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# Optimization of cyanophycin granule polypeptide production of some isolated cyanobacterial species

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**Abstract:** This paper aimed primarily at investigating the potential of cyanophycin production of some selected cyanoprokaryotes. For this purpose, nine different cyanobacteria species namely. Oscillatoria obscura, Anabaena anomala (isolate 1), Anabaena anomala (isolate 2), Anabaena oryzae, Anabaena khannae, Nostoc kihlmani, Nostoc sp, Synechococcus sp, Spirulina platensis, were selected as test species. The cyanobacteria species were grown in standard BG11 nutrient medium and incubated for 30 days under favorable growth condition at 25±2oC and with continuous light of 2.789w-2. Different test cyanobacteria exhibited wide variations in both biomass (0.25 - 0.8 freeze-dry wt g l-1) and cyanophycin content (0.03-0.224g l-1 ). The experimental results indicated that the cyanobacterium Anabaena anomala (isolate 2) maintained relatively higher biomass production and CGP content. Therefore, this cyanobacterium was selected for further screening growth experimental design to define the best growth medium composition (mgl-1). Compared to control, the results indicated that the modified composition of BG11 (2250mg NaNO3, 60mg K2HPO4, 112.5mg MgSO4.7H2O, 54mg CaCl2.2H2O, 3.0mg citric acid, 9mg ferric ammonium citrate, 1.5mg EDTA, 30mg Na2CO3 , 0.031mg H3BO3, 0.255mg MnSO4.H2O, 0.143mg ZnSO4.7H2O, 0.0038mg CuSO4.5H2O, 0.0063mg (NH4)6Mo7O24.4H2O, 25ml Soil solution, 500mg NaCl) increased the biomass production by 86.25% (from 0.8gl-1 to 1.49gl-1) and cyanophycin content by 96.43% (from0.224gl-1to0.44gl-1).Based on these results it has become evident that modified BG11medium composition induced highly significant( $p \leq 0.01$) increase in both biomass production and CGP content of cyanobacterium Anabaena anomala (isolate 2) and open new research window for further large –scale growth experiments.

**keywords**: cyanobacteria, cyanophycin granule polypeptide (CGP), Plackett–Burman, Anabaena anomala,

## 1. Introduction

Cyanobacteria (blue-green algae) are Gram-negative oxygenic photosynthetic autotrophs [1]. Cyanobacteria can perform oxygenic photosynthesis and respiration at the same time; therefore, they can be a model to study important biological activities [2, 3].

Cyanobacteria have been studied for their interesting morphology, diversity, and physiology but in recent years they have gained attention because of their potential applications in biotechnology.

Since cyanophycin granule polypeptides (CGPs) are rich in nitrogen, they are much like cyanobacteria storage material and heterotrophic bacteria. CGP consists of aspartic acid residues linked to the β-carboxylic gatherings of the poly α-aspartic acid vertebrae and arginine residues [4, 5]. Thus, CGP can be seen as poly (β-Asp-Arg) on the other side. A template-independent method of biosynthesis. Cyanobacterial CGP displays large poly differentiate ranging from 25 to 100 kDa in relation to a molecular mass division[6-8]. The purified CGP granules contained in biomass can effortlessly be solubilized in dilute acids (pH < 2) and in alkaline solutions (pH > 9), respectively [9-12].

It has been reported that certain encoding gens are responsible for biosynthesis of CGP in a more or less, similar to genes found in certain heterotrophic bacteria [11, 12].

CGP is likely to be of more ecological significance in the overall nitrogen cycle and may play a significant role in many well-known cyanobacterial symbioses by supplied to its symbiotic partner [13-16], animals [17-19]. with nitrogen containing compounds. Cyanophycin is a temporary reservoir of nitrogen that accumulates during the transition from accelerated to stationary growth and disappears as balance of growth begin [20, 21].

The bio-based, biodegradable, and biocompatible polymeric martials have environmental and industrial importance. CGP and its derivatives are used as a crude material to produce some kinds of these materials [10, 11]. In many industrial applications, cyanophycin is a potential product, in manufacturing of paints, suntan lotions and washing detergents, and can be used as an additive in the paper and oil industries.

Based on the obvious ecological and biotechnological importance of cyanophycin, and the main bottleneck of CGP production in cyanobacteria is the relatively slow growth rate. To overcome this limitation, the idea to initiate the present investigation is originated. The study aimed at investigating the production potential of cyanophycin by certain cyanobacteria species. The isolate(s) with relatively higher growth and cyanophycin content will be selected for further investigations for possible scaling-up and optimizing cyanophycin production.

## 2. Materials and methods

### Isolation andidentificationofcyanobacterial isolates

Raw water samples were collected from Damietta branch of the River Nile at the front of Mansoura University, centrifuged and the greenish pellet was streaked on agar plate containing BG11- medium [22]. Nine different macronutrients stock solutions (100ml each) were prepared by separately dissolving 15 g $NaNO_3$, 0.4g $K_2HPO_4$, 0. 75g $MgSO_4$ .$7H_2O$, 0. 36 $CaCL_2$. $2H_2O$, 0.06g citric acid, 0.06g ferric ammonium citrate, 0.01g EDTA (disodium salt) and 0. 2g $Na_2CO_3$ in glass distilled water. A trace metal stock solution (1000mL) was prepared by dissolving 61mg $H_3BO_3$, 169mg $MnSO_4$. $H_2O$, 287mg $ZnSO_4 \cdot 7H_2O$, 12.5mg $(NH_4)_6Mo_7O_{24}.4H_2O$, 2.5mg $CuSO_4 \cdot 5H_2O$ in glass distilled water.

To one-liter glass – distilled water 10ml of each macronutrients stock solution was add in addition 1.0 ml of trace element, 50ml of soil solution [23] and 20g agar (Difco®Bacto) were added. The nutrient medium was autoclaved, left to cool, poured on to Petri dishes and left to solidify. Plates were streaked with plankton pellet and were incubated at 25±2°C and with continuous light of 2.789$wm^{-2}$. After one week, the separate cyanobacterial filaments or colonies were aseptically picked up with sterilized needle, streaked again on the agar plates containing BG11 medium and left to grow under the same growth conditions. This procedure resulted in obtaining nine different cyanobacteria species, the isolated species were separately grown in 2liter culture flask, each containing 500 ml of BG-11 medium, incubated under same growth condition for 14 days to obtain biomass.

Identification and nomenclature of cyanobacteria isolates were followed [24-30]. The pure and identified cyanobacterial isolates were kept at algal culture collection of Biotechnology International Research and Development Centre (BIRD), Mansoura, Egypt.

### Monitoring of growth and CGP content for cyanobacterial isolates

For each cyanobacterial isolate, 5 replicate culture flasks (each of 1.0 L) were used. A volume of 150ml of BG11 medium enriched with 5%(v/v) soil solution. The culture flasks were autoclaved at 121°C for 20 min. Flasks were left to cool for room temperature before being inoculated. An inoculum (50 ml) of 10 days old cyanobacteria culture was added to each culture flask and incubated in an air-conditioned growth room at 25 ± 2°C with continuous light of 2.789$wm^{-2}$ for 30 days. The algal biomass was harvested by centrifugation at 3000× g for 5 minutes [31]. The wet biomass pellets were collected, freeze dried and weighted.

### Extraction of CGP

A method developed by [32] was used for extraction of CGP. A weight of 1.0g

lyophilized dry biomass was suspended in acetone and agitated strongly in order to dissolve the membrane lipids, to facilitate extraction and remove photosynthetic pigments.

The cells were then centrifuged and washed twice with 50 mM Tris-HCl buffer (pH 7.5) to remove all soluble proteins and other compounds. To dissolve CGP, the washed cells were then suspended in 0.52 ml of 0.1 M HCl, shaken for 30 min at room temperature, and centrifuged for 10 min at 14,000 x g. Then 500 µl of the supernatant was transferred to a clean plastic tube to which 500 µl of precipitation buffer (0.1MTris-HCl [pH 7.5] brought to pH 12 by addition of 0.1 M NaOH) was added to precipitate the CGP from the supernatant at neutral pH.

The mixture was incubated for 10 min on ice to complete CGP precipitation and was then centrifuged at 14,000 x g. The supernatant was discarded, and 500 µl of 0.1 M HCl was added to the CGP containing pellet to dissolve the CGP again before it was centrifuged for 1 min at 14,000 x g to remove insoluble proteins.

**Quantitative analysis of CGP**

The concentration of CGP of different cyanobacteria extraction was measured spectrophotometrically by the method described by [33] and modified by [34]. A volume of 0.3 ml of each CGP suspension of different cyanobacterial isolates was add to a clean dry tested tube, 0.7mL Phosphate buffer saline (PBS) and 5mL of standard dye binding solution (Coomassie brilliant blue G250).

Components were mixed well for 10 minutes to allow complete binding of CGP with the dye and to change the color. Absorbance of different CGP preparation of different cyanobacteria isolates were read at 595nm and the concentration was calculated using a standard curve carried out by a Bovine serum albumin.

**Periodic changes on growth and cyanophycin content of the most promising cyanobacteria isolate**

Based on results of growth of different cyanobacteria species and CGP content, the isolate maintaining the highest biomass and CGP content will be selected for further growth experiment for periodic monitoring biomass production and CGP content. At the same time, the concentration of the photosynthetic pigment chlorophyll a [35] will also be determined as physiological parameter of living biomass.

**Screening the effect of modified BG11 nutrient medium components on biomass production and CGP content by Plackett-Burman experimental design:**

The test cyanobacteria isolate with the highest growth and CGP content will be selected for further experiment, aiming at scaling up and possible optimization of the growth condition using Plackett-Burman screening experiment.

Placket-Burman experimental design [36] [37] was used to screen the effect of seventeen different factors (Table 1). Each factor was tested at three levels: low $(-1)^b$, medium $(0)^a$, and high $(+1)^c$, at concentrations shown in Table (1).

**3. Results and Discussion**

**Cyanobacteria isolates**

The teste cyanobacteria include nine different isolates. These isolates and their taxonomic positions are shown below:

Empire: Prokaryota

**A computer -based validation approach**

A validation approach was carried out to determine the accuracy of the generated models. The optimum conditions were theoretically predicted via response optimizer tool in Minitab computer software (Minitab16). The outcome of the validation approach is the optimum composition of the nutrient medium inducing both maximum biomass and CGP production. The effects of the optimized medium composition will be further tested on growth and CGP content of the selected cyanobacterium species and result will be compared to control (original BG11) nutrient medium.

**Cyanobacteria isolates**

The teste cyanobacteria include nine different isolates. These isolates and their taxonomic positions are shown below:

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**Table 1:** Variables investigated for biomass production and CGP content using Plackett-Burman design:

| Variables | Code | Unit | Center point (0)[a] | Minimum level (-1)[b] | maximum level (+1)[c] |
|---|---|---|---|---|---|
| $NaNO_3$ | $X_1$ | g/100ml | 15 | 7.5 | 22.5 |
| $K_2HPO_4$. $3H_2O$ | $X_2$ | g/100ml | 0.4 | 0.2 | 0.6 |
| $MgSO_4$. $7H_2O$ | $X_3$ | g/100ml | 0.75 | 0.375 | 1.125 |
| $CaCl_2$. $2H_2O$ | $X_4$ | g/100ml | 0.36 | 0.18 | 0.54 |
| citric acid | $X_5$ | g/100ml | 0.06 | 0.03 | 0.09 |
| Ferric ammonium citrate | $X_6$ | g/100ml | 0.06 | 0.03 | 0.09 |
| EDTA (disodium -salt) | $X_7$ | g/100ml | 0.01 | 0.005 | 0.015 |
| $Na_2CO_3$ | $X_8$ | g/100ml | 0.2 | 0.1 | 0.3 |
| $H_3BO_3$ | $X_9$ | mg/L | 61.0 | 30.5 | 91.5 |
| $MnSO_4.H_2O$ | $X_{10}$ | mg/L | 169.0 | 84.5 | 253.5 |
| $ZnSO_4$. $7H_2O$ | $X_{11}$ | mg/L | 287.0 | 143 | 430.5 |
| $CuSO_4$. 5 $H_2O$ | $X_{12}$ | mg/L | 2.5 | 1.25 | 3.75 |
| $(NH_4)_6Mo7O_{24}$. $4H_2O$ | $X_{13}$ | mg/L | 12.5 | 6.25 | 18.75 |
| Soil solution | $X_{14}$ | ml/L | 50 | 25 | 75 |
| Salinity (NaCl) | $X_{15}$ | g/L | 0 | 0.5 | 1 |
| PH | $X_{16}$ | unit | 7 | 6 | 8 |
| dummy | $X_{17}$ | - | 0 | -1 | 1 |

(0) Original concentration of a given components of BG11medium modified

[b](-1) Minus (-)50% of original components concentration

[c](+1) Plus (+) 50% of original components concentration

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**3. Results and Discussion**

**Cyanobacteria isolates**

The teste cyanobacteria include nine different isolates. These isolates and their taxonomic positions are shown below:

Empire: Prokaryota
Kingdom: Eubacteria
Subkingdom: Negibacteria
Phylum: Cyanobacteria
Class: Cyanophyceae

1. Subclass: Oscillatoriophycidae
Order: Oscillatoriales
Family: Oscillatoriaceae

Oscillatoria obscura (Figure 1-a)

2. Subclass Nostocophycidae
Order: Nostocales
Family: Nostocaceae

Anabaena anomala (isolate 1) (Figure 1-b)

Anabaena anomala (isolate 2) (Figure 1-b)

Anabaena oryzae (Figure1-c)

Anabaena khannae (Figure1-d)

Nostoc sp (Figure1e)

Nostoc kihlmani (Figure1-f)

3. Subclass: Synechococcaphycidae
Order: Synechococcales

Synechococcus sp (Figure1-g)

4. Subclass: Oscillatoriophycidae
Order: Spirulinales
Family: Spirulinaceae

Spirulina platensis (Figure1-h)

The variation in freeze-dry weight and the corresponding variation in CGP content, in addition to wt% (CGP/Freeze-dry wight) are listed in (**Table 2)**. Anabaena anomala Variation in dry weight biomass of different cyanobacteria isolates are illustrated in Figure 2, Similarly variation in cyanophycin content are shown in Figure 3. It must be mentioned that all

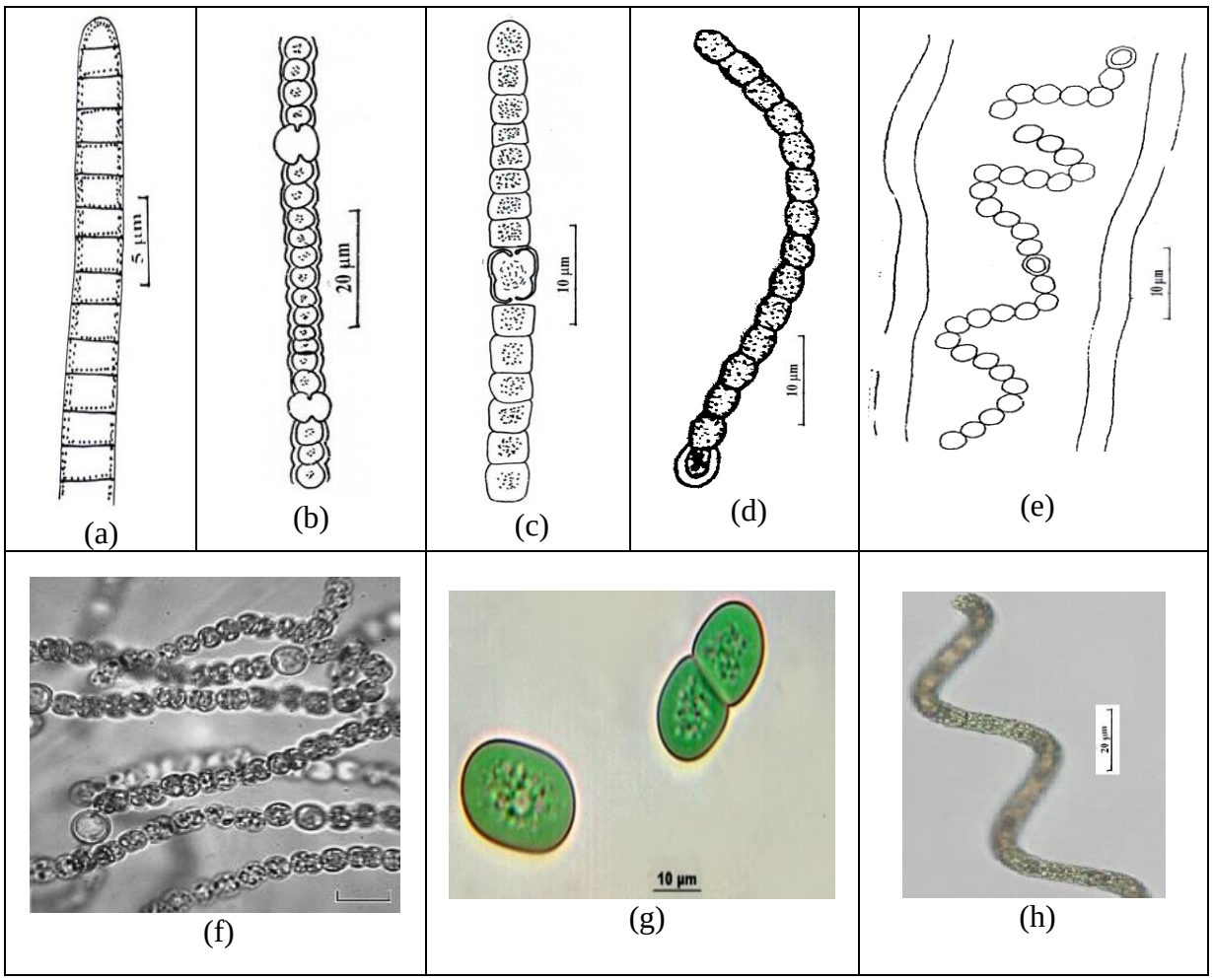



**Fig 1:** Drawings and photographs of different isolated cyanobacteria a,b ,c ([38, 39], d,e [40]), f (I. V. Kozhevnikov et al (2009))[41], g and h ([42])

**Biomass and CGP variation of different test cyanobacteria**

isolated were incubated for 30 days under identically similar growth condition. It is clear that the cyanobacterium *Anabean*

**Table 2**: Variation in freeze dry weight biomass ($gl^{-1}$) and cyanophycin granule polypeptides ($CGPgl^{-1}$) of different cyanobacteria isolates grown for 30 days under standard growth condition

| **Species** | **Freeze- dry weight (g $l^{-1}$) (±SD)** | **Cyanophycin ($CGPgl^{-1}$) (± SD)** | **wt% (CGP/ Freeze dry weigt)** |
|---|---|---|---|
| *Oscillatoria obscura* | 0.25±0.01 | 0.03±0.001 | 12% |
| *Nostoc sp* | 0.3±0.01 | 0.057±0.001 | 19% |
| *Anabeana khanne* | 0.4±0.01 | 0.072±0.003 | 18% |
| *Nostoc kihlmani* | 0.45±0.02 | 0.054±0.003 | 12% |
| *Synechococcease sp* | 0.65±0.04 | 0.13±0.008 | 20% |
| *Spirulina platencs* | 0.7±0.05 | 0.091±0.006 | 13% |
| *Anabaena anomala* (1) | 0.75±0.05 | 0.165±0.001 | 22% |
| *Anabaena anomala* (2) | 0.8±0.02 | 0.224±0.007 | 28% |
| *Anabaena oryzae* | 0.75±0.02 | 0.18±0.005 | 24% |

*anomala* (isolate2) recorded high biomass (0.8 g $L^{-1}$) and CGP content (0.22 g $L^{-1}$) as compared with other isolates (Fig 2-3). Therefore, this cyanobacterium was selected for further growth screening and optimization experiments.

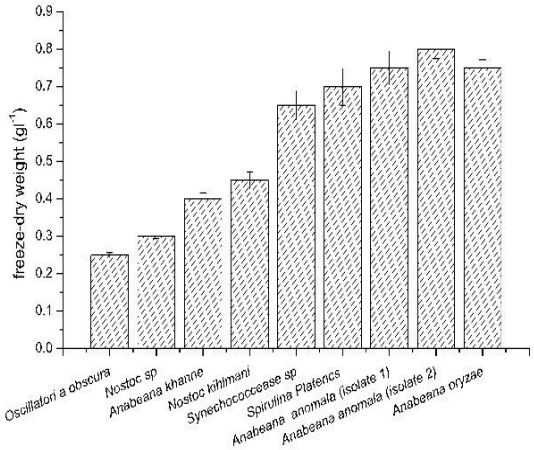
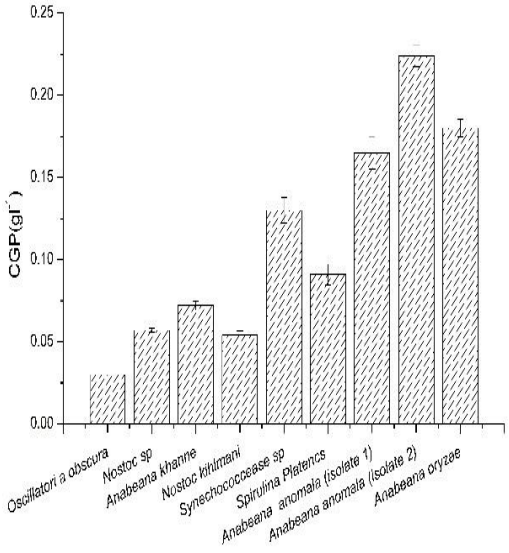



**Fig 2:** Freeze –dry weight ($gl^{-1}$) of different teste cyanobacteria

**Fig 3:** weight of cyanoph




ycin ($gl^{-1}$) of different teste cyanobacteria

**Periodic changes in growth and cyanophycin content of Anabeana anomala(isolate2)**

Compared to other cyanobacterium, *Anabaena anomala* (isolate 2) maintained both relatively higher biomass (Fg.2) and CGP content (Fig.3). Therefore, it has been decided to follow up its growth, CGP production and chlorophyll a with incubation time. As shown in Fig.4, steady increase in both biomass and CGP content observed until the day 30, after which both parameters tended to decline. Figure 5 illustrates that also chlorophyll a content attained its highest level at the day 30. Based on these results, the incubation time 30 days was fixed to carry out further Plackett-Burman experimental design.

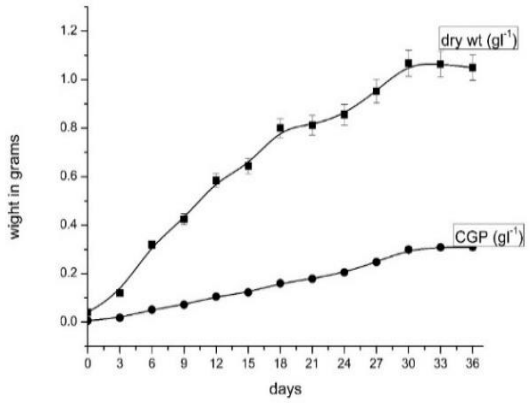



**Fig 4:** Variation in freeze-dry weight ($gl^{-1}$) and CGP ($gl^{-1}$) of *Anabaena anomala* (isolate2) with incubation time(days)

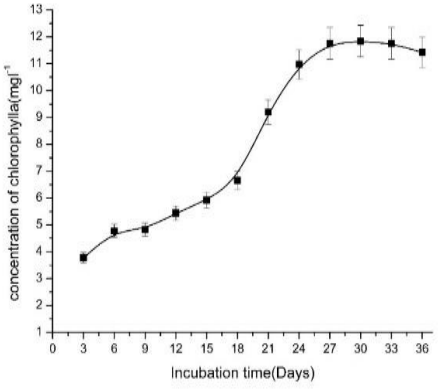



**Fig 5:** Periodic change in chlorophyll a ($mgl^{-1}$) of cyanobacterium *Anabaena anomala* (isolate2) grown for 36 days under standard growth condition

Table 3 clearly illustrates periodic change in biomass and CGP content in addition to variation in wt% (CGP/Freeze-dry weight). It is obvious that the wt% of (CGP/biomass) exhibited increase between 15% (after 3 days of incubation) and 29.2 at days 36 of growth.

**Screening the effect of modified BG11 nutrient medium composition on biomass production of *Anabaena anomala* (isolate 2) by Plackett-Burman design:**

The effects of different nutrients medium components (**Table1**) on growth and CGP content of *Anabaena anomala* was screened using Plackett-Burman experimental design and results are shown in **Table 4.**

The experimental runs (Table1 and 4) were performed in a randomized order and the maximum and minimum levels used for each

variable are indicated as (+) and (-), respectively. The highest growth and CGP content of *Anabaena anomala* (isolate 2) was observed at the run No.8 (Table 4).

The experimental responses were subjected to the analysis of variance and the parameter estimates and results are summarized in Table 5. The P value designates a statistical confidence of a factor estimate, as value of ≤ 0.05 was assigned as a cut-off point indicating the statistical significance of a factor at 95 % confidence level

Pareto charts in Fig 6 show the effect of each of different tested factors on biomass production and CGP content. Bars vertically arranged from the most to the least significance, for instance, bars above and below the reference line represent significant and non-significant factors, respectively. Accordingly, $NaNO_3$, $(NH4)_6Mo_7O_{24}.4H_2O$, $MgSO_4.7H_2O$, $H_3BO_3$ and soil solution are significant factors affecting biomass production (Fig 6a). Similarly, the factors citric acid, soil solution and $Na_2CO_3$ are considered significant factors affecting CGP content. While other factors are non-significant (Fig 6b).

Normal probability plot (Fig .7) illustrates the effect of different teste factors on biomass production and CGP content. The named factors are considered significant, Precisely, the $NaNO_3$ situated on the right of the reference line

(fig.7a) significantly affect positive biomass production at its (+) concentration level (+50% of the original concentration of nutrient medium). However citric acid (Fig.7b) significantly affect positive CGP content at it (-) concentration level (-50% of the original concentration of nutrient medium and so on.

Based on results of the validation experiments, the modified optimized medium composition (mgl-1) to achieve maximum biomass and CGP production is composed of 2250mg $NaNO_3$, 60mg $K_2HPO_4$, 112.5mg $MgSO_4.7H_2O$, 54mg $CaCl_2.2H_2O$, 3.0mg citric acid, 9mg ferric ammonium citrate, 1.5mg EDTA, 30mg $Na_2CO_3$, 0.031mg $H_3BO_3$, 0.255mg $MnSO_4.H_2O$, 0.143mg $ZnSO_4.7H_2O$, 0.0038mg $CuSO_4.5H_2O$, 0.0063mg $(NH4)_6Mo_7O_{24}.4H_2O$, 25ml Soil solution and 500mg NaCl The pH of the medium must be adjusted at 8.0 before autoclaving.

As seen from Table 6 growth and cyanophycin production of *Anabaena anomala* (isolate 2) in optimized medium increased by 86.25% and 96.43%,

respectively, compared to control

**Table3**: Variation in freeze-dry weight biomass ($gl^{-1}$) and cyanophycin content ($CGPgl^{-1}$) of cyanobacterium *Anabaena anomala* incubated for 36 days

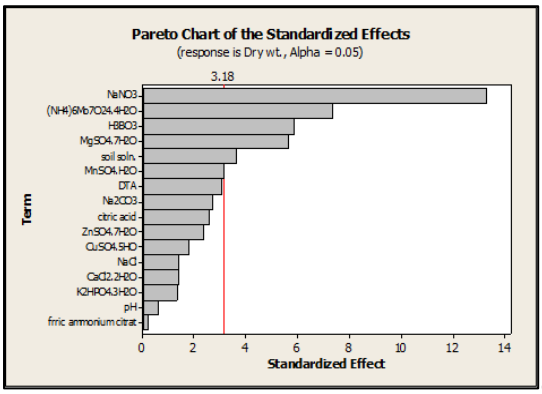
| | **Freeze -dry weight $gl^{-1}$ ($\pm SD$)** | **cyanophycin content ($CGPgl^{-1}$) ($\pm SD$)** | **wt% (CGP/ Freeze- dry)** |
|---|---|---|---|
| 0 | 0.04±0.01 | 0.006±0.001 | 15% |
| 3 | 0.12±0.01 | 0.018±0.001 | 15% |
| 6 | 0.32±0.02 | 0.0512±0.002 | 16% |
| 9 | 0.424±0.02 | 0.07208±0.004 | 17% |
| 12 | 0.584±0.03 | 0.10512±0.01 | 18% |
| 15 | 0.644±0.03 | 0.12236±0.01 | 19% |
| 18 | 0.8±0.04 | 0.16±0.01 | 20% |
| 21 | 0.812±0.04 | 0.17864±0.01 | 22% |
| 24 | 0.856±0.04 | 0.20544±0.01 | 24% |
| 27 | 0.952±0.05 | 0.24752±0.01 | 26% |
| 30 | 1.068±0.05 | 0.29904±0.01 | 28% |
| 33 | 1.064±0.05 | 0.30856±0.02 | 29% |
| 36 | 1.06±0.05 | 0.30952±0.02 | 29.2% |

**Table 4**: The Matrix and the Responses for the screening Plackett-Burman design

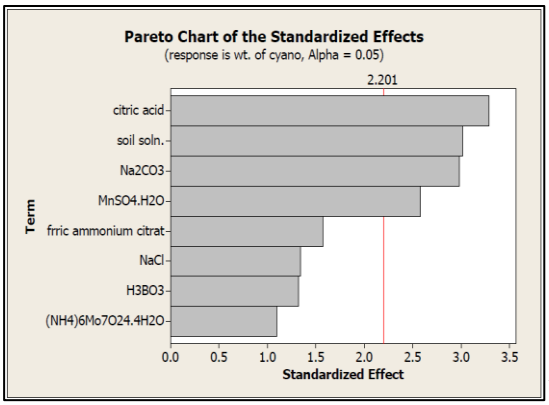
| Run | Variables | | | | | | | | | | | | | | | | | Responses | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $X_1$ | $X_2$ | $X_3$ | $X_4$ | $X_5$ | $X_6$ | $X_7$ | $X_8$ | $X_9$ | $X_{10}$ | $X_{11}$ | $X_{12}$ | $X_{13}$ | $X_{14}$ | $X_{15}$ | $X_{16}$ | $X_{17}$ | Freeze dry Wt.(g/L) | Cyanophycin Wt. (g/L) |
| 1 | 1 | -1 | 1 | 1 | -1 | -1 | -1 | -1 | 1 | -1 | 1 | -1 | 1 | 1 | 1 | 1 | -1 | 0.686 | 0.032 |
| 2 | 1 | 1 | -1 | 1 | 1 | -1 | -1 | -1 | -1 | 1 | -1 | 1 | -1 | 1 | 1 | 1 | 1 | 0.964 | 0.167 |
| 3 | -1 | 1 | 1 | -1 | 1 | 1 | -1 | -1 | -1 | -1 | 1 | -1 | 1 | -1 | 1 | 1 | 1 | 0.614 | 0.063 |
| 4 | -1 | -1 | 1 | 1 | -1 | 1 | 1 | -1 | -1 | -1 | -1 | 1 | -1 | 1 | -1 | 1 | 1 | 0.815 | 0.163 |
| 5 | 1 | -1 | -1 | 1 | 1 | -1 | 1 | 1 | -1 | -1 | -1 | -1 | 1 | -1 | 1 | -1 | 1 | 0.891 | 0.095 |
| 6 | 1 | 1 | -1 | -1 | 1 | 1 | -1 | 1 | 1 | -1 | -1 | -1 | -1 | 1 | -1 | 1 | -1 | 0.759 | 0.095 |
| 7 | 1 | 1 | 1 | -1 | -1 | 1 | 1 | -1 | 1 | 1 | -1 | -1 | -1 | -1 | 1 | -1 | 1 | 1.063 | 0.232 |
| 8 | 1 | 1 | 1 | 1 | -1 | -1 | 1 | 1 | -1 | 1 | 1 | -1 | -1 | -1 | -1 | 1 | -1 | **1.211** | **0.400** |
| 9 | -1 | 1 | 1 | 1 | 1 | -1 | -1 | 1 | 1 | -1 | 1 | 1 | -1 | -1 | -1 | -1 | 1 | 0.686 | 0.163 |
| 10 | 1 | -1 | 1 | 1 | 1 | 1 | -1 | -1 | 1 | 1 | -1 | 1 | 1 | -1 | -1 | -1 | -1 | 0.875 | 0.232 |
| 11 | -1 | 1 | -1 | 1 | 1 | 1 | 1 | -1 | -1 | 1 | 1 | -1 | 1 | 1 | -1 | -1 | -1 | 0.521 | 0.132 |
| 12 | 1 | -1 | 1 | -1 | 1 | 1 | 1 | 1 | -1 | -1 | 1 | 1 | -1 | 1 | 1 | -1 | -1 | 1.040 | 0.195 |
| 13 | -1 | 1 | -1 | 1 | -1 | 1 | 1 | 1 | 1 | -1 | -1 | 1 | 1 | -1 | 1 | 1 | -1 | 0.637 | 0.364 |
| 14 | -1 | -1 | 1 | -1 | 1 | -1 | 1 | 1 | 1 | 1 | -1 | -1 | 1 | 1 | -1 | 1 | 1 | 0.571 | 0.163 |
| 15 | -1 | -1 | -1 | 1 | -1 | 1 | -1 | 1 | 1 | 1 | 1 | -1 | -1 | 1 | 1 | -1 | 1 | 0.637 | 0.232 |
| 16 | -1 | -1 | -1 | -1 | 1 | -1 | 1 | -1 | 1 | 1 | 1 | 1 | -1 | -1 | 1 | 1 | -1 | 0.630 | 0.095 |
| 17 | 1 | -1 | -1 | -1 | -1 | 1 | -1 | 1 | -1 | 1 | 1 | 1 | 1 | -1 | -1 | 1 | 1 | 0.851 | 0.364 |
| 18 | 1 | 1 | -1 | -1 | -1 | -1 | 1 | -1 | 1 | -1 | 1 | 1 | 1 | 1 | -1 | -1 | 1 | 0.700 | 0.063 |
| 19 | -1 | 1 | 1 | -1 | -1 | -1 | -1 | 1 | -1 | 1 | -1 | 1 | 1 | 1 | 1 | -1 | -1 | 0.762 | 0.195 |
| 20 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 0.673 | 0.264 |
| 21 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.036 | 0.095 |

**Table 5:** Estimated effects, coefficients, T values, and p values for the tested variables for *Anabaena anomala* (isolate 2)

| Variables | Freeze -dry wight ($gL^{-1}$) | | | | Cyanophycin content ($gl^{-1}$) | | | |
|---|---|---|---|---|---|---|---|---|
| | Effect | Coefficient | T value | P value | Effect | Coefficient | T value | P value |
| $NaNO_3$ | 0.07550 | 0.03775 | 10.87 | 0.008 | 0.13116 | 0.06558 | 10.72 | 0.009 |
| $K_2HPO_4.3H_2O$ | 0.00750 | 0.00375 | 1.08 | 0.393 | 0.00696 | 0.00348 | 0.57 | 0.627 |
| $MgSO_4.7H_2O$ | 0.03210 | 0.01605 | 4.62 | 0.044 | 0.00564 | 0.00282 | 0.46 | 0.690 |
| $CaCl_2.2H_2O$ | 0.00790 | 0.00395 | 1.14 | 0.373 | 0.01096 | 0.00548 | 0.90 | 0.465 |
| citric acid | -0.01470 | 0.00735 | 2.12 | 0.169 | 0.14764 | 0.07382 | 12.07 | 0.007 |
| ferric ammonium citrate | 0.00110 | 0.00055 | 0.16 | 0.889 | 0.00204 | 0.00102 | 0.17 | 0.883 |
| EDTA | 0.01730 | 0.00865 | 2.49 | 0.130 | 0.00804 | 0.00402 | 0.66 | 0.578 |
| $Na_2CO_3$ | 0.01530 | 0.00765 | 2.20 | 0.159 | 0.14604 | 0.07302 | 11.94 | 0.007 |
| $H_3BO_3$ | -0.03330 | -0.01665 | -4.79 | 0.041 | -0.13736 | -0.06868 | -11.23 | 0.008 |
| $MnSO_4.H_2O$ | 0.01770 | 0.00885 | 2.55 | 0.126 | 0.14396 | 0.07198 | 11.77 | 0.007 |
| $ZnSO_4.7H_2O$ | -0.01310 | -0.00655 | -1.89 | 0.200 | 0.12604 | 0.06302 | 10.31 | 0.009 |
| $CuSO_4.5H_2O$ | 0.01010 | 0.00505 | 1.45 | 0.283 | -0.00064 | -0.00032 | -0.05 | 0.963 |
| $(NH_4)_6Mo_7O_{24}.4H_2O$ | -0.04150 | -0.02075 | -5.97 | 0.027 | -0.01196 | -0.00598 | -0.98 | 0.431 |
| soil solution | -0.02050 | -0.01025 | -2.95 | 0.008 | -0.14624 | -0.07312 | 11.96 | 0.007 |
| NaCl | 0.00790 | 0.00395 | 1.14 | 0.373 | -0.13744 | -0.06872 | -11.24 | 0.008 |
| pH | -0.00330 | -0.00165 | -0.48 | 0.682 | 0.13236 | 0.06618 | 10.82 | 0.008 |

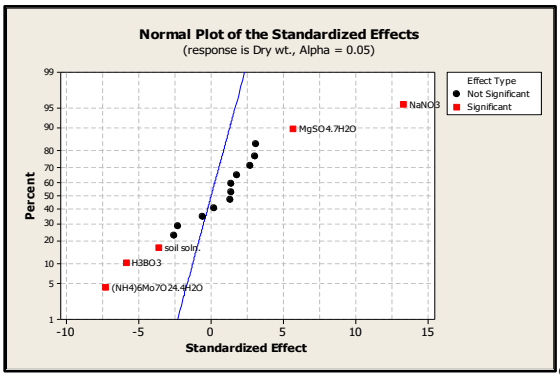



a)

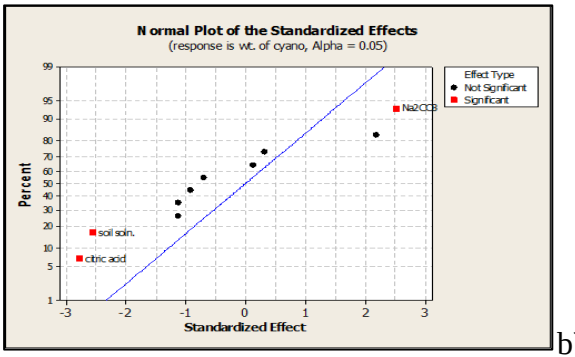



b)

**Fig 6**. Pareto charts of the standardized effect (a) shows the significance of each factor on freeze-dry weight and (b) shows CGP of *Anabaena anomala* (isolate 2)




a)




b)

**Fig.7:** Normal probability plot of the standardized effect (a) shows the significance of each factor on freeze-dry weight and(b) shows CGP of *Anabaena anomala* (isolate 2)

**Table 6:** Growth yield (freeze- dry wight, $gL^{-1}$) and cyanophycin content ($gL^{-1}$) of the cyanobacterium *Anabaena anomala* (isolate 2) grown in original BG11and the optimized nutrient media

| **Parameter** | **Original BG11 (control)** | **Optimized nutrient media (the outcome of validation approach)** | **% increase compared to control** |
|---|---|---|---|
| **Biomass (Freeze -dry wight $gL^{-1}$)** | **0.8$gl^{-1}$** | **1.490 $gl^{-1}$** | **86.25%** |
| **Cyanophycin content ($gL^{-1}$)** | **0.224$gl^{-1}$** | **0.44$gl^{-1}$** | **96.43%** |

## 3. Results and Discussion

The idea to carry out this investigation based on concrete scientific reports indicating that cyanobacteria can synthesize a special protein namely cyanophycin granule poly peptides (CGP)[4, 5]. CGP has been reported to maintain potential ecological importance [13] and can be used for commercial production of useful biodegradable and biocompatible polymers [34].

The results obtained indicated that the

selected test cyanobacteria (Figure1) exhibited different growth production (Figure2) and CGP contents (Figure3).

It is important to stress that all

selected cyanobacteria can synthesis cyanophycin granules poly peptides. This represent a potential result justifying the idea to carry out this investigation.

The growth rate variation among different test cyanobacteria is a normal finding supported by a wealth of publications (e.g; [43, 44]) reported that different microalgae including cyanobacteria exhibited distinct different

growth rates even when grown under similar growth condition.

Figure 3 illustrates distinct variations in CGP content of different test cyanobacteria grown under similar incubation condition. This finding was also reported by other researchers (e.g; [45]).

The results obtained (Table 2) showed that the biomass of different cyanobacteria differ

widely in cyanophycin content ranged between 12% (*Oscillatoria obscura*) and 28% (*Anabaena anomala* (isolate 2)). The results agreed well with these reported by [46].

The relatively higher cyanophycin content (28%gg-1) of the cyanobacterium *Anabaena anomala* justifies the selection of this cyanobacterium for further growth screening experiment using Plackett-Burman design.

As shown in figure 4, progressive increase in growth and cyanophycin content of *Anabaena anomala* was evident until days 36 beyond which the decline growth period started. Also, the results of periodic variation in chlorophyll a (figure 5) indicated that the living biomass starts to decline beyond days 36. These finding maintain prime importance for further mass production of cyanobacterium for commercial production of CGP by adjusting the incubation period at 30 days at which maximum growth and CGP obtained in this study.

It is relevant to mention that a steady progressive increase in the wt% of CGP/freeze -dry weight of cyanobacterium *Anabaena anomala* between 15% at day 3 and 29.2% at day 36. In this respect, [4, 45, 47] reported that CGP content increased with cyanobacteria growth .

It has become evident that the Plackett-Burman design [36] has been extensively used by investigators (e.g; [48-53]) to define growth media composition enhancing maximum biomass production and relatively higher production of vital metabolites of microalgae.

The analysis of the Plackett-Burman screening experiment (Fig. 6-7) indicated that the nutrient compounds $NaNO_3$ and $MgSO_4$ induced significant biomass production of the cyanobacterium *Anabaena anomala* at their higher concentration levels (+50% of the original concentration of nutrient medium) while $H_3BO_3$and $(NH_4)_6Mo_7O_{24}.4H_2O$ induced significant growth at their lower concentration level (-50% of the original concentration of nutrient medium) Also Na2CO3 and citric acid resulted in significant increase in CGP content at their higher and lower concentration levels, respectively These seems novel results for the test cyanobacterium *Anabaena anomala* as no similar previous studies were recorded. However a wealth of publication (e.g; [31, 54, 55]) indicated that algal growth vary significantly with variations in concentration of different essential nutrients, a finding that is additionally supported by the obtained results.

It is a perhaps relevant to mention that several publications (e.g; [56-59]) indicate positive growth effects of soil solution on several test microalgae. According, a decision was taken to study the effect of soil solution on growth of cyanobacterium *Anabaena anomala*, although it is constituent of BG11 nutrient medium. The results obtain (figure 7) confirmed that soil solution (25mlL-1) supported signification biomass production and CGP content of *Anabaena anomala*. This finding supports and justifies the decision to test the effect of soil solution on growth of *Anabaena anomala*.

It is important to mention that, the results listed in Table 6, confirmed an 86.25% and a 96.43% increase in biomass and CGP content of *Anabaena anomala*, respectively, when grow in optimized BG11 medium composition compared to the original BG11 composition (control).

In conclusion the optimized BG11 nutrient medium with composition (2250mg $NaNO_3$, 60mg $K_2HPO_4$, 112.5mg $MgSO_4.7H_2O$, 54mg $CaCl_2.2H_2O$, 3.0mg citric acid, 9mg ferric ammonium citrate, 1.5mg EDTA, 30mg $Na_2CO_3$, 0.031mg $H_3BO_3$, 0.255mg $MnSO_4.H_2O$, 0.143mg $ZnSO_4.7H_2O$, 0.0038mg $CuSO_4.5H_2O$, 0.0063mg $(NH_4)_6Mo_7O_{24}.4H_2O$, 25ml Soil solution, 500mg NaCl) resulted in obvious signification biomass production and CGP content , a finding that may encourage future mass production of *Anabaena anomala* for commercial production the CGP that

maintains a host of biotechnological application.

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