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IMPACT OF TEMPERATURE AND NUTRITION ON BIOMASS AND PHYCOBILIPROTEIN OF *DESERTIFILUM SALKALINEMA* FROM HOT WATER SPRING

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ABSTRACT

Cyanobacteria known for their remarkable adaptability in challenging environments serve as a vital form of the study. The exploration focuses on isolation of micro algal cultures from hi-temperature habitats. The hot spring of Tuva, Gujarat through rigorous morphological and molecular phylogenetic analysis, the isolated strain were identified as *Desertifilum Salkalinema*. The objective is to optimize the growth rate and conditions for enhancing the efficiency of phycobiliprotein (PBP) production exhibiting its maximal growth rate at 30 ⁰C when cultivated in BG11 medium. Notably, Nitrogen concentration experiments within the original BG11 media by increasing the sodium nitrate concentration exhibit both growth rate and PBP as accumulated in these strains. This finding highlights the nitrogen availability and temperature on both growth dynamics and phycobiliprotein. For enhancing the PBP extraction efficiency, the mechanism identified different extortion methods. Wherein the combined extraction protocol involving freeze- through cycles plus ultrasonication exhibited a marked increase in the efficiency of phycobilin protein extraction.

Finally, these observations hold significant implications for the biotechnological application of phycobilin protein, as C-PC are recognized for their antioxidant and antibacterial properties providing valuable potential of these cyanobacteria.

Keywords: Phycobiliprotein, Growth, *Desertifilum sp.,* Hot spring, Extraction mechanism

INTRODUCTION

Microalgae, a diverse group of photosynthetic microorganisms, exhibit adaptability to a broad spectrum of environments **[1]** and have been widely recognized as a cornerstone for bioeconomy and biorefinery development owing that high-applicability as feedstocks for food, feed, bioplastics, bio-fertilizers and bio-fuels as well as waste water treatment and CO_2 mitigation **[2, 3]**. Geothermal features like hot water springs (HWS) started gaining attention for their tendency to survive extreme environment of hot water spring consisting of 45-150ºC. Cyanobacteria, often referred to as blue green algae, have generated significant interest because of their remarkable ability to fix carbon, their rich nutritional contents, and their notable antioxidant attributes. Recently, a new taxon of the order Oscillatoriales was described from the extremely hot and dry Thar desert in north-western India: *Desertifilum tharense* DaDheech et Krientiz **[4]**. The thermophilic cyanobacteria found in yellow stone national park where the first organisms to be extensively studied in this context **[5]**. Although cyanobacteria from various thermal spring have been investigated, numerous such habitat remain unexplored **[6, 7]**. In India, their have been limited reports on the diversity of cyanobacteria in hot water spring, with few studies conducted in the western region of the country covering Bihar, Uttarakhand and West Bengal by Thomas and Gonzalves **[8]**.

Phycobiliproteins (PBPs) serve as primary light harvesting pigment in cyanobacteria. They exhibit natural fluorescence, attributed to the inclusion of covalently linked, linear tetrapyrrole chromophores known as bilins. C-PC is a water-soluble fluorescent pigment containing of two different subunits of polypeptide as a low molecular weight α unit (MW 12-19 kDa) and another large β unit (MW 14-21 kDa) **[9]** and are commonly present in balanced amount holding different class of protein structure consisting of red phycoerythrin (C-PE) and blue green allophycocyanin (C-APC) and C-PC possesses the capability to absorb visible light in the wavelength range of approximately 610-620 nm **[10, 11]**.

Phycocyanin (C-PC) has found diverse application as a natural dark blue dye, serving as a substitute for synthetic pigments in the food and cosmetic industry. It is also utilized as a fluorescent reagent in clinic and research laboratories. Moreover, its versatile properties. Including anticancer, neuroprotective, antioxidant, anti-inflammatory and antimicrobial attributes, make it a promising candidate for potential therapeutic use **[12-14]**. As a result, there is a growing demand for C-PC on a significant commercial scale.

Based on data from 2016, the food colorant market for C-PC was expected to be worth $13 billion, with an annual growth rate of over 6.5%. The market value of C-PC applications in the nutraceutical business has been estimated to range from $6 billion to $60 billion in previous years **[15]**. The primary obstacle to their use in various applications is their low production rate, followed by the purity of C-PC, which establishes an effective use pattern. However, commercialization remains a primary issue. For example, the cost per milligram of food-grade PC is approximately $0.12 USD. In the other hand the analytical grade may cost up to $15USD per milligram **[16]**.

The efficient extraction of C-PC from cyanobacteria accompanied by increased yield, is of utmost importance to accelerate its commercial viability. To achieve the highest possible yield of C-PC, it is crucial to optimize parameters that foster both substantial biomass production and enhanced CPC accumulation within the cell **[10]**. Serval methods and procedures have been employed to isolate C-PC from various cyanobacteria and microalgae, often involving the distribution of cell wall to release CPC into aqueous solution. Challenges in the extraction of CPC arise from the presence of multilayered cell walls and the presence of significant contaminations. However, the cultivation conditions, such as light, temperature and nutrition availability, directly influence the CPC contain within cyanobacteria **[17]**.

Nitrogen(N) is an essential ingredient that is mostly used for protein synthesis and cellular biomass formation. Several variables, including cell growth, biomass output, chlorophyll content, and C-PC accumulation, are directly affected by the concentration of N in the culture medium. The regulation of C-PC accumulation is significantly influenced by the presence of nitrogen **[18]**. And the wet cells with ultrasonication technique can assist phycobiliprotein extraction. Combination of ultrasonication with freezing and thawing resulted in the highest extraction efficiency of APC and C-PC from dried *A. plantensis* biomass **[19-20]**. Light intensity and wavelength significantly influence the constitution of phycobilisomes. It is feasible to significantly increase the C-PC productivity and energy efficiency by carefully adjusting lighting. Complementary chromatic adaptation (CCA) refers to variations in phycobiliprotein synthesis that occur in a cell in response to light. Adjusting the wavelength of light provides a precise means of promoting the accumulation of the targeted pigment in phycobiliprotein **[21]**.

The present study aims to characterize and evaluate the biotechnological potential of isolated species from hot water spring at Tuva, Gujarat. Here, the 16S ribosomal deoxyribonucleic acid (rDNA) sequence found is identical to that the *Desertifilum*. As the biotechnological use of these novel isolate has been formed with this study. However, it's composition in terms of phycobilin was evaluated determining the nutritional potential of this microalgal a new species of the genus *Desertifilum* occurring in hot water spring of Tuva, Gujarat.

METHODOLOGICAL APPROACH

Sampling and Culturing

The cyanobacterium dealt with this study was collected from hot water spring of Tuva, Gujarat during October, 2022. The sampling site is marked with the coordinates: 0º18'23" N, 36º04'43" E. The Physico-chemical properties of the spring water is formed as temperature 60º C, pH-7.86, conductivity-2.7 mS, salinity – 3.4 ppt. Sample collected were précised with three divisions, (i) fixed samples (formaldehyde, final concentration 2.5%), and (ii) fresh samples stored in the water collected from the sampling site, (iii) In media (BG11).

Isolation and Identification

Cyanobacterial species collected as biological mats from the hot spring where repeatedly washed by double distilled water and placed in petri plates and flasks contending BG-11 medium. Water samples were serially diluted (10^1-10^5) with sterile distilled water, and all dilutions were streaked on 2% BG-11 over agar plates. The plated sample were incubated for a period of three week, maintaining a light intensity of 16 hour of light followed by 8 hours of darkness, at a temperature of 25±1º C. After the incubation period, colonies displaying distinct morphologies were carefully selected and subjected to purification using a repeated striking method on fresh BG-11 agar plates, under the same incubation condition **[22]**. For detailed morphological characterization, pure cultures from these colonies were serially diluted and examined under a microscope with a dry field (Nikon ECLIPSE Ci-S) at magnification of 50X. The isolated species were then kept at 4ºC in BG-11 agar slants. Morphological identification of the isolated species was the next step **[23]**.

Molecular analysis of cyanobacterial isolates

The genomic DNA from all the isolates was extracted using the plant genomic kit (Quigen). Subsequently, the isolated DNA was amplified using the 16S rRNA gene. The PCR product of the amplified marker were purified from the gel using a gel extraction kit. To identify the sequences, a comparison was made with the NCBI GenBank database using BLASTn (http://www.ncbi.nlm.nch.gov). Any sequences corresponding to uncultured organism were excluded. The sequence of the marker genes obtained in this study were submitted and deposited in GenBank for reference.

Phylogenetic analysis

To construct the phylogenetic tree, sequences from this study, along with sequences from another genus, where obtained from the NCBI GenBank(http://www.ncbi.nlm.nch.gov).

The phylogenetic tree was constructed using the maximum likelihood method in Mega 11.0 software. To assess homology, the 16S ribosomal RNA reference Gen sequences were analyzed using the BLASTn search program. Spices that exhibit closely related identity (ranging from 97-100 %) were considered to be the closest matches.

Evolution of media, temperature, and Nutrients (Nitrogen) on growth pattern and phycobiliproteins

Towards evaluating the effect of different temperatures on cyanobacterial species, the cultures were incubated at different temperatures of 25, 30, 35 and 40 ⁰C whereas; in the light intensity trial, cultures were exposed to the temperature of 30 ±1 ⁰C. (optimum values obtained through varying temperature experiment). In order to optimized the appropriate culture medium for the selected isolates, they were subjected to three different media with varying nutrient compositions, as (1) Bold basal medium (BBM) (2) BG-11 medium (BGM) (3) For medium. The growth medium was prepared based on their compositions, transferred into 100 mL conical flasks and sterilized at 121 ⁰C for 15 lbs. The selected isolates were inoculated in these three media and later used for laboratory proceedings. Other, experiments were performed using sodium nitrate ($NaNO_3$) as nitrogen source with different concentrations (1.5, 2.5, 3.5 and 4.5 g L^{-1}) and after determining the optimum concentration of $NaNO_3$, the inoculated cultures were gently shaken to accelerate the algal growth. All the tests were carried out in triplicate.

Growth Analysis: The growth of the algal biomass was assessed by means of optical density with two days intervals at 570 nm **[24]** using UV-Vis Spectrophotometer (AnalyticJena-Specord 200/Plus).

Evolution of extraction method for cyanobacterial phycocyanin

50 mg of wet biomass mixed with 15 ml of Na-phosphate buffer (100 – mM, pH 7.0), and soaked for 60 min **[25]**. Then the mixture was distributed using the following methods,

Freeze and thaw combined with ultrasonication

The wet biomass suspended in extraction solution was sonicated for 10 min at 30 kHz, then suspension was subjected to freezing at -20 ⁰C, for 1 h and thawing at 37 ⁰C for 1 h cycle.

Measurement of Phycobiliprotein content

To analyze the PBP in the evaluation of temperature, light and nutrient experiments, the culture was centrifuged and later washed with distilled or deionized dH_2O. A wet biomass of cyanobacteria (100 mg) was emersed in 20 ml of phosphate buffer (pH 7.0; 0.1 M) as part of the extraction process following extraction, the mixture was centrifuging at 6000 rpm for 15 minutes after which the supernatants were collected. The absorbance was measured at various wavelength, including 562 nm, 615 nm, 162 nm and 652 nm using phosphate buffer and water as blank. The quantities of phycocyanin (PC), Phycoerythrin (PE) and allophycocyanin (APC) in the samples were then calculated **[26]**. The total amounts of phycocyanin, Phycoerythrin, allophycocyanin were determined using the details.

$$C - PC\ (mg\ mL^{-1}) = \frac{\{A_{615} - (0.479)A_{652}\}}{5.34}; \dots (1)$$

$$C - APC\ (mg\ mL^{-1}) = \frac{\{A_{652} - (0.208\ A_{615})\}}{5.09} \dots\dots.\dots\dots.(2)$$

$$C - PE\ (mg\ mL^{-1}) = \frac{\{A_{652} - (2.41\ C - PC) - (0.809\ C - APC)\}}{9.62} \dots\dots.(3)$$

Total PBP = (C-PC) + (C-APC) + (C-PE) ….(4)

RESULTS AND DISCUSSION

Strain Characterization

The isolates were collected from hot water spring samples collected from Tuva, Gujarat, India. When cyanobacteria where examine under the microscope for morphologically, it was discovered that the isolates belong to the genes D*esertifilum.* The filaments cells of this isolates ranged in between 4 and 7 m and were identified strain or slightly wavy, motile and unbranched. Additionally, 16 rRNA gene sequences were used for molecular identification, and the resultant sequence was checked against other sequences in the NCBI database using the BLASTn program. With a sequence similarity of 99.99% in the database, the BLAST analysis of the matched sequence revealed that the isolates is closely related to *Desertifilum Salkalinema* BI6852. The sequence was uploaded to GenBank and NCBI (NR177676.1).

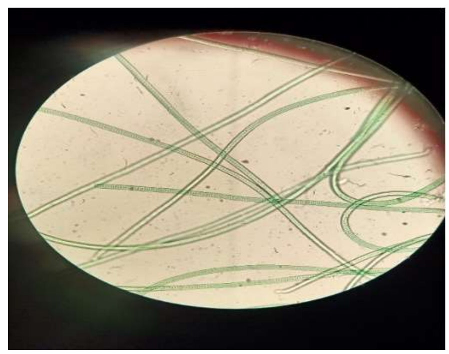

Figure 1: Photomicrographs of *Desertifilum Salkalinema*

Influence of media and temperature

An examination has conducted aiming the effect of temperature and media composition on growth factors within the isolated strains while determining the best culture conditions. The strains' remarkable adaptability was made clear encompassing the temperature range between 25 ºC and 40 ºC and the three different including BG-11, Fog and BBM. Significant oscillations in growth rates C-PC and PBP contents. The intricate orchestration of these parameters was unveiled through meticulous tracking of growth rates dynamics across the diverse temperature, nitrogen and media conditions, culminating in comprehensive visual representations. Simultaneously, the rhythmic patterns of growth rates and phycobiliprotein (PBP) content were observed. The strain *Desertifilum Sp.* showed the maximal growth rate of BG-11 media at 30 ºC, C-PC and PBP and appropriately there is no significant difference ($p > 0.05$) between the growth at 30 ºC and 35 ºC.

Effect of Nitrogen

Experiments were conducted using a medium with an initial $NaNO_3$ concentration of 1.5 g L as a control, aiming to examine how variations in nitrogen source concentration impact both cell growth and accumulation of C-PC within the isolated strain. **Figure 1** shows the results in relation to cell growth and different $NaNO_3$ concentrations in C-PC. 1 the results show a positive relation between cell growth, C-PC content and $NaNO_3$ concentration, with the subsequent as with the increase in $NaNO_3$ concentration from 1.5 to 3.5 g L1. The subsequent increase in $NaNO_3$ concentration to 4.5 g L1 resulted in negative impact in cell development. However, the highest results for cell growth rate were 0.88 at 2.5 g/l and the lowest were 0.64 at 4.5 g/l. Interestingly, the total phycobiliprotein content in the standard BG-11 medium was measured as 23.61%. Remarkably, when the medium was enriched with the nitrogen source with 3.5 g/l the content of total PBP was found as 25.47%. Furthermore, when the nitrogen source increased with 4 mg/ml then the growth rate, CPC and PBP were found decreasing.

Efficiency of cell disruption method

In this study, two cell disruption were applied using wet and oven-dried biomass and their efficiency for C-PC extraction using NA-phosphate buffer was assessed. By monitoring the amount of released C-PC, the impact of various cell disruption techniques on C-PC extraction was examined bearing the yield of C-PC and extracted as a function of cell disruption methods. The concentrations of C-PC and PBP varied significantly when different extraction methods were employed. From the array of cell disruption methods and extraction solutions investigated in this study, the combination of freeze and thaw cycles (-20 ºC, 1h, four cycle), along with ultrasonication (5 min) for both wet and dry biomass proved to be highly effective in extracting C-PC. Among theses methods, the maximum yield of C-PC was observed with dry biomass, followed by wet biomass when utilizing freeze-thaw plus ultrasonication.

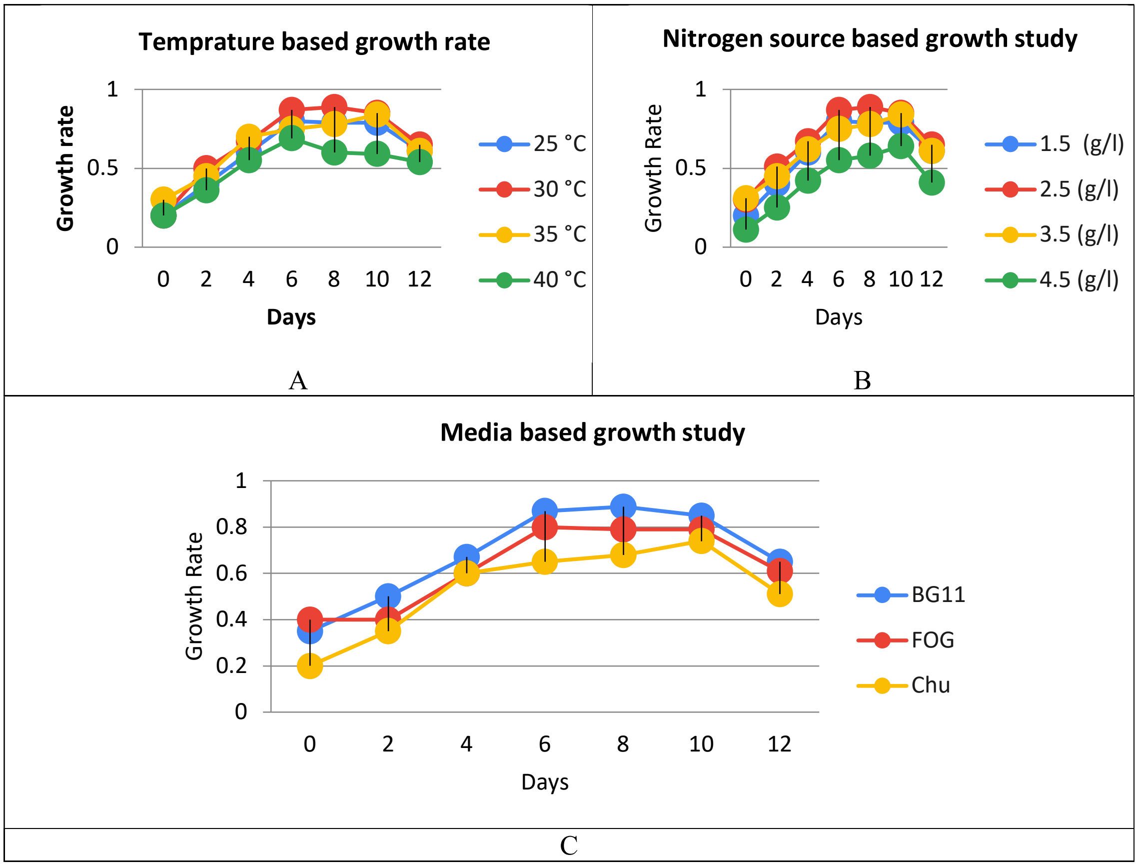

Figure 2: The growth response of *Desertifilum Salkalinema* to different (A) Temperatures, (B) Nitrogen (C) Media

The outcome of present study suggested the need for further and more comprehensive examinations of the tested cyanobacterial strains as well as the influence of other environmental stress on their pigment composition.

Based on the 16s RNA gene sequencing, *Desertifilum Salkalinema* was recognized as the cyanobacteria isolate. Previously, the 16S rRNA gene sequence was used to isolate the *D. Salkalinema* strain from the alkaline water **[23]**. The taxonomic analysis based on molecular phylogenic relationship closely links this cyanobacterial strain to *Desertifilum*. It was first discovered in extremely hot and arid Thar Desert in India and in a warm spring in East Africa, where it forms crusts and biofilms. During the process additional analysis was also conducted using other primer such as ITS and rbcL which discriminates the isolated strain for identification of the strain of the ITS **[27]**.

PBPs composition is affected by a number of environmental conditions, and modifications to this structure may result from nutrient availability **[29]**. It is well known that phycobiliprotein have evolved inti molecules that serve as secondary sources of intracellular nitrogen storage **[29, 30]**. The pigments content displayed significant variation, primarily depending on the strains under examination and the specific growth condition. Both qualitative and quantitative aspects content of total and individual phycobilin differed, clearly indicating the presence of distinct characteristics in the pigment composition of each strain **[31]**.

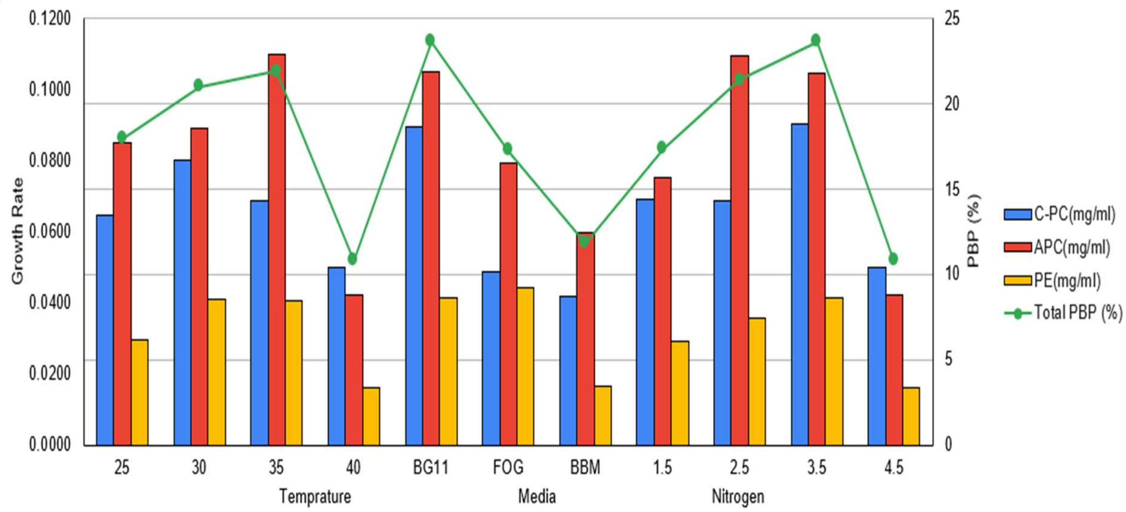

Figure 3: Phycobiliprotein content Phycocyanin (PC), Allophycocyanin (APC) and Phycoerythrin (PE) and total Phycobiliprotein content

Every strain under study holds varying qualitative and quantitative phycobiliprotein contents depending on the type of nutrient supply and environmental factors, according to the study that examined the content of these proteins while on other hand, it might be of great importance for the use of biotechnological strains in the creation of particular types of pigments. Changes in growth conditions can exert an impact on the pigment content within the tested strains. Optimal conditions that result in high phycobiliproteins levels can be achieved by combining appropriate factors. However, it is found feasible that using other factors will also enhance the percentage of phycobiliprotein, necessitating the continued research. Turbidity was used to monitor the growth rates of cyanobacteria cultures when efforts were onto optimizing the culture conditions, including the use of various media, temperatures and sources of nitrogen. Consequently, for particular cultivation circumstances, the amount of chlorophyll can be unrendered substitute to measure quantity of biomass **[32]**. Within *Desertifilum,* the accumulation of Chl-a is influenced by factor such as temperature, light, nitrogen and iron source during the late exponential phase (18-day-old cultures), as has been demonstrated in prior studied of cyanobacteria **[18]**. The growth of cyanobacteria was reliant on suitable supply of important macronutrients as nitrogen, iron and phosphorus. But an excessive amount can be unfavorable to cell metabolism. The present study's chosen cyanobacteria's growth may have been slowed by the commercial media's high nutrition concentration. On the other hand, the main nutrient needed for growth of microalgae is nitrogen.

One of the key factors that significantly impact the cyanobacteria cell development and metabolic processes is temperature **[27]**. Both lower and higher temperature have detrimental effects on cellular metabolic processes, biochemical compositions and PBP production. Among the three main pigments of phycobiliprotein, phycocyanin is the most valuable natural blue pigment used in food and pharmaceutical industry owing to their color, fluorescence and antioxidant properties **[32]**. Similar to many other metabolic processes, PBP production in cyanobacteria is regulated by temperature, and the optimal temperature for maximum PBP production varies depending on the strain **[33]**. According to existing literature, temperatures between 25ºC to 36ºC have been identified as optimal for PBP production in cyanobacteria **[34, 35]**. In a study of *Desertifilum sp.* temperature of 28ºC and 32 ºC were found to be suitable for growth and PBP accumulation **[18]**.

Depending on the cyanobacteria species, the biomass, buffer and cell disruption method along with the PBP production might vary in terms of efficiency, quantity and purity. Besides the aforementioned cell disruption method, mechanical methods are widely used for pigment extraction from microalgae, both at pilot-scale and commercial levels. A cooling system appears essential when extracting the heat-sensitive items since the heat generated during the mechanical disturbance can harm the finished products. The installation and operation of a cooling system will incur equipment and energy expenditures, which will increase the process cost **[36]**. An alternative technique is Ultrasound-assisted extraction, which avoid some of the challenges associated with the conventional mechanical disruption methods. The process is straightforward and easy to set-up, resulting in higher purity to the final product **[18]**.

In this study for the cell disruption method, ultrasonication plus freezing and thawing methods were assessed because ultrasonication, freezing and thawing individually cannot obtain phycobilin protein. On the other hand, the number of cycles required to extract the essential components that improve cell rupture in both wet and oven-dried biomasses. While wet biomass was also shown to give greater results, employing dried biomass led to 50% decrease in the C-PC content **[37]**. Ultimately, it can be concluded that because wet biomass does not lose pigments, it is a better option for C-PC extraction than dried biomass.

CONCLUSION

The present study provides one cyanobacteria and which has been confirmed by 16srRNA gene sequence and found determined as identical to that of *Desertifilum Salkalinema* confirming its phylogenetic position within the genus *Desertifilum* a prominent genus of *Desertifilum Salkalinema.* This strain was explored first time in India and the study on this strain to production and the source of phycobiliprotein content.

Media and cultured conditions directly affect growth and C-PC content. The optimal conditions for achieving higher biomass growth and enhanced phycocyanin production were found to be temperature of 30 ºC and twice the strength concentration of sodium nitrate in the original BG-11 media. Additionally, the combination of freeze-thaw and ultrasonication extraction methods demonstrated promising extraction efficiency for recovering phycocyanin compared with other methods. Cultivation of these cyanobacteria offers promising prospects for the development of microalgae products. Further research is needed to explore the antioxidant, antimicrobial and anticancer potential of phycocyanin and phycobiliprotein content.

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