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Optimizing Nano-silver Formation by *Fusarium oxysporum* PTCC 5115 Employing Response Surface Methodology

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Abstract: *Fusarium oxysporum* was grown in medium containing malt, yeast extracts and glucose on shaker at 25±1ºC and180 rpm for 96 h. The mycelia were used to convert silver nitrate solution into nano-silver. The bioconversion was optimized through response surface methodology-central composite design .The factors which affected the process were pH, temperature, agitation rate, concentration of silver nitrate, time of reaction and weight of *Fusarium oxysporum* mass. The R^2 was calculated to be 98% indicating the accuracy and ability of the polynomial model to be suitable and reasonable. Positive coefficient of the factors like concentration of silver nitrate solution (E) and weight of *Fusarium oxysporum* biomass (F), quadratic terms A^2, B^2, F^2 and interaction term BC affected linearly formation of nano-silver whereas negative coefficient of pH(A), temperature (B), rate of agitation(C), time(D) along with quadratic terms C^2, D^2, E^2, and interaction terms such as BF decreased the nano-silver formation.

Key words: *Fusarium oxysporum*, optimization, nano-silver formation, response surface methodology -central composite design

INTRODUCTION

The nano-scale silver will play roles in understanding and ability to manipulate biological processes which will be the central theme to present biomedical and biological issues that need a nano-science or nanotechnology approach[1]. The flourish of this technology in nano-medicine is clearly obvious with the possibility to diagnosis the diseases. Properties when compared with the bulk material the extremely small size of nano-particles results in the particles having a large surface area relative to their volume. In the case of silver nano-particles this allows them to easily interact with other particles and increases their antibacterial efficiency[2]. Until now, we have witnessed a wide range of prokaryotes as prospective nano-particle synthesizers[3-5]. Klus-Joerger and co-workers have shown that the bacterium *Pseudomonas stutzeri* AG259 isolated from a silver mine, that was capable of producing silver crystals within the periplasmic space of the bacteria[5-6]. One major advantage of having prokaryotes as nanoparticle synthesizers is that they can be easily modified using genetic engineering techniques for over expression of specific enzymes, apart from the ease of handling. However, the use of eukaryotes, especially fungi, is potentially exciting since they secrete large amounts of proteins, thus increasing productivity, and their easy usage in laboratory works is a suitable option in production of metalic nanoparticles among other microorganisms [7-10]. Moreover the process can be easily scaled up, economically viable with the possibility of easily covering large surface areas by suitable growth of mycelia. One of the novel works defining the use of fungus for nanoparticle synthesis was carried out by Mukherjee *et al* in 2002[11]. For the intracellular production of silver nanoparticle using *Verticilium*, (AAT-TS-4). *Verticillium*, when exposed to aqueous $AgNO_3$, caused the reduction of the metal ions and formation of silver nanoparticles of about 25 nm diameters[12]. *Fusarium oxysporum* or

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other fungi have been employed to synthesize nanosilver in aqueous media[13]. In this article, attempts are made to synthesize nanosilver which can be employed in optoelectronic devices, biological sensors, drug and gene delivery, antimicrobial protection, water treatment and textile industry by two level fractional factorial design [14-16]. This methodology can be used to optimize biotechnological processes in the advance of bioprocess engineering[17-19]. Response Surface Methodology (RSM) is a collection of techniques that are developed as a mean to find out optimal conditions of input factors which maximize/minimize the output variables i.e. measured responses[20-21]. Furthermore, Central Composite Design (CCD) contains an imbedded factorial or fractional factorial design with center point that is augmented with a group of star points that allow estimation of the curvature. The mathematical representation of RSM in this study is the second order (quadratic) with the following model:

$$Y = b_0 + \sum_{i=1}^{K} b_i + x_i + \sum_{j=2}^{K} \sum_{i=1}^{j-1} b_{ij} x_i x_j + \sum^{k} b_{ii} x^2$$

Where Y is the predicted response variable, b_0, b_i, b_{ii} and b_{ij} are regression coefficient of the model, x_i, x_j represent the independent variables (reaction composition) in the form of real values.

MATERIALS AND METHODS

Chemicals: Glucose, yeast extract, peptone, silver nitrate were of Merck (Germany) Malt extract was procured from Himedia (India). Sterile distilled water was used throughout the experiments.

Microorganism: *Fusarium oxysporum* PTCC 5115 was obtained from Persian Type Culture Collection IROST, Tehran, Iran. It was maintained on Sabouraud's dextrose agar slants at 25 ºC, ±1ºC. It was sub-cultured every 4-6 week.

Medium: The medium composed of malt extract 3 g, glucose 10 g, yeast extract 3 g and peptone 5 g per in one litter of distilled water. The medium was designated as MGYP and autoclaved at 121±1ºC for 15 minutes. The fungus was grown in 500-ml ErlenMeyer flasks each containing 100 ml MGYP medium at 25±1ºC and180 rpm for 96 h. After 96 h of growth, mycelia were separated from the culture broth by centrifugation (3500 rpm) at 10 ^{o}C for 20 min and the settled mycelia were washed three times with sterile distilled water and freezed until use.

Table 1: The design matrix showing the number of experiments with five central points

No.	Exp. Run	pH	Temp (ºC)	Agitation (rpm)	Time (h)	Con (mM)	Biomass weight(g)	Δ OD λ 410nm
1	12	10.0	40.0	250.0	132.0	0.5	5.0	1.36
2	24	10.0	40.0	250.0	12.0	3.0	5.0	1.89
3	1	10.0	40.0	100.0	132.0	0.5	15.0	0.465
4	25	10.0	25.0	250.0	12.0	3.0	15.0	0.736
5	18	6.0	40.0	100.0	132.0	3.0	15.0	0.465
6	26	10.0	25.0	250.0	132.0	0.5	15.0	0.296
7	20	6.0	40.0	250.0	12.0	0.5	5.0	0.92
8	23	10.0	40.0	100.0	12.0	3.0	15.0	0.605
9	31	10.0	25.0	100.0	132.0	0.5	5.0	0.56
10	11	6.0	25.0	250.0	12.0	0.5	15.0	0.66
11	17	6.0	40.0	100.0	12.0	0.5	15.0	0.24
12	7	10.0	25.0	100.0	12.0	3.0	5.0	1.25
13	5	6.0	25.0	100.0	132.0	3.0	5.0	0.96
14	27	6.0	25.0	250.0	132.0	3.0	15.0	0.75
15	30	6.0	40.0	250.0	132.0	3.0	5.0	1.875
16	32	6.0	25.0	100.0	12.0	0.5	5.0	0.98
17	6	4.0	32.5	175.0	72.0	1.75	10.0	1.59
18	4	12.0	32.5	175.0	72.0	1.75	10.0	1.53
19	21	8.0	17.5	175.0	72.0	1.75	10.0	1.2
20	29	8.0	47.5	175.0	72.0	1.75	10.0	0.89
21	2	8.0	32.5	25.0	72.0	1.75	10.0	0.51
22	28	8.0	32.5	325.0	72.0	1.75	10.0	ND
23	9	8.0	32.5	175.0	-48.0	1.75	10.0	ND
24	8	8.0	32.5	175.0	192.0	1.75	10.0	ND
25	16	8.0	32.5	175.0	72.0	-0.75	10.0	ND
26	3	8.0	32.5	175.0	72.0	4.25	10.0	0.65
27	33	8.0	32.5	175.0	72.0	1.75	0.0	0.76
28	15	8.0	32.5	175.0	72.0	1.75	20.0	1.256
29	19	8.0	32.5	175.0	72.0	1.75	10.0	0.785
30	14	8.0	32.5	175.0	72.0	1.75	10.0	0.785
31	10	8.0	32.5	175.0	72.0	1.75	10.0	0.785
32	13	8.0	32.5	175.0	72.0	1.75	10.0	0.785
33	22	8.0	32.5	175.0	72.0	1.75	10.0	0.785

Table 2: Factors and actual low and high values

High actual	Low actual	Type	Units	Name	Factors
10	6	Numeric		pH	A
40	25	Numeric	ºC	Tem	B
250	100	Numeric	RPM	Agitation	C
132	12	Numeric	H	Time	D
3	0.5	Numeric	mM	Con	E
15	5	Numeric	G	Weight	F

Experimental design: Response Surface Methodology (RSM) was employed to investigate the influence of factors like, pH, temperature (ºC), agitation rate (rpm), time of incubation (h), concentration of silver nitrate (mM), and weight of fungal biomass (g) on the conversion of silver nitrate to nano silver by *Fusarium oxysporium* PTCC 5115 by CCD using six factors at three levels with five replicates at the center point to fit the data on a second order polynomial (quadratic) model. The experimental matrix is shown in Table 1

Statistical analysis: Data from the CCD obtained from the experimental matrix Table 1 are computed for the

determination of regression coefficient of the second order multiple regression model. The analysis of regression and variance was performed by Design Expert (Ease State Version 6.0).

RESULTS AND DISCUSSION

The biomass obtained by the growth of *Fusarium oxysporium* PTCC 5115 in MGYP medium was able to convert silver nitrate to nano silver through an enzyme known as nitrate reductase under the conditions of temperature, pH and time of the reaction, agitation rate and fungal biomass. The conversion of silver nitrate to nano silver in an aqueous medium was followed by scanning the optical density of the reaction filtrate at wave length ranged from 310, 325, 350, 375, 410, 510 and 610 nm spectrophotometrically .Figure 1 , 2 and 3 show UV-Vis spectrum of nano silver formation ,change in the color of the reaction mixture from pale yellow to dark brown and the transmission electron micrograph of the nano-silver synthesized by *Fusarium oxysporium* PTCC 5115 respectively . This conversion was brought about by nitrate reductase requiring NADH/NADPH as coenzyme. The enzyme and coenzyme are provided by the mycellial biomass and the conversion unlike *Verticillium* is extracellular[22]. The bioformation of nano silver was optimized through RSM/CCD to observe the influence of the factors like pH, temperature (ºC), agitation rate (rpm), time of incubation (h), concentration of silver nitrate solution (mM), and weight of fungal biomass (g) under study. Table 2 indicates the actual low and high values taken

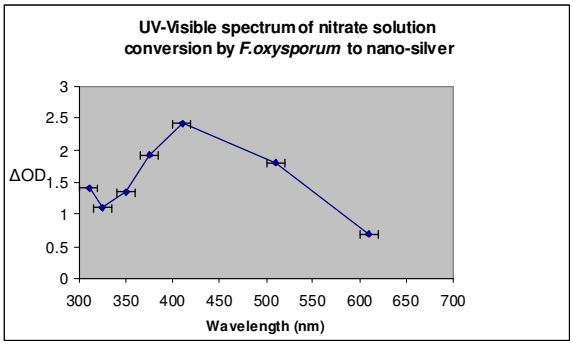

Fig. 1: UV- Vis spectra recorded with respect to time after the reaction of 3 mM $AgNO_3$ solution with 5 g *Fusarium oxysporum* PTCC 5115 wet biomass at pH 6and 40°C 132 h

Fig. 2: Conversion silver nitrate to nano silver by *Fusarium oxysporum* PTCC 5115

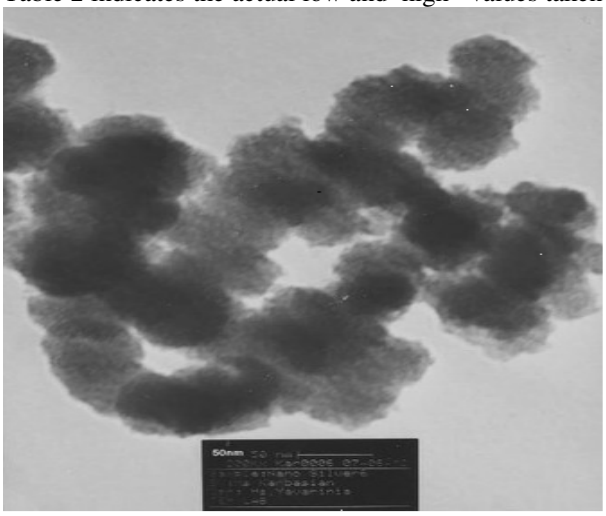

Fig. 3: TEM was used to analyze the size and distribution of nano silver synthesized by *Fusarium oxysporum* PTCC 5115.The size of biosynthesized nanosilver was about 50 nm

Table 3: Model summary

PRESS	Predicted R-squared	Adjusted R-squared	R-squared	Std Dev	Source
10.44	-0.2466	0.0562	0.2332	0.49	linear
110.40	-12.7019	0.368-	0.5297	0.59	2F1
233.85	-28.0245	0.8727	0.9801	0.18	Quadratic (suggested)
+		1.00	1.00	0.00	Cubic (Aliased)
<0.0001	6.366 E+007	0.16	1	0.16	

in designing the experiments. However by following the table 3 shows the selected model is appropirate. Furthermore, table 4 reveals the regression analysis of a

second order polynomial model for optimizing nano-silver formation by *Fusarium oxysporum.* By comparing the data obtained, it is obviously clear that the runs 24 and 30 are to be optima for nano-silver formation. Run 24 has been taken as optimum, because the time of reaction is 12 h whereas in run 30 the time of reaction is 132 h. As this bio-transformation is mediated by enzyme and further studies are required to find out the kinetic parameters as such K_m, $K_{cat.}$ By employing the enzyme it can be possible to lower the time of reaction, there are three possible ways that the reaction taken place in 132 h is the same as 12 h one; a) the substrate might have no more been available to proceed further, b) the enzyme being inhibited by the product i.e. nano-silver, c) the enzyme might have been denatured during the catalysis of silver nitrate solution to nano-silver. To answer these questions, it is essential to study the kinetic parameter with isolated enzyme. In optimizing process, the ratio of minimum to maximum response is about 8 . The ratio less than 10 does not require transformation [18]. The R^2 is 98% indicating the accuracy and ability of the polynomial model seems to be suitable and reasonable Table 3. Positive coefficient of the factors like concentration of silver nitrate (E) and weight of *Fusarium oxysporum* biomass (F),quadratic terms A^2, B^2, F^2 and interaction term BC affect linearly formation of nano-silver whereas negative coefficient of pH(A), temperature (B), rate of agitation(C), time(D) along with quadratic terms C^2, D^2, E^2, and interaction terms such as BF decreased the nano-silver formation.

Table 4: Results of regression analysis of a second order polynomial model for optimizing nano-silver formation by *Fusarium oxysporum*

Source	Sum of squares	DF	Mean square	F - value	Pro>F	Significant
Model	7.9	27	0.29	9.13	0.0107	*
A^2	1.67	1	1.67	52.14	0.0008	*
B^2	0.34	1	0.34	10.58	0.0226	*
C^2	0.25	1	0.25	7.85	0.0379	*
D^2	0.72	1	0.72	22.62	0.0051	*
F^2	0.28	1	0.28	8.82	0.0312	*
BC	1.19	1	1.19	37.17	0.0017	*
BF	0.52	1	0.52	16.27	0.0100	*

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