# Development of cost-effective fertilizer-based media for the microalgae cultivation aimed at effective biomass production

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## Abstract

In the diversification of the world's energy portfolio, microalgae biofuels have gained particular attention as a means of providing carbon-neutral hydrocarbon-based fuels. The progress on this front has been hindered because of expensive and unreliable microalgae cultivation systems, where the preparation of synthetic culture media poses a monetary strain. This study aims to investigate a new microalgae growth media that is readily available and cost-effective. The new microalgae fertilizer-based media (FBM) were designed and compared with Bold Basal media (BBM) in terms of growth parameters, production of lipid and value-added algal by-products (carotenoid and phycobiliprotein). The growth parameters such as cell count and specific growth rate were investigated for FBM and BBM. FBM showed a significant growth rate of 7 million/mL-per day as compared to 2 million/ml per day. A similar growth trend was observed for chlorophyll (a), (b) and carotenes which increased to the values of 5microgram/gram formula weight (µg/gfw), 3 µg/gfw and 2 µg/gfw for FBM. On the other hand, lipid and phycobiliprotein showed a decrease of 18.75 % and 16.66 %, respectively, compared to BBM. Even though some growth parameters are negatively affected by fertilizer-based media, the cost reduction is substantial to allow this drawback to be overlooked. The medium's cost is reduced by a factor of 7 and provides ground for the use of the FBM in the large-scale cultivation of algae for biomass production.

**Keywords:** Microalgae, Energy, Biofuels, Nutrient sources, Algal Growth kinetics

## Introduction

With the inevitable depletion of traditional fossil fuels, switching to renewable primary energy resources is the next logical step for sustainable growth [1, 2]. Biodiesel presents itself as a bridging fuel that can provide sustainable, less emission fuel from the alternative resources [3, 4]. Microalgae are a large and diverse group of aquatic organisms that lack the complex cell structures found in higher plants. They can be found in diverse environments, with some species thriving in freshwater while others in saline conditions like seawater [5, 6]. Most species are photoautotrophic, converting solar energy into chemical forms through photosynthesis. Microalgae have received considerable interest as a potential alternative feedstock for biofuel production due to the high growth rates of algae with appropriate lipid contents and large consumption of $CO_2$. They can produce useful quantities of polysaccharides (sugars) and triglycerides (fats), which are the raw materials for producing bioethanol and biodiesel transport fuels. Furthermore, microalgae can also produce proteins as sources of animal feed and commercially valuable compounds such as pigments and pharmaceuticals [7].

Microalgae are one of the most promising third-generation sources of biofuels due to their ability to accumulate substantial amounts of lipids, divide rapidly, absorb $CO_2$, growth in low-quality water and non-arable land [8, 9]. There is a wealth of literature that documents the commercial-scale growth of various microalgae species for natural products as well as the progression of both basic and applied biological research, improvements to photo-bioreactor (PBR) and pond design (6) and lifecycle analyses of microalgae biofuels [10].

While cultivating microalgae in an artificial environment (e.g., outdoor or pond), growth factors must be plentiful to maximize growth rates [11]. While $CO_2$ can be acquired from the atmosphere, it is commonly fed into microalgae growth media to improve production [12]. In addition to $CO_2$, nitrogen and phosphorus are the primary nutrients required for microalgae growth [13, 14]. Microalgae need phosphates, nitrates and silicates, and specific vitamins, proteins, and other trace elements for their growth [15]. Conventionally synthetic growth media are employed for lab-scale culturing of microalgae. However, these are expensive and not suitable for the mass culturing of microalgae [16]. For large-scale culturing, raw sources of nutrients are used as they are low-cost. Still, it significantly reduces the microalgae biomass yield along with the negative impact the lipid production. Therefore, there is a need for an economic microalgae growth media that would be suitable for mass culturing and promoting the growth of bioactive compounds. Microalgae can carry out many of the post-translational mechanisms needed to produce many complex proteins and are relatively easy and cheap to culture. They require fundamental media to grow on, such as minimal or acetate-based media with a few trace elements and the ability to use sunlight as an energy source; hence the cost of goods is low [17].

In this study, a novel microalgae growth media, Fertilizer Based Media (FBM), was developed using essential nutrients from cost-effective sources like agricultural fertilizers, market available multivitamins and egg proteins. Local microalgae strain Dictyosphaerium iso 6-8 was later cultivated in both FBM and control growth media (BBM) under ambient growth conditions to compare the growth kinetics and production rates bioactive value-added compounds. It is expected that the

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development of such cost-effective growth media will encourage the advancement of microalgae cultivation and, ultimately, the production of value-added bioproducts.

## Material and Methods

### Control & FBM Growth Media Preparation

Bold's Basal Media (BBM) was selected as a synthetic control growth media and was prepared using the recipe by [18] which had the following composition: $K_2HPO_4$ (75 mg/L), $MgSO_4$ (75 mg/L), NaCl (25 mg/L), $CaCl_2$ (25 mg/L), $NaNO_3$ (250 mg/L), $KH_2PO_4$ (105 mg/L), $FeCl_3$ (0.194 g/L), $MnCl_2$ (0.082 g/L), $CoCl_2$ (0.16 g/L), $Na_2MoO_4.2H_2O$ (0.008 g/L), and $ZnCl_2$ (0.005 g/L). The initial pH was 6.6, which was brought in 7 before the commencement of experimentation. BBM is a highly enriched media with low salinity, which is ideal for culturing a wide variety of freshwater microalgae strains. This growth media is similar to the standard microalgae media 3N-Basal Bold Medium (3N-BBM), except that it has higher nitrate concentrations than standard BBM [19]. Water-soluble Phycobiliprotein pigments, including C-phycocyanin (CPC), Allophycocyanin (APC), and C-phycoerythrin (CPE), were extracted from fresh microalgae sample (1 g) with 0.05M phosphate buffer (10ml, pH 6.8).

Fertilizer Based Media (FBM) was the novel, cost-effective microalgae culture media developed using different ratios of agriculture grade fertilizers, mainly Diammonium phosphate (DAP), Urea, Calcium ammonium nitrate (Fuji Fertilizer Company, Pakistan), Revitale multi tablets (GlaxoSmithKline) and egg white solution. The Egg white solution was prepared by dissolving egg white in water with 10% NaOH solution (Merck) known as sodium albumate. It was used as a protein source for microalgae. All the media constituents were readily available from the local market and the entire media preparation cost was seven times less than that of a conventional microalgae growth media.

### Isolation, growth and testing of Local Algae Strain (Dictyosphaerium iso 6-8)

Local algae strains were collected from Kallar Kahar Lake, located in Jhelum, Pakistan [20]. They were later subjected to isolation and preservation at Biofuel laboratory at USPCAS-E, NUST, Islamabad, Pakistan, using standard protocols [7]. In brief, the strains were first revived prior to isolation in BBM at ambient temperature (20 ± 5 ºC) with constant $CO_2$ supply having a flow rate of 0.3 L/min. Illumination was provided by natural sunlight and pH was maintained at 7 using NaOH solution. Afterward, a sample was taken, serially diluted and cultured on agar plates. The isolation protocol and regular observations under microscope continued until uni-microalgae culture was acquired. The resulting isolated microalgae strain was identified as Dictyosphaerium iso 6-8 which were later cultivated in both control (BBM) and novel FBM under identical ambient growth conditions to establish a comparative study.

Samples were taken from inoculated experiment and control media cultures daily for measuring pH (pH meter, Fisher Scientific accumetAE150 pH Benchtop Meter Education Set), turbidity (Fisher Scientific 2020WE Portable Turbidity Meter) and cell count, which was measured using a hemocytometer (Neubauer Hemocytometer, China). While dry biomass weight (g) and chlorophyll-a (mg/L) were determined every three days. Chlorophyll-a was determined spectrophotometrically after extraction by 90% acetone as reported by [21]. Dry biomass weight (g/L) was calculated using protocol by Irving T [22], in which 10 mL of microalgae culture was filtered through pre-heated, pre-weighed glass microfiber filters (Whatman GF/C, 47 mm). After filtration, the filters containing microalgae suspension were dried at 103 °C to a constant weight, cooled in a desiccator (Fisherbrand) and weighed on an electronic balance (Sartorius Cubis MSE Precision Balance).

Protocol by Bligh and Dyer [23] was used to extract lipids from microalgae biomass [24]. Microalgae culture was centrifuged (4000 g, 10 min) to obtain a wet pellet, mixed in 4 mL distilled water and extraction solution (MeOH/chloroform, 1:2 v/v) and left for an overnight on a shaker at 300 rpm moderate speed. The following day, distilled water and chloroform (1:1 v/v) were introduced in the sample and allowed to mix for 5-6 hrs. Finally, the sample was centrifuged (4000 g, 10 min) to produce a biphasic layer in which the bottom layer contained lipids dissolved in chloroform. This layer was extracted using a micropipette and dried (50 ºC, 2 hrs) in the oven to obtain lipid content. Carotenoid content was measured by UV spectrophotometer by the method of Jaspers [22][14]. The water-soluble Phycobiliprotein pigments, including C-phycocyanin (CPC), Allophycocyanin (APC) and C-phycoerythrin (CPE) were extracted from fresh microalgae sample (1 g) with 0.05 M phosphate buffer (10 mL, pH 6.8). The absorbance (A) of the solution was measured at 650 nm, 620 nm and 565 nm and the concentrations were calculated using Eq. (1) to (3) [25]:

$$CPC(mg/ml) = \left[ A620 - \left( \frac{0.72 \times A650}{6.29} \right) \right] \quad (1)$$

$$CPC(mg/ml) = \left[ A620 - \left( \frac{0.191 \times A620}{5.79} \right) \right] \quad (2)$$

$$CPC(mg/ml) = \left[ A565 - 2.41 \times \left( \frac{Conc.\ \text{of CPC} \times 1.4 \times Conc.\ \text{of APC}}{13.02} \right) \right] \quad (3)$$

The calculated dry biomass weight from the exponential growth phase of each growth study was used to determine the growth kinetics of established experimentation. The specific growth rate (μ, $d^{-1}$) was calculated using Eq. (4) [20].

$$\mu = \left[ \left( \frac{\ln X_2 - \ln X_1}{t_2 - t_1} \right) \right] \quad (4)$$

Where, $X_2$ and $X_1$ were the dry biomass weight (g/L) at time $t_2$ and $t_1$, respectively. The maximum specific growth rate ($\mu_{max}$, $d^{-1}$) was determined from all the different μ values

calculated, while the maximum biomass obtained was designated as $X_{max}$ (g/L). Cell doubling time ($t_d$, d) was estimated using Eq. (5) [20].

$$t_d(d) = \left[\left(\frac{\ln 2}{\mu_{\max}}\right)\right] \quad (5)$$

## Experimental setup

The response of microalgae to fertilizer nutrients, multivitamins and sodium albumate was tested sequentially in three experimental phases, where the best performing of each previous experiment was subjected to further investigation in the second phase. The microalgae were grown in flasks (1 L) with water and 10% microalgae inoculum, making 1 L of algae culture in each bottle (0.3 L/min $CO_2$, 7.5 pH). The growth conditions are summarized in Table 1. The ranges tested for each nutrient ingredient are shown in Table 2. The results were compared with BBM as a control. The results include microalgal growth parameters, bioactive compound analysis and growth kinetic parameters of Dictyosphaerium iso 6-8.

**Table 1: Growth conditions for microalgae during the experimental setup**

| Parameters | Ranges |
|---|---|
| Average temperature (°C) | 26 ± 5 |
| Average pH | 9 ± 0.5 |
| Light Intensity (LUX) | 700 ± 50 |
| Working volume (L) | 1 |
| Inoculum concentration (%) | 10 |
| % $CO_2$ | 0.02 (via air pump) |

**Table 2: Nutrient ranges tested in three phases of the experimental setup. The best combinations in previous phases were used in the next phase.**

| Phase 1 | | Phase 2 | | Phase 3 | |
|---|---|---|---|---|---|
| Urea, DAP & CAN | | Multi vitamin | | Sodium albumate | |
| Sample identification | Nutrient ratio (Urea:DAP:CAN) | Sample identification | Nutrient conc. (g/L) | Sample identification | Nutrient (%) |
| S1 | 6:1:3 | V1 | 10 | P1 | 1 |
| S2 | 5:2:3 | V2 | 20 | P2 | 2 |
| S3 | 4:3:3 | V3 | 30 | P3 | 3 |
| S4 | 3:4:3 | V4 | 40 | P4 | 4 |
| S5 | 2:5:3 | V5 | 50 | - | - |

**Table 3: Breakdown and comparison between total phycobiliproteins produced by local algae strain Dictyospherium iso 6- under control (BBM) and experiment (FBM) mediaconditions**

| Media condition | CPC (mg/mL) | APC (mg/mL) | CPE (mg/mL) | Total phycobiliproteins (mg/mL) |
|---|---|---|---|---|
| FBM | 0.12 | 0.01 | 0.02 | 0.15 |
| BBM | 0.13 | 0.00 | 0.05 | 0.18 |

## Results and Discussion

### Development of cost-effective FBM growth media

During the experimental Phase 1, although microalgae strain was provided with major essential nutrients of nitrogen, phosphorus and calcium, it still showed poor results compared to synthetic growth media (BBM). The main reason would be additional vitamins such as vitamin B1, vitamin B12 and vitamin H. Most microalgae species do not require all three vitamin types for growth. However, there seems to be no harm caused by adding a nonessential vitamin. In addition to the three common vitamins, some recipes call for other vitamins. For example, nicotinamide (nicotinic acid amide, niacinamide) is added to the culture media for Phacotus lenticularis (Ehrenberg) Stein. The S1 with 6:1:3 ratio of Urea, DAP, CAN respectively show the cell count of about 11 million cells per mL at approximately 30$^{th}$ day of an experiment which is much higher than the other four samples. S1 also shows the growth rate of 0.106 $d^{-1,}$ doubling time of 2.94 days and maximum biomass concentration of 0.32 g, which is higher than all other samples as shown in Figure 1(a-c) and Figure 2, so this sample was further evaluated in the next phase.

In phase two, S1 was further tested under five different compositions of a multivitamin tablet to improve the overall cell count and dry biomass amount of microalgae. The addition of multi-minerals improved the cell count and as shown in Figure 2 (a), which also concludes that multivitamins have a positive effect on microalgae growth [26]. Among all five samples, V2 (fertilizers (6:1:3) + Revitale 20 g) showed enhanced results. However, the results were still not that satisfactory to compete with conventional fertilizers and to improve it further sample V2, which contains the best fertilizer combination and the best multivitamin combination. Now in phase 3, proteins were tested along with this best combination.

The turbidity was measured for all the three samples shows in Figure 3(b), where we can see in the P1, which shows the highest.

In the third phase of the experiments, we tried to nurture the microalgae with some protein-rich media. To make media protein sufficient, egg white was dissolved in the media with the help of NaOH and tested at four different concentrations. All four protein samples were tested with S1 and V1 under similar growth conditions. Sample P2, which contains Fertilizers in the ratio of 6:1:3, 200 g of multivitamins and 2% egg albumate gave the maximum cell count of 25 million cells per mL, the maximum specific growth rate of 0.11 $d^{-1}$ (Figure 3(c) and this sample gave maximum biomass concentration of 0.78 g/L. The doubling time is also shown in Figure 3(d), demonstrating the highest for the fertilizer media.

### Comparison between novel FBM and control growth media

Sample P2 showed the best results and these results were verified in further experiments with the same conditions and compared with the result of synthetic media for growth parameters. Cell count data of both experiment and control is as shown in Figure 3, along with the dry biomass content. The cell count for BBM on the 22$^{nd}$ day is 32 million cells per mL, while in cheap fertilizer media as FBM, it is 25 million cells per ml on the 26$^{th}$ day. Bligh & Dyer method yielded the lipid content shown in Table 4, which shows a difference of only four units, showing the promise of the FBM. Chlorophyll (a, b) and carotenes, determined by UV spectrophotometer technique, under UV absorption at 662, 644 and 470 nm, showed higher levels of these components for FBM, which can lead to higher absorption of the incident light, thus increasing the overall conversion efficiency of solar energy to chemical energy. The absorbance (A) of the solution was measured at 650 nm, 620 nm and 565 nm and the concentrations calculated as shown in Table 3. Not much difference is seen in the amounts of phycobiliproteins yielded under both cases. The difference of 0.3 mg/ml is negligible. Table 4 summarizes the differences among various growth parameters for the two media, BBM and FBM and yields favorable economic results for the case where FBM is used.

Table 4 Effect on growth kinetic parameters under Control and Experiment media conditions

| Growth kinetics parameters | Control (BBM) | Experiment (FBM) |
|---|---|---|
| Max. cell count (million cells/mL) | 25 | 32 |
| Max turbidity (NTU) | 680.94 | 710 |
| Specific growth rate ($d^{-1}$) | 0.11 | 0.21 |
| Doubling time (d) | 2.90 | 2.01 |
| Maximum biomass Conc. (g/L) | 0.78 | 1.1 |
| Lipids (g/L) | 16 | 13 |
| Chlorophyll a (µg/gfw) | 48 | 53 |
| Chlorophyll b (µg/gfw) | 18 | 21 |
| Carotenes (µg/gfw) | 21 | 23 |
| Total phycobiliproteins (mg/mL) | 0.18 | 0.15 |
| Cost ($) | 3.4 | 0.5 |

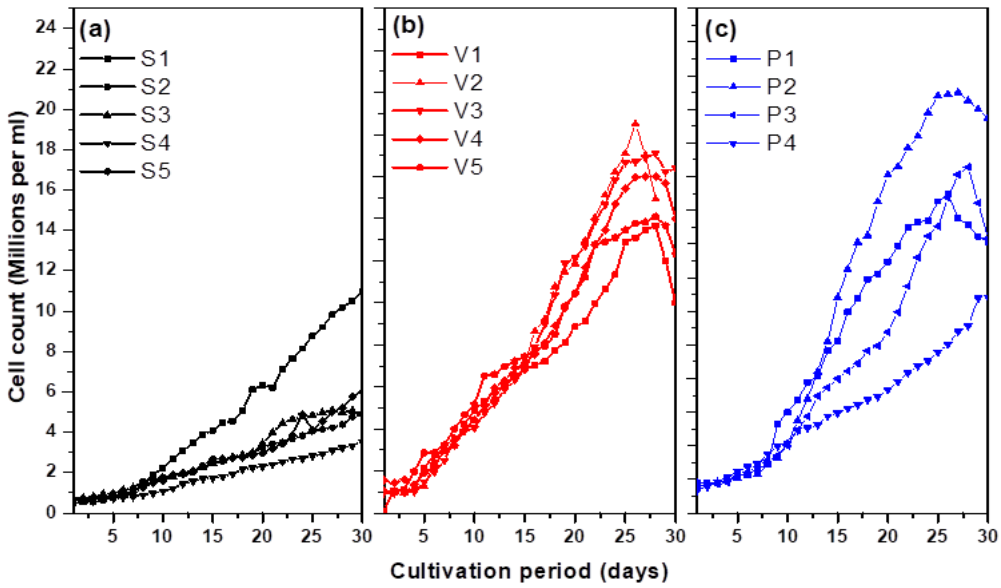


**Figure 1: Effect on the cell count (million cells per mL) of the local algae strain (Dictyospherium iso 6-8) by (a) change in the added fertilizer ratios, (b) amounts of added vitamins, and (c) amount of added protein.**

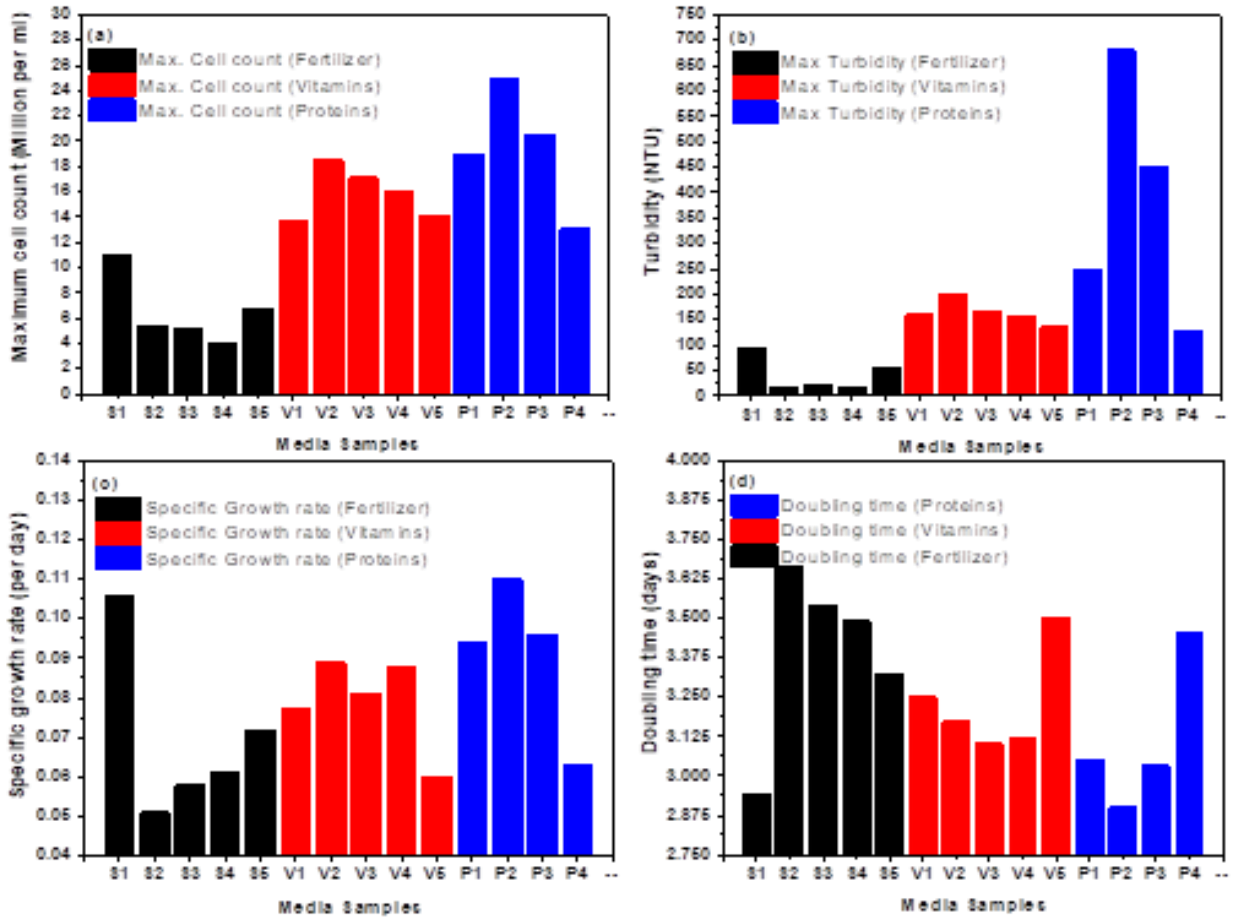


**Figure 2: Growth kinetic parameters for local algae strain Dictyosphaerium iso 6-8**

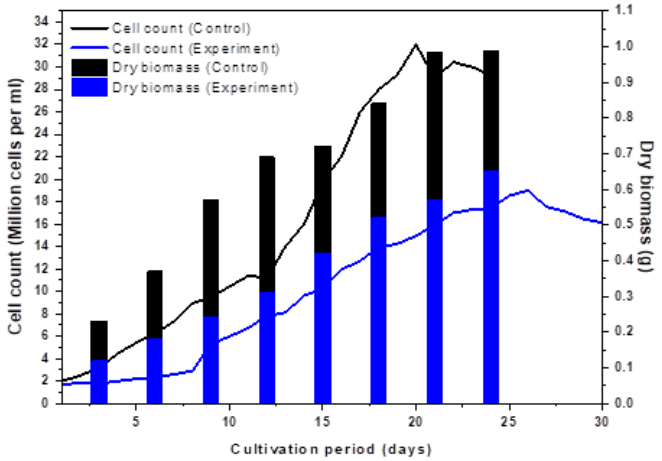


**Figure 3: Comparison of cell count of local algae strain Dictyospharium iso 6-8 under control (BBM) and experiment conditions (FBM)**

## Conclusion

Local microalgae strain KKL-5 showed upright growth in both Bold's Basal Media and newly designed FBM. The productivity in FBM was found to be slightly lower than BBM. However, the low-cost FBM still makes a viable choice for large-scale microalgae production, such as in raceway ponds or photo-bioreactors. The fertilizer media cost is about half a dollar, while the synthetic Bold's Basal media cost about 3.4 US dollars. FBM can be commercially viable for the production of algae biomass for biofuel production.

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