Statistical Optimization and Chemical Characterization of Newly Extracted Siderophore from *Azotobacter chroococcum*

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Abstract

A bacterial strain capable of producing siderophore was isolated from the soil rhizosphere of Sadat city, Egypt. The new isolate was identified as *Azotobacter chroococcum* by various biochemical and phylogenetic analyses. The siderophore production by the isolated strain was qualitatively and quantitively assayed via overlay-CAS and CAS-shuttle methods. The factors affecting the production of the siderophore were verified throughout two-steps statistical optimization course. An initial screening of eight variables was performed by applying the Plackett-Burman Design (PBD). Subsequently, three significant variables (pH, incubation time and K2HPO4 concentration) were matrixed using central composite design (CCD) with a wider range of levels. The optimization of the fermentation conditions increased the siderophore production to reach 89.33%. The chemical structure of the extracted siderophore was characterized suggested using colorimetric, HPLC, FT-IR and NMR Mass spectroscopy analytical techniques. The results revealed that the *A. chroococcum* produces a hydroxamate-type siderophore. This is the first report on statistical optimization and chemical characterization of siderophore production by *A. chroococcum*.

Keywords: *Azotobacter chroococcum*, hydroxamate siderophores, response surface methodology, chemical structure, submerged fermentation.
Introduction

The iron element is essential for most microorganisms in trace quantities to perform several vital biochemical metabolic processes, i.e. oxygen reduction for ATP synthesis, heme formation, reduction of DNA precursors and detoxification of oxygen radicals. Hence, microbial cells need 0.4-1 µM of iron for their optimum growth in surrounding medium. Even though iron is highly available in the Earth’s crust, the concentrations of dissolved iron are low (1). These ecological limitations and biological requirements have actuated microbial cells to produce siderophore molecules to bind the ferric iron, an available element but biologically inaccessible.

Siderophores are produced by several microbial cells when iron is limited, to capture ferric ions with great affinity. They are quite low-molecular mass (0.5-1 KDa) molecules with iron-chelating abilities (2). They make iron biologically available by solubilizing the insoluble nearby iron. According to their structural properties, siderophores include two basic types, the catecholates and hydroxamates (3).

Microbial cells capable of producing siderophores own molecular structures to shuttle iron via siderophore-iron complexes inside the cells using iron regulated outer membrane proteins (IROMP) (4). These proteins specifically distinguish the soluble ferric-siderophore complex and dynamically transfer the complex inside the cell and the iron is finally released in the cytoplasm (5).

Siderophores have several applications, particularly, in the agricultural field. With the escalating consciousness of the adverse health difficulties and ecological concerns due to protracted synthetic chemical utilization, there is a growing demand for the development of different and benign methods to control agricultural diseases (6). Biological control has arisen as a widespread substitute as it provides a method of managing pathogens without the application of any chemicals (7). It was reported
that the siderophore mediator can compete with soil borne pathogens for iron
representing a vital biological control mechanism as most of plants can uptake iron
from the soil using the microbial iron siderophore complexes with a competitive
benefit under iron shortage and therefore hamper the propagation and root
colonization by means of phytopathogens (8). Moreover, siderophores have several
medical applications for iron overload therapy and improved target antibiotics (9).

The microbial routes to produce siderophores are industrially required as they
are produced under mild conditions and without toxic compounds, compared to
analogous chemical routes (10). Nevertheless, low amounts of these compounds are
usually excreted by microbial cells. Therefore, several approaches should be followed
to tackle this challenge through screening novel bacterial strains with high
productivity along with the optimization of the suitable growth and/or reaction
conditions for high yield. In the present study, a novel Azotobacter strain was isolated
with capability to produce siderophore under submerged fermentation conditions. The
maximum production of this siderophore was adjusted through multiple statistical
approaches by investigating set of different fermentative conditions. Moreover, the
new siderophore was extracted and a preliminary identification was performed by
high pressure liquid chromatography (HPLC). After chromatographic analysis, the
atomic components and morphological features of the extracted siderophore were
fully characterized by detailed spectroscopic and microscopic techniques,
Materials and methods

2.1 Chemicals

All the microbial growth media components were obtained from Sigma-Aldrich™. The solution of acetonitrile (HPLC grade) was purchased from Sigma-Aldrich™. Promega™ PCR Master Mix kit (Madison, WI, USA) was used to perform the PCR reaction. Nucleospin® Extract II kit (MACHEREY-NAGEL GmbH & Co. KG, Germany) was used to purify the PCR Products. The primers 27F (5'-GAG AGT TTG ATC CTG GCT CAG -3') and 1495R (5'-CTA CGG CTA CCT TGT TAC GA-3') were used for the amplification of the 16S rRNA gene. Distilled water was used in all experiments. All other chemicals were of analytical grade.

2.2 Isolation and characterization of the soil bacterium

All the bacterial strains were isolated from the rhizospheric soil of a farm located in Sadat City (30.446369 N, 30.624044 E, Menofia Governorate, Egypt). Cells were isolated on Jensen’s medium containing per liter of distilled water: 20 g sucrose, 0.5 g NaCl, 0.1 g K₂SO₄, 1 g K₂HPO₄, 0.5 g MgSO₄·7H₂O, 0.005 g Na₂MoO₄, 20 g agar, pH 6.9. A suspension of soil sample was prepared (0.1 g/mL) in sterilized water. One drop of the prepared suspension was inoculated onto Jensen’s medium plates. After incubation, the grown bacterial isolates were identified based on morphological and biochemical features by means of standard methods and the obtained results were interpreted using Bergey’s Manual of Systematic Bacteriology (11).

Moreover, the bacterial isolate was recognized up to the species level based on sequencing of 16S rRNA gene. The total genomic DNA was separated from the
bacterial cells and the 16S rRNA gene was then amplified by a polymerase chain reaction (PCR) (Applied Biosystems Inc., USA). The resulted PCR products were analyzed by electrophoresis using 1.5% (w/v) agarose gel and DNA sequencing was performed by a 310A DNA sequencer (Applied Biosystems Inc., USA). The sequence was aligned with reference sequences existing in the GenBank database by the advanced Blast search program (http://www.ncbi.nlm.nih.gov/BLAST). The obtained sequences were aligned using DNAman 5.1 software (Lynnon Biosoft, CA, USA). A phylogenetic tree was then built from a group of pairwise genetic distances applying the maximum-parsimony algorithm and the neighbor-joining method.

2.3 Fermentative optimization of siderophore production

Optimization of siderophore production by the isolated strain was performed via two-step statistical approach using modified Jensen’s medium. Initially, eight variables, including the concentrations of some medium components (glucose, sucrose, mannitol, MgSO₄, K₂HPO₄), pH, medium volume and harvesting time, were screened through the application of Plackett-Burman design (PBD), in which three levels were used under each factor; designated as -1, 0 and +1 for low, middle and high levels. Plackett-Burman is a type of fractional factorial designs which is based only on key effects. It is a screening design that is employed to recognize significant factors that significantly impact the tested response (8). PBD was applied for the scanning and examination of the significance among the tested variables which impact the siderophore production according to the weight percentage of the studied variables (12). The achieved PBD results were statistically analyzed by JMP® software (SAS Institute Inc., Cary, NC, USA). All the runs were performed in triplicate and the average siderophore productivities were calculated as a response. The PBD comprised
45 experiments where the levels of the eight independent variables were deviated (Table 1).

The optimization was additionally extended to a central composite design (CCD) depending on the PBD results. The recognized significant variables from the PBD (harvesting time, pH and K$_2$HPO$_4$ concentration) were tested as the key variables for CCD. These variables, with three levels (-1, 0 and +1), were diverged, in which the design involved 14 runs (Table 1). CCD is a factorial design which can be a complement for PBD or can be applied alone. This design is based on main, interaction and quadratic effects, and is employed to optimize significant factors recognized by PBD. A second order polynomial equation was tailored to correlate the relation between the response and the tested independent factors to predict the optimal point (13). Moreover, the regression analysis of the obtained results was done by JMP® software and the coefficient of determination ($R^2$) was calculated to reveal the degree of the polynomial model equation fitting. The average value was calculated from three replicates for each CCD fermentative run.

2.4 Detection of bacterial hydroxamate siderophore

The siderophore production by the isolated strains was qualitatively and quantitively assayed via overlay-CAS (14) and CAS-shuttle methods (15), respectively. The qualitative assay was initially carried out by formulating CAS medium (Chrome azurol S (CAS) 60.5 mg, piperazine-1,4-bis (2-ethanesulfonic acid) (PIPES) 30.24 g, hexadecyltrimethyl ammonium bromide (HDTMA) 72.9 mg and 1 mM FeCl$_3$·6H$_2$O in 10 mL of HCl (10 mM) and 0.9% agarose. For the detection of siderophore production, 10 mL overlays of CAS medium were used to cover the pre-cultivated agar plates containing the isolated bacterial cells. A change in color from
blue to orange will be detected, after a period of 0.5 h, in the overlaid medium, which surround the producer strain. The chemical detection of hydroxamate siderophore was performed via FeCl₃ Neilands assay, while catechol siderophore was detected using the Arnow assay (14). The type of the produced siderophore was determined using spectrometer (Perkin Elmer Instruments).

Quantitative estimation of siderophores was carried out following the CAS-shuttle assay, in which the isolates were grown in a Jensen’s broth medium on a rotary shaker (120 rpm) incubated at 28 °C for 24 h. The bacterial cells were separated by centrifugation at 3000 xg for 15 min. The obtained supernatant (0.5 mL; 10⁷ cells/mL) was mixed with CAS solution (0.5 mL) and shuttling sulfosalicylic acid solution (10 mL). Absorbance of the resulting mixture was measured at 630 nm, after incubation at room temperature for 20 min. This measurement was performed against a reference containing a mixture of CAS reagent (0.5 mL) and uncultured broth (0.5 mL). The percentage of siderophore units was calculated per Equation (1).

\[
\% \text{ Siderophore units} = \left( \frac{A_r - A_s}{A_r} \right) \times 100
\]

(1)

Where \( A_r \) and \( A_s \) are the absorbance of the reference and sample, respectively, at 630 nm.

2.5 Extraction of the hydroxamate siderophore

The siderophore was partially purified using the cell-free supernatant. The cells were separated by centrifugation (6000 xg for 20 min) and the obtained supernatant liquid was concentrated at 35°C, then 5 g/L was supplemented with
FeCl₃, and ammonium sulfate was added to obtain 50% saturation (16). An equal volume of phenol/chloroform mixture (1:1 v/v) was added to the supernatant in a funnel which was shaken with sufficient venting, and left to separate in the dark. The aqueous phase was discarded while the organic phase was kept. A mixture of ether/water (1:1 v/v) was added to the organic phase (2:1 v/v), and the mixture was shaken, vented and separated in the dark. The organic phase was removed and ether was added to the left over aqueous phase (1:1 v/v), in which the funnel was left to separate under shaking and venting. Ether was used to wash the aqueous phase several times in a similar way until separation took place (17).

2.6 Characterization of the bacterial siderophore

HPLC analysis

Chromatographic examination of the extracted siderophore was performed by HPLC instrument YL9100 (Gyeonggi-do, Republic of Korea) using a Nucleosil 100 C column (250 × 4 mm i.d., 5 µm film thickness) (Knauer, Berlin, Germany). A mobile phase of aqueous 50% (v/v) acetonitrile (contains 0.1 % (v/v) trifluoroacetic acid) was applied. A linear gradient of 1-30% acetonitrile in 0.5 h with a flow-rate of 1.5 mL/min was utilized. An amount of sample (10 µg) was dissolved in 10 µL of aqueous 50% (v/v) acetonitrile and injected for each liquid chromatographic analysis. The column was re-equilibrated for 10 min between injections.

Fourier transform infrared spectrometry (FTIR)

The Fourier-transform IR spectrum was determined for the bacterial siderophore after lyophilization. Lyophilized sample was pelleted using potassium bromide (KBr) and analyzed by FTIR spectroscopy (Jasco 4100 A, Tokyo, Japan) to determine its
functional groups. The recorded spectrum was determined in the range of 4000 to 400 cm\(^{-1}\) (18).

Spectroscopic analysis

The atomic components of the extracted siderophore were identified by \(^1\)H and \(^{13}\)C- Nuclear magnetic resonance (NMR) and mass spectroscopy analyses. The NMR and mass spectroscopy data were recorded on JEOL-ECA (MA, USA) and Shimadzu QP-2010 plus (Kyoto, Japan) spectrometers at (400 and 100 MHz using deuterium oxide (D\(_2\)O) and dimethyl sulfoxide (DEMSO) for \(^1\)H and \(^{13}\)C) and respectively. While mass spectroscopy estimated at spectrometers at 50 and 860 MHz,

Scanning electron microscope (SEM)

The surface and morphological features of the lyophilized sample of the extracted siderophore were investigated by SEM imaging.
3 Results

3.1 Isolation and identification of a siderophore-producing bacterium

Bacterial cells were isolated from the soil samples to be scanned for siderophore production by employing the CAS medium. Only one, out of total of 20 isolates changed the medium color producing orange shades, while no changes in color were detected in the control. This result indicates the presence of hydroxamate-type siderophores. The overlay-CAS method was used to enable the growth of different microorganisms and exclude the toxicity of HDTMA to Gram-positive bacteria (19). The change in color is ascribed to the development of siderophore iron dye complex (14). The chemical nature of siderophores, produced by the isolate, was determined using a variety of colorimetric methods. The results showed that the isolate produced hydroxamate type siderophore as evidenced by the positive reaction in the universal CAS assay solution and modified Neilands spectrophotometric assay at 420 nm and negative reaction in the Arnow assay for catecholates detection. This isolate was morphologically and biochemically identified. The colonies were Gram-negative, motile, aerobic ovoid to rods or coccobacilli that was positive to catalase test (Fig. 1). The isolate successfully fermented mannitol, glucose and sucrose. Based on these results, the isolate was initially identified as *Azotobacter chroococcum* (11). Additionally, the designated isolate was recognized with 16S rRNA homology analysis. The phylogenetic tree was created by some neighbors that have the close homologous gene sequences (Fig. 2). Sequence of the isolated bacterial strain was closely similar (94%) to the 16S rDNA sequence of *Azotobacter chroococcum* KM043465.1. Although the production of siderophores is a regular metabolic mechanism for getting iron complex from the soil by different microorganisms, e.g. *Bacillus* sp., *Pseudomonas* sp., *Klebsiella* and *E. coli* (20), the
Azotobacter chroococcum had not been investigated for siderophore production. Azotobacter exhibits dual benefit for the plants by siderophores production and nitrogen fixation (21). Accordingly, the characteristics of this novel isolate were further studied.

3.2 Optimization of siderophore production

The PBD was applied for the screening of several variables that can significantly influence the siderophore production by A. chroococcum (Table 2). The siderophore production fluctuated from 26.33 to 42.33%, which emphasizes the importance of the screening process of the tested variables, which significantly affect the siderophore production. The minimum siderophore production was perceived in the 10th run, while the maximum one was observed in the 5th run. This improvement in the siderophore production was obtained after 6 days under pH 8 with 50 mL of growth medium containing 5 g/L sucrose, 10 g/L glucose, 5 g/L mannitol, 2 g/L K$_2$HPO$_4$ and 0.2 g/L MgSO$_4$. Additionally, the key effects of the experimental variables on the siderophore production were calculated and demonstrated as a Pareto chart (Fig. 3). The calculated regression coefficients revealed that all the experimental variables had a positive influence on the siderophore production, except glucose concentration, medium volume and mannitol concentration.

These significant independent variables were additionally optimized via CCD comprising the creation of regression equation and linking the response to the coded levels of the tested independent variables. The significant variables, viz. the pH ($X_4$), incubation time ($X_5$) and K$_2$HPO$_4$ concentration ($X_6$), were matrixed in the CCD using three levels for each variable, while the non-significant variables were steadied at their high/low levels according to their positive/negative effect values, respectively.
The obtained results revealed that all tested factors, except pH significantly increased the A. chroococcum siderophore production (Table 3), with maximum production (89.33%) obtained in the 11th run after 3 days under pH 8 upon using concentration of 3 g/L for K$_2$HPO$_4$. This production was twofold more than that achieved in the PBD. Moreover, a reasonable correlation between the predicted and actual values was noticed ($R^2 = 0.64$), indicating the extent of precision regarding the comparison of the tested variables and simultaneously confirm the model significance. For the prediction of tested variables optimum point; a second-order polynomial model was fitted using the obtained experimental siderophore production (Eq. 1).

\[
\begin{equation}
Y = 84.08 + 7.033X_4 + 0.567X_5 + 3.533X_6 - 9.787X_4^2 + 1.546X_5^2 + 3.046X_6^2 - 0.708X_4X_5 - 0.958X_4X_6 + 0.708X_5X_6
\end{equation}
\]

Where $Y$ is the siderophore production response, while $X_4$, $X_5$ and $X_6$ represent pH, incubation time and K$_2$HPO$_4$ concentration, respectively. The obtained model was also validated by the scatter plot (Fig. 4). Most points were close to the regressed diagonal line due to the reasonable $R^2$.

The optimum quantity and type of carbon/nitrogen substrates have been the aim of numerous industrial studies to achieve economical fermentation media components. This kind of research is particularly imperative when the optimum fermentation conditions of the microbial producers are unknown. These fermentation factors have been observed to regulate the optimum growth of microbial cells and their ability to produce cellular products (22). Therefore, one aim of this study was to optimize fermentation medium components (K$_2$HPO$_4$ concentration) and growth conditions (pH and incubation time), in order to maximize the siderophore production.
by A. chroococcum. In general, the optimization of the bacterial siderophore production has been investigated in very few studies. The comparison of our results with the previously performed studies on siderophore production from different bacterial strains, revealed that A. chroococcum siderophore production surpassed those results. In two studies conducted to optimize the siderophore production by Pseudomonas fluorescence and Enterococcus sp. using different nitrogen/carbon sources, the maximum production obtained was 72.33 and 65% (2) (5). Similarly, the Pseudomonas putida siderophore production was optimized, and the maximum production was 71%. Moreover, the siderophore produced by Pseudomonas aeruginosa was 68.41% (23).

3.3 Characterization of the produced siderophore

HPLC was used to analyze the retention times (RT) of peaks with comparable heights (Fig. 5). Dominant peaks appeared at 1.77 and 1.67 min for the standard hydroxamate (deferroxamin mesylate) and the extracted siderophore, respectively. Moreover, a small peak at 1.93 min was observed in the extracted siderophore profile which could be attributed to the presence of another component in the sample. The functional groups of the obtained hydroxamate siderophore were determined by FTIR in the frequency range of 4000-400 cm\(^{-1}\) (Fig. 6). IR spectra of the hydroxamate siderophore showed sharp peak at 3450 and 3234 cm\(^{-1}\) corresponding to hydroxyl (-OH) and amino (-NH2) groups, respectively. Small absorption peak at 1670 cm\(^{-1}\) corresponding to carbonyl group (C=O) stretch matched with the absorption at 1627 cm\(^{-1}\) from the standard sample of Desferal \(^\circ\). Characteristic peaks at 1408, 1091 and 615 cm\(^{-1}\) were observed correspond to asymmetric stretched N-O, stretched C-N and (primary amines N-H Wag groups, respectively.
These characteristic peaks are in agreement with previous studies (1) (23). The magnetic properties of the extracted siderophore were obtained by NMR (Table 4). The proton NMR spectrum of the A. chroococcum hydroxamate siderophore displayed three peaks at 2.7 and 2.8 ppm due to CH$_2$-C=O and NH-CH$_2$, respectively. Multiple peaks at 3-3.6 ppm indicate CH$_2$ and single peak at 4 ppm correspond to NH$_2$ (Fig. 7a). The $^{13}$C NMR spectral data of the A. chroococcum extracted siderophore revealed the presence of a peak at 40 ppm which designated CH$_2$. Moreover, a peak was observed at 64 and 73 ppm related to C-N and C-O, respectively (Fig. 7b). The NMR spectra of the A. chroococcum siderophore illustrated the occurrence of a hydroxamate-type siderophore as previously described in several studies (24) (25). Results (Fig. 8), were observed that the mass fragment 53 m/z may be due to ferric ion lost which is bound to siderophore. Based on the fragmentation patterns of the MS/MS spectrum of the dehydrated siderophore, it was confirmed that the produced bacterial extract contained hydroxamate type siderophores as reported in Storey et al. (2006) (26). The obtained powder form of the extracted siderophore was examined by SEM. This analysis was performed to document for the first time the morphological shape of the siderophore. The morphological shape of the siderophore was obviously visible under microscopic inspection, in which images demonstrated that siderophore appeared like small tubular architecture in aggregates upon magnification to 2000 x - 4000 x (Fig. 9A & B). The length of these tubes varied from 4 – 50 µm.
4 Discussion

The production of siderophores and metabolites, contributing to antibiosis by some bacterial cells has been the aim of several studies devoted to examining plant growth-promoting rhizobacteria (PGPR) (4). The uptake of ferric ion by siderophore is mainly exploited by soil pathogenic/non-pathogenic microbial cells, as iron is a vital element for various biological metabolic processes. Accordingly, this study was carried out with the aim to isolate a novel bacterial strain that is capable of producing siderophore. The isolated *Azobacter* has not been investigated for the ability to synthesize siderophores. The statistical optimization confers an effective and practical approach, where siderophore production by the isolated strain was doubled with the second step of optimization (CCD) compared to the first step (PBD).

The pH is one of the most important parameters for siderophore production, as it has an important role in iron solubility in the fermentation media and siderophore production. Due to the insolubility of iron at neutral to alkaline pH, it leads to increase in the production of siderophore (2). In agreement with our results, the highest siderophore production of 93% units was obtained at pH 8 using *Streptomyces fulvissimus* (27). Moreover, the incubation period can greatly affect the production of siderophore, and it depends on the producer strain. A range of periods (36 – 96 h) were reported in different studies (28) (2). The maximum production of siderophore units (89.33%) in our study was obtained at an incubation period of 72 h, which falls inside the reported incubation period range.

This study aimed not only to isolate siderophore-producing soil *Azobacter* strain and optimize its production, but also to characterize and identify the produced siderophore type. The obtained *Azobacter* siderophore developed a stable complex with Fe$^{3+}$ (Neilands assay) revealing that it belongs to the hydroxamate type. The
result of the colorimetric assay was further confirmed by analyzing the chemical
structure of the produced siderophore. The obtained functional groups are
characteristic for hydroxamates, which are type of organic compounds including the
functional group R-C-O-N-OH-R', where R and R' represent organic residues while
CO is a carbonyl group (29) (30).
5 Conclusions

It has recently become obvious that siderophores are vital organic compounds for iron chelating in several microorganisms. Studying the chemical compositions of various types of siderophores and optimization of their production, particularly for novel ones, has opened novel routes of research. Accordingly, in this study we reported the isolation of A. chroococcum, as a novel record for the Egyptian flora. Furthermore, this is a pioneer report for the capability of A. chroococcum to produce hydroxamate type siderophore. The potential applications of siderophores are clear, and they can offer an important role in the ecological fields, however there are many questions are remained. More research is still required to discover efficient ways to employ siderophores in bioremediation applications. The variation of siderophore, based on their structural and functional features and their relation to microorganisms must be intensively studied to enhance the role of siderophores in environmental fields. The integration of metagenomics with chemical characterizations could result in vital information that may help to enhance the present ecological applications and open the door for novel potential applications of siderophores.
References


