940	35.2506	57.88
5	2.95538	1.4053	48.0401	252.35
6	5.54760	1.8940	46.3767	208.37
7	3.46373	1.4900	57.1466	720.00
8	5.94207	1.9820	62.5291	2338.00
9	1.18369	1.1460	38.1697	81.00

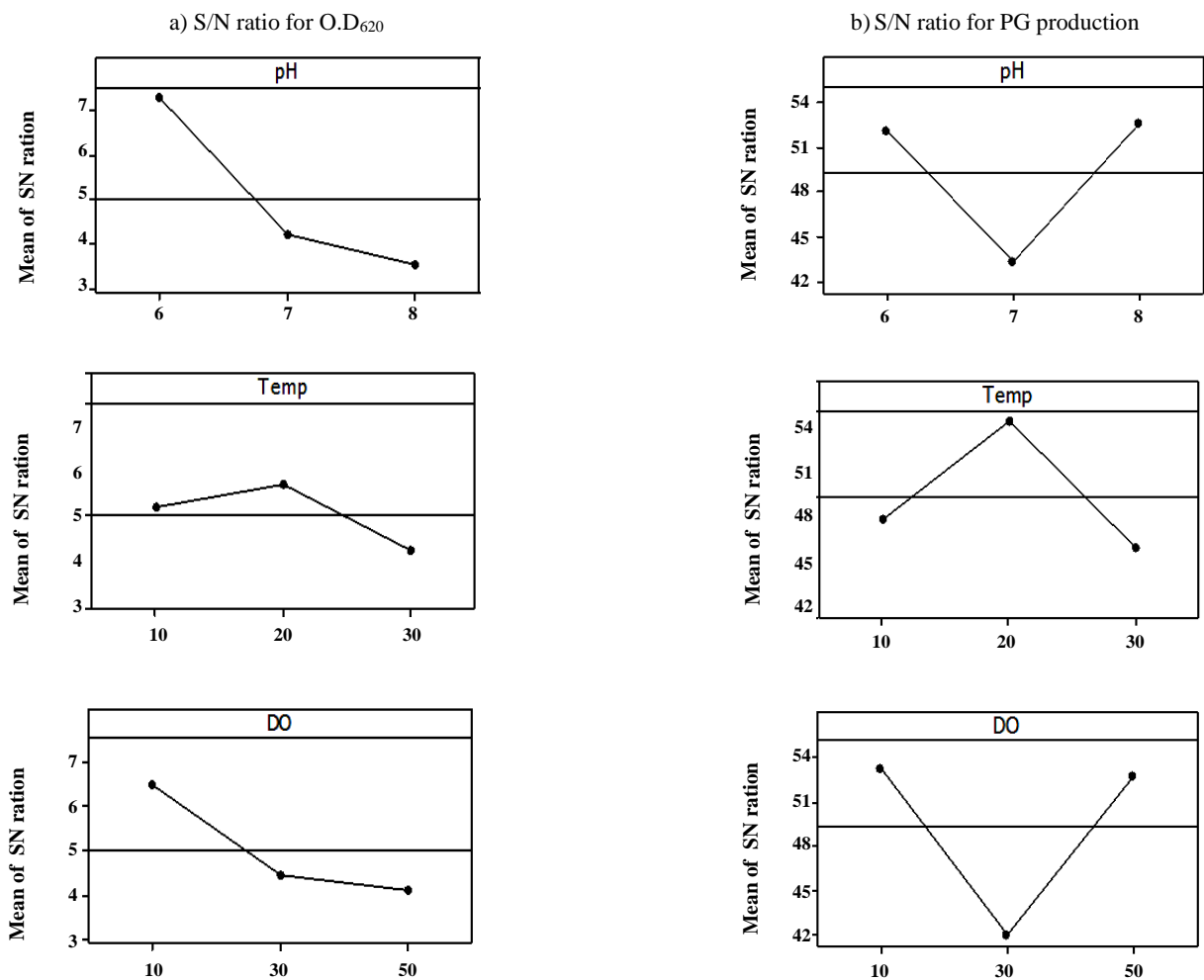


Fig. 1: Main effect plots for S/N ratios with larger-the-better objective function of Taguchi optimized fermentation process parameters. a) Main effect plot for growth and b) Main effect plot for pigment production.

Table 4: Impact of fermentation factors and their assigned levels on bacterial growth and PG production.

Factor	O.D ₆₂₀				PG conc. (mg/L)			
	Level 1	Level 2	Level 3	Rank	Level 1	Level 2	Level 3	Rank
pH	7.285	4.184	3.530	1	52.12	43.22	52.62	2
Temperature (°C)	5.146	5.661	4.192	3	47.74	54.35	45.86	3
Dissolved oxygen (%)	6.471	4.440	4.088	2	53.24	41.97	52.74	1

The experimental data revealed that; selected level 1 of pH and dissolved oxygen were observed to be optimum for bacterial growth whereas for temperature, selected level 2 was observed to be preferred for bacterial growth.

Taguchi experimental design involves a study of a given system by a set of factors over a specific region of interest (levels) by identifying the influence of individual factors, establishing the relationship between variables and also the performance at the optimum levels. By studying the main effects of each of the factors, the levels of factors to produce the best results can be predicted [19].

The experimental data revealed that level 3 of pH, level 2 of temperature and level 1 of dissolved oxygen were observed to be optimum for PG production. Using the Response Optimizer tool in the software (a tool applied to predict the optimum conditions); the optimum suggested by the software to attain the maximum growth and productivity was; temperature 20°C, pH= 8.5 and dissolved oxygen= 5%

In Taguchi approach, multi-way ANOVA is used to analyze the results of the performed experiments and determine how much variation that each factor has contributed. By studying the main effects of each of the factors, the general trends of the influence of the factors towards the process can be distinguished. Analysis of the data for the determination of significant parameters was performed and the results are summarized in Table 5.

From the calculated ratios (F), pH was found to be the most significant factor followed by dissolved oxygen (DO) and temperature respectively.

The growth and productivity curve within the bioreactor, under the suggested optimum conditions, were calculated and the result obtained was summarized in Fig. 2, as the maximum production of PG was approximately 7g/L achieved after 19hrs from inoculating the pilot scale bioreactor.

Taguchi experimental design was proven to enhance the production yield of different microbial products

with fewer experimental runs including protease production by *Bacillus subtilis* HB04, alkaline protease production by *Bacillus clausii*, xylanase production by *Bacillus cereus* BSA1 and extra sucrase production from *Weissella confuse* Cab3 [20-22] leading to considerable economy in time and cost for the process optimization.

The scaling up process from the shake flask to bioreactor level was achieved and Batch cultivation was carried out at 25°C, 180 rpm, controlled dissolved oxygen at 10 % and pH uncontrolled. The result showed a remarkable increase in PG production (7316 mg/L compared to 138.9 mg/L prior to optimization) representing approximately 70-fold increase in the amount of the product. The maximum production achieved after 19 h from inoculating the bioreactor which represent the end of log phase of growth. The data concluded that application of statistical optimization via DOE tools is very useful in optimizing PG produced by this local bacterial isolate.

Scanning spectrophotometer for the produced pigment

After extraction and purification of the produced pigment; the pigment was analyzed by scanning spectrophotometer in a UV-Visible spectrophotometer to detect the maximum absorbance of Prodigiosin and the scanning range from (400-600nm). The prodigiosin pigment showed a characteristic peak at 535nm in absolute ethanol, as shown in Fig. 3, which is in line with the literature [12].

Chemical structure of Prodigiosin by ¹H-NMR spectroscopy

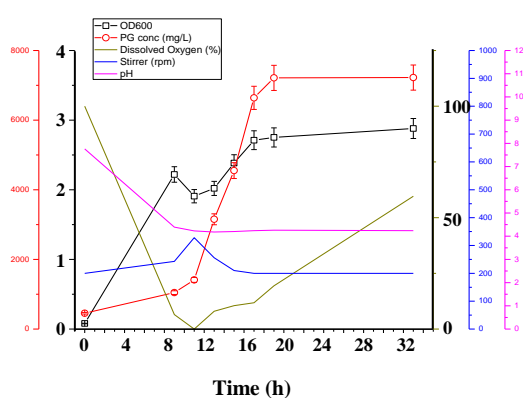
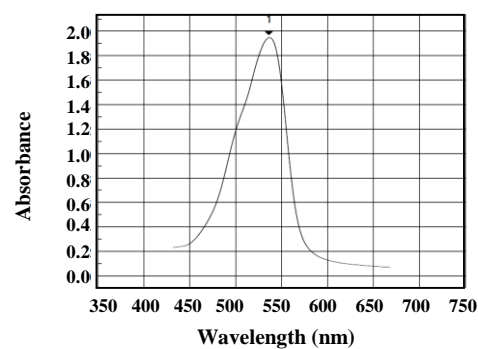
For determination the structural composition of prodigiosin, proton NMR analysis was applied. The ¹H-NMR spectra were detected for prodigiosin which produced by the isolate under investigation as shown in Table 6. The ¹H-NMR spectroscopic data verified the pigment to be prodigiosin. The ¹H-NMR data were summarized as ¹H-NMR (CDCl₃, 500 MHz), 4.1 (s, 3H, OCH₃),

Table 5: Analysis of variance (ANOVA) in $L_9(3^3)$ orthogonal array of designed experiments.

Source	DF	SS	MS	F-Value	Prop. (P)>F	Percentage contribution %
pH	2	27519	13760	1.93	0.341	34.99 %
Temperature (°C)	2	17785	8892	1.25	0.445	22.62 %
Dissolved oxygen (%)	2	19088	9544	1.34	0.427	24.27 %
Residual Error	2	14234	7117			
Total	8	78626				

Table 6: Data of NMR spectra (d, ppm) of prodigiosin, obtained in this study and those reported in literature

PigmentM 323.5 Da[23]	Pigment M 323 Da[24]	Pigment M 322.9 Da[25]	Pigment M 323.1 Da The present study
12.44	-	-	-
12.54	-	-	-
-	-	7.55	7.52
7.23	7.26	7.27	-
6.95	6.99	6.99	-
6.92	6.95	-	-
6.69	6.71	6.68	-
6.35	6.39	6.35	-
6.08	6.11	6.08	-
4.01	4.04	4.00	4.18
2.76	2.58	2.55	2.32
2.39	2.44	2.40	2.30
1.53	1.59	1.60	1.9
1.30	1.34	1.33	1.33
1.32	1.31	1.31	1.30
0.89	0.93	0.93	0.90

**Fig. 2: Time trajectory for growth and productivity of *Serratia* sp within In-Situ Sterilization bioreactor on the optimized production medium under precise condition (20°C, controlled dissolved oxygen 5% and pH 8.5)****Fig. 3: Absorption spectrum of red pigment with maximum absorbance at 535nm produced by *Serratia* AM8887.**

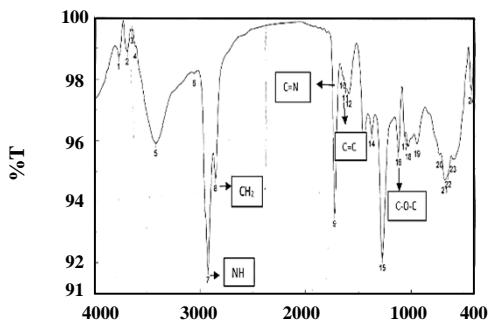


Fig. 4: FT-IR spectrum of the Prodigiosin produced by the isolate *Serratia* AM8887.

2.31 (s, 3H, CH₃), 1.51-1.65 (m, 8H, 4CH₂), 0.9 (s, 3H, CH₃), 5.31 (s, 1H, CH), 7.25-7.56 (m, 5H, Ar-H) which confirmed the structure of prodigiosin (5[(3-methoxy-5-pyrrol-2-ylidene-pyrrol-2-ylidene)-methyl]-2-methyl-3-pentyl-1Hpyrrole).

Fourier Transform InfraRed (FT-IR) spectroscopy for the produced Prodigiosin

The FTIR range for the purified PG (as shown in Fig. 4) demonstrated groups at 2909 cm⁻¹ (methylene group), 1565 cm⁻¹ (pyrrole ring) and 3463.65 cm⁻¹ (amide group). Tops at 3400-3445 cm⁻¹ are allotted for aliphatic alcohols, essential amines and amides while assimilation band at 1655 cm⁻¹ related to (C=C) extending vibrations. From the range, the fundamental utilitarian gatherings of rough PG were pyrrole, methylene, alkane and alkene showing a high similarity to the PG produced by different *Serratia* strains [26, 27].

Mass Spectrum of the produced prodigiosin

The molecular mass of the pigment was determined by using mass spectrometry which corresponding to 323.1 Da as shown in Fig. 5 which agreed with the result obtained previously [14].

Evaluation of in vitro antimicrobial activity of produced prodigiosin

By applying the disc-agar diffusion technique, it was observed that the prodigiosin produced by the isolate *Serratia* AM8887 was able to inhibit the growth of some antibiotic resistant bacterial strains. Fungistatic activity was observed against *Candida albicans*. Prodigiosin

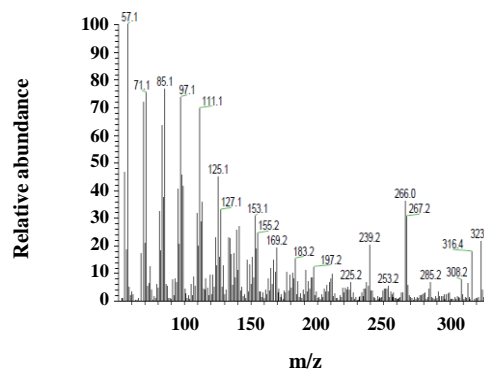


Fig. 5: Mass spectrum of Prodigiosin produced by *Serratia* AM8887.

was found to possess an antibacterial activity against gram positive bacteria like *Bacillus subtilis*, Methicillin Resistant *Staphylococcus Aureus* (MRSA) and *streptococcus pyogenes* and against gram negative bacteria like *Escherichia coli*, *Enterobacter cloacae*, *klebsiella pneumonia* and *Erwinia carotovora*.

Antioxidant activity of the produced prodigiosin by using DPPH radical (1, 1-diphenyl-2-picrilhydrazyl) assay

The role of antioxidant is to remove free radical by donating hydrogen to free radicals in its reduction to an unreactive species. Prodigiosin which extracted from *Serratia* sp AM 8887 was found to have an antioxidant activity by reducing DPPH radical (violet in color) into diphenylpicrylhydrazine (yellow in color) by donating hydrogen or electron. There was a significant association could be found between the concentration of prodigiosin and percentage of inhibition. These values were plotted against mg of pigment to show the amount of antioxidant necessary to decrease the initial DPPH[·] concentration by 50% (IC₅₀). Catechine, ascorbic acid and gallic acid were used as references as shown in Table 7.

According to the obtained results; the amount of prodigiosin necessary to decrease the initial DPPH[·] concentration by 50% (IC₅₀) was 0.034mg/mL indicating that the Prodigiosin considered as an antioxidant agent stronger than gallic acid and ascorbic acid.

Applying PG in food industry could be beneficial in adding a natural food colorant with antimicrobial and antioxidant capabilities which could be advantageous and require no more additives for food protection.

Table 7: Antioxidant activity of Prodigiosin (PG).

Substances	Conc (mg/ml)
Prodigiosin (PG)	0.034 (± 0.002)
Catechine	0.018 (± 0.001)
Ascorbic acid	0.044 (± 0.001)
Gallic acid	0.038 (± 0.001)

Cytotoxic Activity of the produced Prodigiosin against breast cancer cell line (MCF-7) and liver cancer cell line (HepG-2)

Prodigiosin from *Serratia* sp AM 8887 has exerted visible cytotoxic effect against breast cancer cell line (MCF-7) and liver cancer cell line (HepG-2).

Calculation of the IC_{50} required the use of concentration gradients ranged from (zero – 50) $\mu\text{g/mL}$. The IC_{50} values were calculated for the pure Prodigiosin and found to be 5.4 $\mu\text{g/mL}$ (± 0.5) against HepG-2 cell line and 8.73 $\mu\text{g/mL}$ (± 0.9) against the MCF-7 breast cell line.

With an IC_{50} value of 5.4 $\mu\text{g/mL}$ for (HepG-2) cell line and 8.73 $\mu\text{g/mL}$ for the (MCF-7) breast cell line; the PG produced by the isolate was found to be more efficient than the PG produced by the isolate *Rugamonas rubra* RR62 which showed no apoptotic activity against HepG-2 cell line when the pigment concentration is below 125 $\mu\text{g/mL}$ [28] and more efficient than the PG produced by the isolate *Serratia marcescens* which has an IC_{50} of 40.76 $\mu\text{g/mL}$ against the MCF-7 cell line [29].

Recently, the PG mode of action, as an anticancer, was revealed and it was reported to induce protein kinase B inhibition to down-regulate S-phase kinase-associated protein 2 (SKP2) in an independent manner. Our findings further implicate the potential for emerging PG as a novel class of SKP2-targeting anticancer agent [30].

CONCLUSIONS

A novel PG producer, isolated from Egyptian local habitats, was found to be a potential high producer of the renowned microbial pigment with unique antimicrobial characteristics. The amount of the pigment was significantly increased upon transferring the production process from shake flask level to bioreactor level. The scaling up process was achieved via applying Taguchi orthogonal array ensuing approximate 7 gm/L compared to 0.1 g/L prior to optimization. The purified pigment

was found to possess high biological activity especially as an antioxidant, anticancer and antimicrobial.

Received : Jan. 7, 2018 ; Accepted : Apr. 30, 2018

REFERENCES

- [1] Cang S., Sanada M., Johdo O., Ohta S., Nagamatsu Y., Yoshimoto A., [High Production of Prodigiosin by *Serratia Marcescens* Grown on Ethanol](#), *Biotechnology Letters*, **22**(22): 1761-1765 (2000).
- [2] Sumathi C., MohanaPriya D., Swarnalatha S., Dinesh M., Sekaran G., [Production of Prodigiosin Using Tannery Fleshing and Evaluating Its Pharmacological Effects](#), *The Scientific World Journal*, **2014**:1-8 (2014).
- [3] Maheswarappa G., Kavitha D., Vijayarani K., Kumanan K., [Prodigiosin as Anticancer Drug Produced from Bacteria of Termite Gut](#), *Indian Journal of Basic and Applied Medical Research*, **1**: 257-266 (2013).
- [4] Tomás R.P., Ruir C.D., Montaner B., [Prodigiosin Induces Cell Death and Morphological Changes Indicative of Apoptosis in Gastric Cancer Cell Line HGT-1](#), *Histology and Histopathology*, **16**(2): 415-421 (2001).
- [5] Elrazak A.A., Ward A.C., Glassey J., [Polyunsaturated Fatty Acid Production by Marine Bacteria](#), *Bioprocess and Biosystems Engineering*, **36**(11): 1641-1652 (2013).
- [6] Fei N.C., Mehat N.M., Kamaruddin S., [Practical Applications of Taguchi Method for Optimization of Processing Parameters for Plastic Injection Moulding: A Retrospective Review](#), *ISRN Industrial Engineering*, **2013**: 1-11 (2013).
- [7] Osman Y., Elrazak A.A., Khater W., [Bioprocess Optimization of Microbial Biopolymer Production](#), *Journal of Biobased Materials and Bioenergy*, **10**(2):119-128 (2016).
- [8] Venil C., Lakshmanaperumalsamy P., [Taguchi Experimental Design for Medium Optimization for Enhanced Protease Production by *Bacillus subtilis* HB04](#), *e-Journal of Science & Technology*, **4**(4): 1-10 (2009).
- [9] Yong-jun X., Lian-zhong A., [Determination of Prodigiosin in Fermentation Broth by HPLC](#), *Modern Food Science and Technology*, **29**(11): 2772-2776 (2013).

- [10] Venil C.K., Lakshmanaperumalsamy P., [An Insightful Overview on Microbial Pigment, Prodigiosin](#), *Electronic Journal of Biology*, **5**(3): 49-61 (2009).
- [11] Naik C., Srisevita J., Shushma K., Farah N., Shilpa A., Muttanna C., Darshan N., Sannadurgappa D., [Peanut Oil Cake: a Novel Substrate for Enhanced Cell Growth and Prodigiosin Production from *Serratia marcescens* CF-53](#), *Journal of Research in Biology*, **2**(6): 549-557 (2012).
- [12] Mohammed H., Naseer J., Aruna K., [Study on Optimization of Prodigiosin Production by *Serratia marcescens* Msk1 Isolated from Air](#), *International Journal of Advanced Biotechnology Research*, **2**: 671-680 (2012).
- [13] Namazkar S., Garg R., Ahmad W.Z., Nordin N., [Production and Characterization of Crude and Encapsulated Prodigiosin Pigment](#), *International Journal of Chemical Sciences and Applications*, **4**(3): 116-129 (2013).
- [14] Casullo De Araújo H.W., Fukushima K., Takaki G.M.C., [Prodigiosin Production by *Serratia Marcescens* UCP 1549 Using Renewable-Resources As A Low-Cost Substrate](#). *Molecules*, **15**(10): 6931-6940 (2010).
- [15] Ibrahim D., Nazari T.F., Kassim J., Lim S.H., [Prodigiosin-an Antibacterial Red Pigment Produced by *Serratia Marcescens* IBRL USM 84 Associated with a Marine Sponge *Xestospongia Testudinaria*](#). *Journal of Applied Pharmaceutical Science*, **4**(10): 1-6 (2014).
- [16] Vora J., Jain N., Modi H., [Identification and Characterization of Pigment Producing Strain *Kocuria* KM243757 & JO1 KM216829 from Kharaghoda Soil](#), *International Journal of Current Microbiology and Applied Sciences*, **4**(9): 850-859 (2015).
- [17] Mosmann T., [Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays](#), *Journal of Immunological Methods*, **65** (1):55-63 (1983).
- [18] Vijayan P., Raghu C., Ashok G., Dhanaraj S., Suresh B., [Antiviral Activity of Medicinal Plants of Nilgiris](#). *Indian Journal of Medical Research*, **120** : 24-29 (2004).
- [19] Wang D.S., Koo T.Y., Lin I.-P., Liu H.-R., Chou C.-Y., [Note: Determination of Nattokinase Production Condition Using Taguchi Parameter Design](#), *Food Science and Technology International*, **12** (3): 215-220 (2006).
- [20] Oskouie S.F.G., Tabandeh F., Yakhchali B., Eftekhari F., [Enhancement of Alkaline Protease Production by *Bacillus Clausii* Using Taguchi Experimental Design](#), *African Journal of Biotechnology*, **6**(22):2559-2564 (2007).
- [21] Mandal A., Kar S., Dutta T., Pati B.R., Mondal K.C., Mohapatra P.K.D., [Parametric Optimization of Submerged Fermentation Conditions for Xylanase Production by *Bacillus Cereus* BSA1 Through Taguchi Methodology](#), *Acta Biologica Szegediensis*, **59** (2): 189-195 (2015).
- [22] Shukla S., Goyal A., [Development of Efficient Fermentation Process at Bioreactor Level by Taguchi's Orthogonal Array Methodology for Enhanced Dextranase Production From *Weissella Confusa* Cab3](#), *Advances in Microbiology*, **2** (3): 277-283 (2012).
- [23] Alihosseini F., Lango J., Ju K.S., Hammock B. D., Sun G., [Mutation of Bacterium *Vibrio Gazogenes* for Selective Preparation of Colorants](#), *Biotechnology Progress*, **26** (2), 352-360 (2010).
- [24] Song M.J., Bae J., Lee D.S., Kim C.H., Kim J.S., Kim S.W., Hong S.I., [Purification and Characterization of Prodigiosin Produced by Integrated Bioreactor from *Serratia* Sp. KH-95](#), *Journal of Bioscience and Bioengineering*, **101**(2): 157-161 (2006).
- [25] Guryanov I., Karamova N., Yusupova D., Gnezdilov O., Koshkarova L., [Bacterial Pigment Prodigiosin and Its Genotoxic Effect](#), *Russian Journal of Bioorganic Chemistry*, **39** (1): 106-111 (2013).
- [26] Ahmad W.A., [Production and Characterization of Crude and Encapsulated Prodigiosin Pigment](#), *International Journal of Chemical Sciences and Applications*, **4** (3):116-129 (2013).
- [27] Aruldass C.A., Venil C.K., Zakaria Z.A., Ahmad W.A., [Brown Sugar As A Low-Cost Medium for the Production of Prodigiosin by Locally Isolated *Serratia Marcescens* UTM1](#). *International Biodeterioration & Biodegradation*, **95**:19-24 (2014).

- [28] Hassani H.H., Cytotoxicity and Dna Fragmentation in Cancer Cell Lines, Hep-2 and Amn-3, Induced by a Novel Maroon Pigment From Mutant Strain of *Rugamonas Rubra* Rr62, *Journal of Al-Nahrain University*, **14**(3): 110-114 (2011)
- [29] Athavale M.A., Somaiya S.S., More G.A., Satish S., Srivastava S.A., [Comparitive In-Vitro Cytotoxicity of Red Pigment Extract of *Serratia Marcescens* on Breast and Prostrate Cancer Cell Lines](#), *International Journal of Current Pharmaceutical Research*, **5**(2): 140-143 (2013)
- [30] Hsieh H.Y., Shieh J.J., Chen C.J., Pan M.Y., Yang S.Y., Lin S.C., Chang J.S., Lee A.Y.L., Chang C.C., [Prodigiosin Down-Regulates SKP2 to Induce P27kip1 Stabilization and Antiproliferation In Human Lung Adenocarcinoma Cells](#), *British Journal of Pharmacology*, **166** (7): 2095-2108 (2012).