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Research Article

Application of Plackett- Burman design for optimization of silver nanoparticles produced by *Streptomyces sp.* DW102

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ABSTRACT

Background and Objective: Multidrug-resistant (MDR) *Staphylococcus aureus* is considered more significant because of its ability to cause both nosocomial and community infections resulting in serious threat to public health. The aim of this study is to synthesize silver nanoparticles (AgNPs) using *Streptomyces sp*. DW102 in optimized conditions for obtaining improved antimicrobial activity against MDR *S. aureus.*

Methods: Plackett-Burman design is used for optimization of the media for improving the antimicrobial activity of the AgNPs. AgNPs are produced using a basal control and twelve factors including soluble starch, K_2HPO_4, KNO_3, NaCl, Casein, $MgSO_4$. $7H_2O$, CaCO3, $FeSO_4.7H_2O$, pH, temperature, agitation and medium volume by *Streptomyces sp*. DW102and its antibacterial activity is tested against MDR *S. aureus*.

Results: As a result, AgNPs produced using the optimized conditions of (g/L);10 soluble starch, 2 K_2HPO_4, 2 KNO_3, 2NaCl, 0.1 Casein, 0.02 $MgSO_4.7H_2O$, 0.02 $CaCO_3$ and 0.01 $FeSO_47H_2O$, pH 7.5, 37^0Ctemperature, 200rpm agitation speed and 75ml of medium volume were predicted to increase the antimicrobial activity of *Streptomyces sp*. DW102. A higher inhibition zone(28 mm) was obtained using optimized media comparing to inhibition zone (22mm) produced by AgNPs produced by basal control media against MDR *S. aureus.*

Conclusion: So the AgNPs produced by *Streptomyces sp*. DW102 can be efficiently used for controlling MDR pathogens after further investigation.

Keywords: *Streptomyces sp*. DW102, silver nanoparticles, multidrug-resistant, Plackett-Burman design, optimized.

INTRODUCTION

Multi-drug resistant *Staphylococcus aureus* is responsible for causing severe nosocomial infections such as endovascular infections, soft-tissue infections, sepsis, pneumonia and most feared toxic shock[1,2]. Methicillin resistant *Staphylococcus aureus* (MRSA) isolates are resistant to β-lactam drugs such as penicillin which reduces the option of treatment with standard available drugs[3]. World Health Organization report says that 64% of the patients affected by MRSA have an increased risk of mortality than the patients infected with normal strains of the same bacteria[4].It is essential to study the antimicrobial susceptibility pattern,

since the antibiotic susceptibility pattern of pathogenic bacteria have been changing over the past years[5]. Today, because of emerging antibiotic resistance among the pathogens due to the over usage of existing antibiotics, there is a need for developing a technology for discovery of new antibiotics[6].The broad spectrum antibiotics currently being used are more toxic and less effective. So in the modern era, the scientists are trying to modify the antimicrobial compounds to improve the antimicrobial activity and reduce the toxicity[7]. By using nanotechnology the synthesis of nanoparticles can be achieved in nanosize which improves the antimicrobial activity[8].

The researchers have found that silver nanoparticles can solve the problem by having higher surface area to volume ratio which leads to increased antimicrobial activity[9].Green synthesis method which involves biological sources for the synthesis of nanoparticles is not only reduce the toxic substances, it is also less expensive and ecofriendly[10].

Streptomyces sp. belongs to actinomycetes having filamentous structure which have the potential to produce most of the antibiotics being used[11]. Studies have shown that when *Streptomyces* biomass is incubated with silver nitrate in orbital shaking conditions it reduces the Ag+ ions and leads to extracellular production of silver nanoparticles[8,12-13]. Same studies have shown that the AgNPs produced higher inhibition zones against the pathogens tested.

Media composition plays an important role in not only increasing the growth and metabolism of the organism but also improve the quality of the metabolite produced. Media selected should easily available at a low cost and have the capacity to support the growth of the organisms and efficiency of the product. To improve a biological process optimization of medium components and cultural conditions is considered as very important task[14]. According to Gresham and Inamine (15), two step level is considered for optimization of media in which the components were initially screened to check the growth of the organism and some important variables are selected and optimized. Kumar et al. (16)**,** found that by limiting the nutrient concentrations the growth metabolism and product secretion can be influenced in secondary metabolites. But the optimization of fermentation conditions, using classical methods has some disadvantages since it is time consuming and expensive[17]. In modern era, optimization is carried out using statistical methods which have more advantages when compared to classical methods[18]. Therefore recently, Plackett- Burman design[19] and Response surface methodology[20]have been used for determining the components or variables that have more significant effect on the production of antibiotic or other metabolites and optimized statistically.

In this study, using Plackett- Burman design we have designed an optimization strategy to study the effect of the different variables influencing the production of silver nanoparticles by *Streptomyces* isolate DW102 and its antimicrobial activity against *Staphylococcus aureus*.

MATERIALS AND METHODS

Microorganisms and cultural conditions

The strain *Streptomyces sp.* DW102 and the Silver nanoparticles synthesized were kindly provided by Dr.Gouse Basha Sheik (Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, Dawadmi, Saudi Arabia). The isolate was maintained on slopes containing starch-casein agar medium of the following composition (g/L): Starch20; Casein 0.3: KNO_3 2; K_2HPO_4 2; $MgSO_4 \cdot 7H_2O$ 0.05; NaCl 2; $CaCO_3$0.02; $FeSO_4 \cdot 7H_2O$ 0.01; Agar 20 and distilled water up to 1 L.

Inoculum preparation

Streptomyces sp. DW102 was inoculated in Starch casein broth (basal medium) taken in 250 mL Erlenmeyer flask containing 50ml of (g/L):

Starch 20; Casein 0.3: KNO_3 2; K_2HPO_4 2; $MgSO_4 \cdot 7H_2O$ 0.05; NaCl 2; $CaCO_3$0.02; $FeSO_4 \cdot 7H_2O$ 0.01 and distilled water up to 1 L. pH is adjusted to 7 and incubated at 30^0C in a rotary shaker at 200 rpm for 3 days. After incubation, the mixture is centrifuged at 5000rpm for 30 min and supernatant is used for subsequent experiments

Synthesis of AgNPs using basal conditions for antimicrobial activity

The synthesis of AgNPs was done by using modified method of Abd-Elnaby et al. (21). The cell free filtrate is used for the extracellular synthesis of AgNPs by mixing 50ml of cell-free filtrate with 50ml of 1mM $AgNO_3$ solution in a 250ml Erlenmeyer flask. Then the mixture is incubated at basal conditions inside a rotary shaker at 200rpm in dark conditions at 35^0C for 5 days for reduction. A control flask containing 50ml of cell-free filtrate without AgNPs was also incubated under same conditions for comparison. After the development of the brown color, characterization of AgNPs was performed by using UV–Vis Spectrophotometer (UV-2550). The silver nanoparticles obtained from basal conditions were used to determine antibacterial activity against *Staphylococcus aureus.*

Selection of the significant variables using Plackett- Burman design

This design is used for determining various factors influencing the production of silver nanoparticles by *Streptomyces sp.* DW102 and to increase its antimicrobial activity against MDR *Staphylococcus aureus*. In general this design is a two factorial one, which identifies the critical parameters required for elevated antibiotic production by screening n variables in n+1 experiment[19]. A total of 12 independent (assigned) were screened in Plackett–Burman experimental design of 16runs (Table 2).Using this design silver nanoparticles are produced using 12 independent variables which differ in their media components and fermentation conditions. Each variable is examined in 6 trials in high levels denoted by (+) and6 trials in low level denoted by (-). The 12 different variables and the basal control were performed in duplicate. The 12 different variables chosen for the present study were soluble starch, K_2HPO_4, KNO_3, NaCl, Casein, $MgSO_4.7H_2O$, $CaCO_3$, and FeSO4.7H2O, pH, temperature, agitation speed and medium volume(Table 1). Using this design the main effects, percentage coefficient, p-value and percentage confidence can be determined[22].

Table 1: Experimental Independent variables at two levels affecting antibacterial activity of AgNPs

Symbol	Independent Variables	Levels		
		-1	**0**	**1**
A	Soluble Starch	10	20	25
B	K_2HPO_4(g/L)	1	2	2.5
C	KNO_3(g/L)	1	2	2.5
D	NaCl(g/L)	1	2	2.5
E	Casein(g/L)	0.1	0.3	0.5
F	$MgSO_4.7H_2O$(g/L)	0.02	0.05	0.07
G	$CaCO_3$(g/L)	0.01	0.02	0.04
H	$FeSO_4.7H_2O$(g/L)	0.001	0.01	0.02
I	pH	7	7.0	7.5
J	Temperature	33	35	37
K	Agitation	150	200	200
L	Medium volume	50	50	75

1- higher level; -1-lower level; 0-basal control

Table 2: Plackett Burman design for evaluation of 12 independent variables (A-L) for antimicrobial activity against *Staphylococcus aureus*

Run Order	A	B	C	D	E	F	G	H	I	J	K	L	Inhibition Zone(mm)
1	-1	1	1	1	-1	-1	-1	1	1	1	-1	-1	21
2	1	1	1	-1	-1	-1	1	1	1	-1	-1	1	19
3	1	1	-1	-1	-1	1	1	1	-1	-1	1	1	23
4	1	-1	-1	-1	1	1	1	-1	-1	1	1	-1	18
5	-1	-1	-1	1	1	1	-1	-1	1	1	-1	-1	26
6	-1	-1	1	1	1	-1	-1	1	1	-1	-1	1	20
7	-1	1	1	1	-1	-1	1	1	-1	-1	1	1	28
8	1	1	1	-1	-1	1	1	-1	-1	1	1	1	17
9	1	1	-1	-1	1	1	-1	-1	1	1	1	-1	15
10	1	-1	-1	1	1	-1	-1	1	1	1	-1	-1	23
11	-1	-1	1	1	-1	-1	1	1	1	-1	-1	-1	21
12	-1	1	1	-1	-1	1	1	1	-1	-1	-1	1	19
13	1	1	-1	-1	1	1	1	-1	-1	-1	1	1	16
14	1	-1	-1	1	1	1	-1	-1	-1	1	1	1	22
15	-1	-1	1	1	1	-1	-1	-1	1	1	1	-1	21
16	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	14
Basal control	0	0	0	0	0	0	0	0	0	0	0	0	22

(A-L) 12 variables soluble starch, K_2HPO_4, KNO_3, NaCl, Casein, $MgSO_4$. $7H_2O$, $CaCO_3$, $FeSO_4.7H_2O$, pH, temperature, agitation and medium volume respectively.

Determination of antibacterial activity of AgNPs by *Streptomyces sp.* DW102

MDR *Staphylococcus aureus* was obtained from Dawadmi General Hospital, Dawadmi, Saudi Arabia and was used to determine the antibacterial activity of the AgNPs produced by *Streptomyces sp.* DW102using well diffusion method[23].AgNPs by using 12 different variables and basal control were used for determining antibacterial activity. Agar wells of 6mm diameter were made on Muller Hinton agar (MHA) plates and swabbed with 24 hour broth culture of MDR *Staphylococcus aureus*. 30 µl of 30 µg/ml biosynthesized AgNPs were added to the wells in each plate. After overnight incubation at 37ºC, the plates were observed for zone of inhibition and measured.

Verification experiment

The average of antibacterial activity(Zone of inhibition) against MDR *Staphylococcus aureus* produced by the silver nanoparticles synthesized by using optimized media is compared with the ZOI of AgNPs produced by basal conditions[21]. This experiment was performed in duplicates.

RESULTS AND DISCUSSION

Extracellular Synthesis of AgNPs by *Streptomyces sp.* DW102

Production of AgNPs by the culture supernatant of *Streptomyces* isolate DW102with 1mM $AgNO_3$ solution was observed by seeing color change from

yellow to brown (Fig.1) which is an indication of the extracellular formation of AgNPs. There was no color change in the control without $AgNO_3$solution which indicates no reaction (Fig.2). Characterization of AgNPs was done using UV Vis spectrophotometer at wavelength

Fig. 1 AgNPs production Fig.2 Control: No AgNPs production

200nm-500nm and peak was observed at 430nm which confirms silver nanoparticles production. Several other studies have also shown the peak in the range of 400-450nm which confirms the AgNPs production[12,24].

Evaluation of the factors affecting silver nanoparticles activity using Plackett Burman design

Plackett-Burman experimental design is most suitable and well-established system for screening multiple variables and selection of most significant media components and fermentation conditions for better results. This design is also very simple and less time consuming when compared with other statistical methods used for medium designing. In our study, a total of 12 independent variables were screened using Plackett-Burman experimental design. The experiment was conducted in 16 runs to study the effect of the selected variables on the production of silver nanoparticles by *Streptomyces* isolate DW102and its antibacterial activity against MDR *Staphylococcus aureus* (Table 2). All the 16 trials were performed in duplicate and the average Zone of inhibition in millimetres were treated as responses. In our study, Plackett-Burman experimental design showed a markedly wide variation (14-28mm) in inhibition zone. This variation reflected the importance of medium optimization to increase the activity of silver nanoparticles. The maximum ZOI(28mm) was achieved in the run number 7, while the minimum ZOI (14mm)was observed in the run number 16.In a similar study conducted by El-Naggar et al. (25),15 different variables were screened with 20 runs to study the effect of metabolites produced by *Streptomyces lienomycini* NEAE31 against MDR *Pseudomonas aeruginosa* in which ZOI showed a higher variation (17-37mm). Similar results were obtained in another study conducted by Mohamedin et al (26) in which 15 different variables were screened with 20 runs to study the *Streptomyces psammoticus* strain M19 against *Staphylococcus epidermidis* and ZOI also showed a higher variation (6mm-37mm).

The Fig. 3 and Table 3show the main effect of each variable on the silver nanoparticles production. Main effect allows the determination of the effect of each variable. A large effect either positive or negative indicates that a factor has a large impact on production; while an effect close to 0 means that a factor has little or no effect. With respect to the main effect we can see that six variables out of twelve namely, $CaCO_3$, $FeSO_4.7H_2O$, pH, temperature, agitation speed and medium volume have positive affect whereas other six variables namely, soluble starch, K_2HPO_4, KNO_3, NaCl, Casein and $MgSO_4.7H_2O$ have negative effect on silver nanoparticles production by *Streptomyces sp.* DW102and its antibacterial activity against MDR *Staphylococcus aureus*. The percentages

contributions of the variables are given in Table 3. The results revealed thatKNO_3, soluble starch, medium volume, temperature and $CaCO_3$ are the most contributing components with ranks 16.51, 14.59, 14.25, 14.21 and 10.94 % respectively. Also, it was clear that among the five variables, KNO_3 and soluble starch exerted negative effects whereas medium volume, temperature and $CaCO_3$ exerted negative effect on silver nanoparticles production and its antimicrobial activity. A study conducted by El-Naggar et al. (25), shows that among the variables tested, pH, $CaCO_3$, yeast extract, temperature, KNO_3, $FeSO_47H_2O$ and inoculum size showed significant positive effect against microorganisms tested. In another study conducted by Mohamedin et al (26), 14 variables were screened and found that peptone concentration, medium volume and inoculation age have significant effect on AgNPs production. In another study conducted by Mohamedin et al (13) in which 15 variables were screened and found that agitation speed, KNO_3, NaCl and yeast extract have significant effect on the production of metabolites.

The Pareto chart illustrates the order of effects of the variables affecting silver

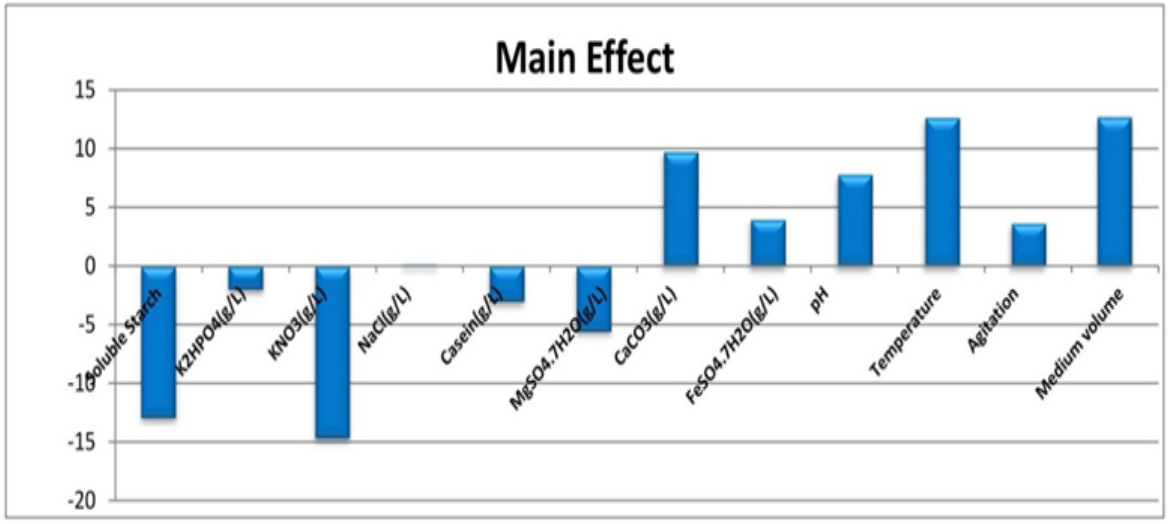

Fig. 3: Main effects of the different variable factors affecting the AgNPs

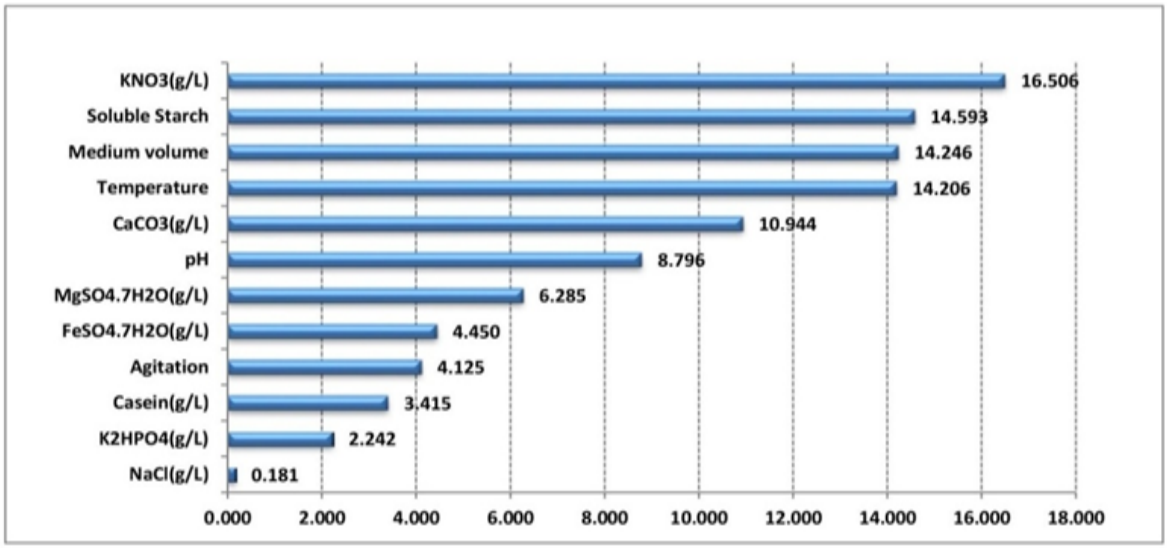

Fig. 4: Pareto Chart showing the amount of influence of each factor on the AgNPs production

Table 3: Regression coefficients, main effect, P-value and confidence for the AgNPs production

Independent Variables	Coefficients	Main Effect	Rank(%)	P-value	Confidence (%)
Soluble Starch	-6.5	-13.0	14.59	0.01	98.64
K_2HPO_4(g/L)	-1.0	-2.0	2.24	0.21	78.80
KNO_3(g/L)	-7.3	-14.7	16.51	0.01	99.01
NaCl(g/L)	0.1	0.2	0.18	0.94	6.36
Casein(g/L)	-1.5	-3.0	3.41	0.10	90.00
$MgSO_4.7H_2O$(g/L)	-2.8	-5.6	6.28	0.05	94.82
$CaCO_3$(g/L)	4.9	9.7	10.94	0.01	98.79
$FeSO_4.7H_2O$(g/L)	2.0	4.0	4.45	0.05	94.53
pH	3.9	7.8	8.80	0.02	97.54
Temperature	6.3	12.6	14.21	0.01	98.50
Agitation	1.8	3.7	4.12	0.06	93.79
Medium volume	6.3	12.7	14.25	0.01	98.54

nanoparticles production and its antibacterial activity (Fig. 4). The data also shows 8 significant and 4 non-significant variables (Table 3) affecting the production of silver nanoparticles and its antimicrobial activity. $KNO_3$was the most significant variable at 99.01% confidence (p-value = 0.01) followed by $CaCO_3$at confidence 98.79% (p-value = 0.01), soluble starch (p-value = (0.01) at confidence 98.64%, medium volume at confidence 98.54 (p-value = 0.01), temperature at confidence 98.50 (p-value = 0.01), pH at confidence 97.54 (p-value = 0.02). $MgSO_4.7H_2O$ at confidence 94.82 (p-value = 0.05) and $FeSO_47H_2O$ at confidence 94.53 (p-value = 0.05). The non-significant variables are, Agitation speed at confidence 93.79 (p-value = 0.06), Casein at confidence 90.00 (p-value = 0.10), K_2HPO_4 at confidence 78.80 (p-value = 0.21) and NaCl at confidence 6.36 (p-value = 0.94). In a study conducted by El-Naggar et al. (25), pH showed highest confidence level 99.94% followed by $FeSO_47H_2O$ at 99.88% and KNO_3 at 99.86%.In another study conducted by Mohamedin et al. (26) in which agitation speed showed highest confidence level 99.96% followed by KNO_3at confidence 99.94% and NaCl at confidence 99.77%.

Verification experiment

This experiment was carried out to compare the average zone of inhibition (antibacterial activity) produced by optimized medium with the ZOI produced by basal conditions to validate the obtained optimized medium. *Streptomyces sp.* DW102 was grown on the optimized medium of the following components (g/L): 10 soluble starch, 2 K_2HPO_4, 2 KNO_3, 2NaCl, 0.1 Casein, 0.02 $MgSO_4.7H_2O$, 0.02 $CaCO_3$ and 0.01 $FeSO_47H_2O$. The pH maintained was 7.5 at 37^0C temperature, 200rpm agitation speed and 75ml of medium volume was used. It resulted in larger inhibition zone (28 mm) (Fig. 4) when compared to ZOI in the basal conditions (22mm) when tested against MDR *Staphylococcus aureus*. The present result confirmed the validity of the optimized conditions. In a similar study conducted by Abd-Elnaby et al. (21) showed that the optimized conditions produced increase in inhibition zone(24 mm) when compared to the basal conditions (22mm) when tested against *Vibrio fluvialis.* In another study conducted by El-Naggar et al. (25) showed that the optimized media produced ZOI of 36mm whereas it is only 25mm when prepared by basal conditions against *Pseudomonas aeruginosa.*

CONCLUSION

This study shows that the optimized media produced by altering 12 different variables, increased the antibacterial activity (Zone of inhibition) of the silver nanoparticles produced by *Streptomyces sp*. DW102 against MDR *Staphylococcus aureus*. So the AgNPs produced by Streptomyces sp. DW102 can be efficiently used for controlling MDR pathogens after further investigation.

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CONFLICT OF INTEREST

No conflict of interest was declared.

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