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Research Article

ISOLATION AND IDENTIFICATION OF CYANOBACTERIA FROM DAIRY EFFLUENT FOR REMOVAL OF NUTRIENT WITH EXTRACTION OF HIGH VALUE COMPOUNDS FROM BIOMASS

KABARIYA J.H.[1] AND RAMANI V.M.*[2]

Department of Dairy Microbiology, College of Dairy Science, Kamdhenu University, Amreli, 365601, Gujarat, India
*Corresponding Author: Email-vimalmramani@gmail.com

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Abstract- Dairy is one of the major industries causing water pollution with its large water consumption. In present study cyanobacterial species were isolated from dairy industries effluent and explored for removal of nutrients from dairy effluent simultaneously biomass produced again used for extraction of high value compounds. The percentage of nitrogen (N) and phosphate (P) reduction was 78.12%, 88.91%, 78.66% and 87.33%, 79.68%, 78.57% by *spirulina, oscillatoria* species and its consortium, respectively after 18 days. In case of high value compounds maximum amount of C-phycocyanin 0.431 mg/L and phycoerythrin 0.165 mg/L was extracted from *oscillatoria* species. The purification of the crude extract done by ammonium sulfate fractionation and Sephadex-G50 size exclusion chromatography. The lipid content from *oscillatoria* species was about 125.65 mg/l^{-1} which can be used for biofuel production.

Keywords- Dairy effluent treatment, Cyanobacteria, C-phycocyanin, Phycoerythrin and Lipid content

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Introduction

Dairy industry is one of the major food industries in India. India ranks first for the maximum milk producing nation. Water pollution from dairy effluent has been major problem of environmental concern in countries around the world. This industry generates large volume of effluent with its high nutrient characteristics, that's why dairy industrial effluent is more polluting. In dairy industries approximately two to three times greater amount of effluent generates for the processing of each liter of milk [1]. The dairy industry on an average has been reported to generate three to five liters of waste water per liter of the milk processed. It is estimated that about 2% of the total milk processed is wasted into drains. Due to the high pollution load of dairy effluent some of the milk-processing industries discharging untreated/partially treated effluent which can cause serious environmental problems like water and soil pollution. Dairy industries generate approximately 50 m^3 effluent daily with considerable concentration of organic matter (fat, protein and carbohydrates) and nutrients mainly (nitrogen and phosphorous) originating from the milk and the milk products. High amount of organic and inorganic compound in dairy effluent can produce excess growth of algae, eutrophication and also effect on biodiversity that cause decreased water quality level that may affect human and animal health. In recent years, environmentalists and government are looking for efficient, economic and long-lasting solutions for dairy effluent treatment and recycling. The traditional method of dairy effluent treatment is available but it is unavoidably, costly, time consuming process and it cannot be affordable in all industries especially in developing countries like India. In dairy effluent plant (ETP) involves high energy cost by mechanical aeration to provide oxygen to microorganism to degrade the organic matter in effluent. Dairy effluent treatment process produced large amount of sludge and it must be remove. The removal of this sludge is usually the more cost component in process of dairy effluent treatment. One kWh of electricity requires for aeration to remove approximately one kg of BOD in activated sludge process, which generate one kg fossil CO_2 for one kWh of electricity production [2]. By contrast, one kg of BOD removed by photosynthetic oxygenation requires no energy inputs and produces enough algal biomass to generate methane that can produce one kWh of electric power [3]. Hence in recent years, the importance of biological treatment systems has attracted the attention of investigators in all over the world and it help to develop the efficient and low-cost dairy effluent treatment systems. Low-cost dairy effluent treatment methods are prime need for the developing countries. Dairy effluent contains sufficient nutrients for cyanobacterial growth. The role of cyanobacteria in the removal of various kinds of organic, inorganic and other related substances has been studied by several workers during the last several years. Nutrient removal in particular nitrogen and phosphorus from dairy effluent is a growing regulatory need and the use of cyanobacteria cultivation could create a unique combination between dairy effluent treatment and from produced cyanobacterial biomass extract high value compounds. The present study aims to evolve effective and economic biological treatment method for dairy effluent using cyanobacteria and to produce its biomass for their economical applications. In this study we have isolates the cyanobacteria from dairy effluent plant and dairy industry which having different product production range like, ice cream, paneer, ghee, cheese production and packaged milk. In next step isolated cyanobacteria were used for dairy effluent treatment by removal of nutrients and separate the generated biomass for the extraction of high value compounds. From such cyanobacterial biomass, some of the important high value compounds such as biofuel, antioxidant compounds, pigments, nutraceuticals like omega 3, carotenoids, astaxanthin, single cell protein (*spirulina*). The antioxidant compounds such as phycobiliproteins, C-Phycocyanin (C-PC) and Phycoerythrin (PE) have high economic value like, $110-500/mg, $1050/mg and $30-724/mg, respectively [4].

Material and Method

Collection of dairy effluent sample

Treated and untreated dairy effluent sample collection was carried out from area

of Anand district, Gujarat. The dairy industry which having different product production range like, ice cream, paneer, ghee, cheese production and packaged milk. Which also have slight different environmental condition and dairy product range compare to other region of Gujarat and dairy industry. So, chance to availability of diverse cyanobacterial species is high. The dairy effluent sample collection was taken from the effluent entry point (untreated-primarily tank) and at final discharge tank (treated-secondary clarifier) of effluent treatment plant. The sample collection was carried out in sterilized glass and plastic sampling bottle and after collection of sample it reach to laboratory within 4-6 h at 4 ^{o}C.

Isolation of cyanobacteria from dairy effluent

Isolation of cyanobacteria from dairy effluent sample was carried out using modified method [5]. First shake the dairy effluent sample and allow to sediment suspended particle, repeat this process for three time to removed suspended particle and then add 100 ml sterile distilled water and filtered it by filter. Then filter was place on BG-11 (Blue Green-11, Hi-Media, India) medium containing petri plate. Incubate petri plate with filter paper for 15 days under continuous lighting (2000 lux) 14:10 h light: dark system at 28 ±2 ^{0}C. After incubation period different species of cyanobacteria were grow in medium. Aseptically each species was picked and sub-cultured in 500 mL BG-11 medium for growth of organism and incubated under same condition. Isolated cyanobacterial species was purified then identified and check the capacity of nutrient removal from dairy effluent and extract the high value compound from produced biomass.

Purification of cyanobacterial culture

Purification of cyanobacteria was carried out according to work done by Elango, *et al*., 2008 [6]. 100 mL of tap water and distilled water was taken and checked the pH separately before and after sterilization. 1 mL of cyanobacterial culture as an inoculum was added into the sterilized water which used as a medium for the removal of bacteria, planktons and other contaminations. Then it was incubated at 27 ± 2 ^{o}C with light intensity of 2000 lux in 14/10 h light and dark cycle. After 5 days of incubation examine the cyanobacterial culture under microscope for contamination. Then remove the contaminating dead cells which adsorbed on cyanobacterial species by washing the culture with sterilized double distilled water. Sub-culturing was carried out in the same medium which helps to obtain good effective results. The purity of the culture was checked under light microscope.

Morphological identification of cyanobacteria

Identification of cyanobacteria was carried out using a trinocular microscope (Nikon Eclipse-N*i*) with NikonDS-Fi2 camera. Identification was carried out up to genera level by compare with Algae Identification lab guide Agriculture and Agri-Food Canada Agri-Environment Services [7].

Physico-chemical characterization of dairy effluent

Physico-chemical characterization of dairy effluent was carried out by using standard method [8,9]. The parameters include, pH, temperature, colour, dissolve oxygen(DO), biochemical oxygen demand(BOD), chemical oxygen demand(COD), chloride, sulphate, nitrate and phosphorus were measured according to standard method. These methods were checked the amount of nutrient content before and after inoculation of cyanobacterial culture into dairy effluent

Inoculation of cyanobacterial culture into dairy effluent

Spirulina species, *Oscillatoria* species and its consortium were used as inoculum for dairy effluent treatment. 10% cyanobacterial cultures were used as an inoculum for growth in Erlenmeyer flask (500 ml) containing 250 ml of untreated and treated dairy effluent. The culture was incubated at 27 ^{o}C ± 3 ^{o}C temperature for 18 days and measured physico-chemical analysis at interval of every 6 days [10].

Separation of cyanobacterial biomass for dairy effluent

Take the 250ml of cyanobacterial treated dairy effluent and centrifuge it at 10,000 rpm for 10 minutes, take the supernatant solution for physico-chemical analysis and cyanobacterial biomass which settle down as a pellet. Use this biomass for further extraction of high value compounds.

Extraction of phycobiliproteins from cyanobacterial biomass

The extraction of phycobiliproteins from cyanobacterial biomass of s*pirulina* species, *oscillatoria* species and its consortium were carried out by freezing and thawing method [11]. Cyanobacterial culture was rinsed two times with double distilled water and lyophilized (ANM FD-307K) it with sucrose which was used as a cryoprotectant. 1 gm of lyophilized biomass was added into 100 mL of 0.1 M sodium phosphate buffer (pH 7.0) which contain 1 mM sodium azide. Then this solution was sonicated (LABMAN PRO-SONICATOR-500) for 60 to 80 seconds and freeze it at -20 ^{o}C then it was thawing at room temperature in the dark condition. This procedure was repeated for three time. The mixture was centrifuge at 10,000 rpm for 30 minutes at 4 ^{o}C and collect the clear supernatant containing phycobiliproteins.

Estimation of phycobiliproteins

The amount of phycobiliproteins present in clear supernatant was measure by UV-Visible spectrophotometer (ELICO SL-244) at wavelengths 565, 620 and 650nm for the calculating the concentration of C-phycocyanin and phycoerythrin by using following equations:

$$Phycoerythrin\ (PE)\ (mg/ml) = (0.123A_{565} - 0.068A_{620}) + 0.015A_{650}$$

$$C\text{-}Phycocyanin(C\text{-}PC)\ (mg/ml) = (0.162A_{620} - 0.001A_{565}) - 0.098A_{650}$$

Extraction of lipid from cyanobacterial biomass

The cyanobacterial biomass of s*pirulina* species, *oscillatoria* species and its consortium were separated by centrifuge at 10000 rpm for 10 minutes. Supernatant was discarded and freeze the biomass at -20 ^{o}C for overnight and then it was freeze dried by vacuum freeze dryer (ANM FD-307K). The next step of the experiment was cell disruption by ultrasound system. Take 1gm of freeze dried cyanobacterial biomass in 50ml of double distilled water and mix it vigorously. Then this mixture was sonicating (LABMAN PRO-SONICATOR-500) at 45 amplitudes for five minutes. After this, mixture was used for the extraction of lipid by Bligh and Dyer (1959) [12]. The sonicated mixture was transfer into separating funnel and add mixture of chloroform: methanol (2:1 v/v), mix it vigorously for 10-15 minutes. Top milk layer was removed from separating funnel and left it for overnight for the solvent evaporations. Then measured the weight of extracted crude lipid from cyanobacterial biomass.

Result and Discussion

Collection and isolation of cyanobacteria from dairy effluent sample

Collection of dairy effluent sample was carried out from dairy industries around the Anand district, Gujarat. Four dairy effluent samples were collected from dairy industry, two sample of untreated effluent and two sample of treated dairy effluent. Total four cyanobacterial species were isolated using BG11 medium from dairy industries effluent around the Anand district. These two species were used to preliminary study of dairy effluent treatment by cyanobacteria and extraction of high value compounds from generated cyanobacterial biomass.

Morphological identification of cyanobacteria

The morphological identification was carried out by using trinocular microscope (Nikon Eclipse-N*i*) with NikonDS-Fi2 camera attachment. Cyanobacterial species were identified on the basis of morphological characteristics up to genus level. The identified species of cyanobacteria was *spirulina* and *oscillatoria* genus which shows in [Fig-1]. The *oscillatoria* species with filamentous, unseriated, unbranched trichomes and its name *oscillatoria* on its oscillation movement while *spirulina* has distinctive morphology, the degree of spiralization and the arrangement of the spirals. In this study we have used two species of cyanobacteria, among two species one was *spirulina* and second one was *oscillatoria* species. Pathade K. N.(2012), [13] reported that the *oscillatoria* genus present dominantly in dairy effluent. Our result is at par to this.

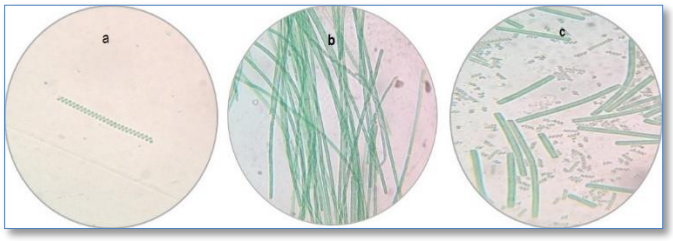


Fig-1 Isolated species of cyanobacteria from dairy effluent a) *Spirulina spp.* b) *Oscillatoria spp.* and c) consortium

Table-1 *Physico-Chemical Parameters of Diary effluents*

Sr. No.	Parameters	Untreated Dairy effluent (mg/L)	Treated Dairy effluent (mg/L)
1	Colour	off white	Transparent
2	Temperature	28.1	28.5
3	pH	9.21	8.85
4	DO	1.2	5.5
5	BOD	166.11	10.08
6	COD	96	16
7	Phosphate	1.5	0.55
8	Nitrogen	4.48	2.24
9	Chloride	99.96	29.99
10	Sulphate	19.0	2.90

Characteristics of dairy effluent

The analysis of physico-chemical characteristic of treated and untreated dairy effluent were carried out for the comparison of traditional method with our cyanobacterial based dairy effluent treatment and checked their efficiency. Various parameters such as pH, temperature, color, DO, BOD, COD, phosphate, nitrogen, chloride and sulphate were measured which shows the difference between treated and untreated dairy effluent shown in [Table-1]. The enough amount of nutrient was present in untreated dairy effluent to support cyanobacterial growth and it also considered as advantageous for pigment, lipid and another metabolites production. While in treated dairy effluent, the nutrient level was quit less compare to untreated dairy effluent. The treated dairy effluent nutrient data was used to compare the efficiency of reduced amount of nutrient by traditional and cyanobacteria based dairy effluent treatment. Munawar M. (1970a) and Munawar M. (1970b) [14,15] suggested that nutrient rich dairy effluent especially calcium, nitrogen, phosphorus and other nutrient enhance the flourishing growth of blue green algae. Uslu, *et al*., (2011) [16] found that the amount of nitrogen was critical factor for the lipid and other metabolites production in cyanobacterial cell.

Nutrient removal from dairy effluent treatment by cyanobacterial species and its consortium

The higher amount of nutrients such as nitrogen and phosphate were present in dairy effluent. The experiment of cyanobacteria based dairy effluent treatment and simultaneously removal of nutrients was carried out for the 18 days with untreated dairy effluent. In this experiment, checked the presence of initial concentrations of total nutrients and examined the concentration of nutrient at the interval of every 6 day after inoculation of *spirulina* and *oscillatoria* species and its consortium in untreated dairy effluent shown in [Table-2-4]. After 18 days of experiment, cyanobacteria reduced the overall nutrients concentration upto 87% of untreated dairy effluent. At zero day the amount of nitrogen (N) was 4.48 mg/L and it reduced to 0.98 mg/L, 0.91 mg/L and 0.96 mg/L after 18 days [Fig-2] by *spirulina*, *oscillatoria* species and its consortium, respectively. The overall nitrogen reduction percentage was 78.12%, 88.91% and 78.57% by *spirulina*, *oscillatoria* species and its consortium, respectively. The initial phosphate (P) concentration was 1.5 mg/L was reduced to 0.19 mg/L, 0.21 mg/L and 0.20 mg/L after 18 days by *spirulina*, *oscillatoria* species and its consortium, respectively. The overall phosphate reduction percentage was 87.33%, 79.68% and 86.66% by *spirulina*, *oscillatoria* species and its consortium, respectively [Fig-2].

The overall growth of the cyanobacterial biomass was 99%, 99.80% and 99.26% *spirulina*, *oscillatoria* species and its consortium, respectively [Fig-3]. Similar or higher nutrient removal efficiencies were observed by other researchers for algae-based treatment. The dissolve oxygen of dairy effluent after 18 days of treatment was increase overall 93%, which support the aquatic life. [10] reported that the algae removed 96% of nutrient present in the municipal and dairy effluent. Our cyanobacterial species efficiency to reduce nutrient is affirmation to this.

Table-2 Dairy effluent treatment by cyanobacterial *spirulina* species with reduction of nutrient present in dairy effluent

Sr. No.	Parameters	Untreated dairy effluent Zero Day mg/L	6th Day mg/L	12th Day mg/L	18th Day mg/L	Overall result after 18 days (%)
1	Colour	off white	slight off white	transparent	transparent	-
2	Temperature (°C)	28.1	28.6	28.8	29.3	-
3	pH	9.21	9.2	8.25	7.91	85.88
4	DO	1.01	1.9	3.1	4.2	+83.16
5	BOD	166.11	28.45	14.21	3.98	97.6
6	COD	96	23.21	18.86	6.97	92.73
7	Phosphate	1.5	0.65	0.33	0.19	87.33
8	Nitrogen	4.48	2.76	1.89	0.98	78.12
9	Chloride	99.96	38.98	17.53	12.55	87.44
10	Sulphate	19.05	11.04	6.12	3.01	84.19
11	Biomass (gm/100mL)	2.5	7.99	12.75	14.85	+99.00

Table-3 *Dairy effluent treatment by cyanobacterial oscillatoria species with reduction of nutrient present in dairy effluent*

Sr. No.	Parameters	Untreated dairy effluent Zero Day mg/L	6th Day mg/L	12th Day mg/L	18th Day mg/L	Overall result after 18 days (%)
1	Colour	off white	slight off white	transparent	transparent	-
2	Temperature (°C)	28.1	28.5	28.8	29.2	-
3	pH	9.21	9.02	8.56	8.04	87.29
4	DO	1.01	1.78	3.14	4.01	+99.25
5	BOD	166.11	25.12	13.01	3.76	92.28
6	COD	96	29.1	21.07	7.41	86.00
7	Phosphate	1.5	0.77	0.41	0.21	79.68
8	Nitrogen	4.48	3.11	1.72	0.91	88.91
9	Chloride	99.96	41.17	18.51	11.08	83.14
10	Sulphate	19.05	13.41	7.3	3.21	97.73
11	Biomass (gm/100mL)	2.5	7.64	12.56	14.97	+99.80

Table-4 *Dairy effluent treatment by consortium of cyanobacterial species (spirulina & oscillatoria) with reduction of nutrient present in dairy effluent*

Sr. No.	Parameters	Untreated dairy effluent Zero Day mg/L	6th Day mg/L	12th Day mg/L	18th Day mg/L	Overall result after 18 days (%)
1	Colour	off white	slight off white	transparent	transparent	-
2	Temperature (°C)	28.1	28.5	28.8	29.2	-
3	pH	9.21	9.1	8.41	7.93	86.1
4	DO	1.01	1.84	3.16	4.01	+99.25
5	BOD	166.11	26.88	13.93	3.81	97.70
6	COD	96	25.93	20.06	7.17	92.53
7	Phosphate	1.5	0.71	0.37	0.2	86.66
8	Nitrogen	4.48	2.85	1.71	0.96	78.57
9	Chloride	99.96	39.12	17.1	11.01	88.98
10	Sulphate	19.05	12.34	6.74	3.12	83.62
11	Biomass (gm/100mL)	2.5	7.89	12.65	14.89	+99.26

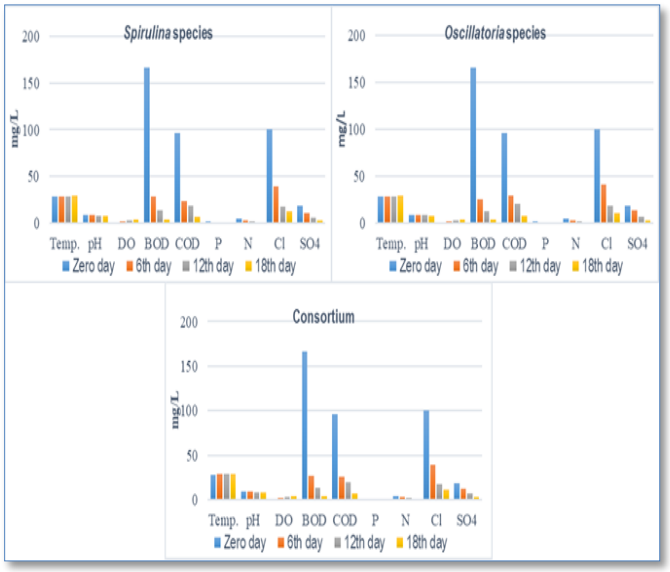


Fig-2 Physico-chemical analysis of Dairy effluent before and after inoculation of *oscillatoria*, spirulina species and its consortium

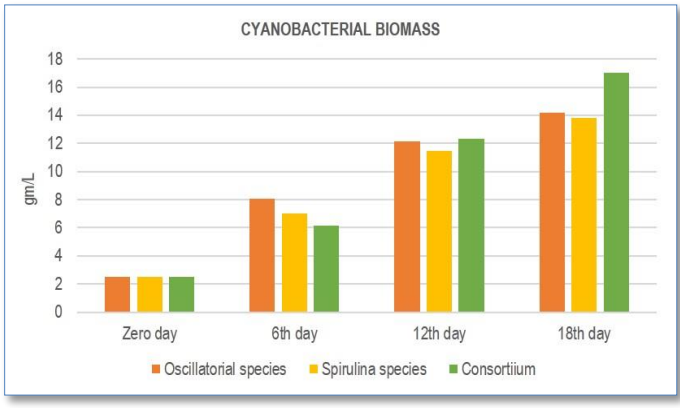


Fig-3 Cyanobacterial biomass produced after 18 days of dairy effluent treatment

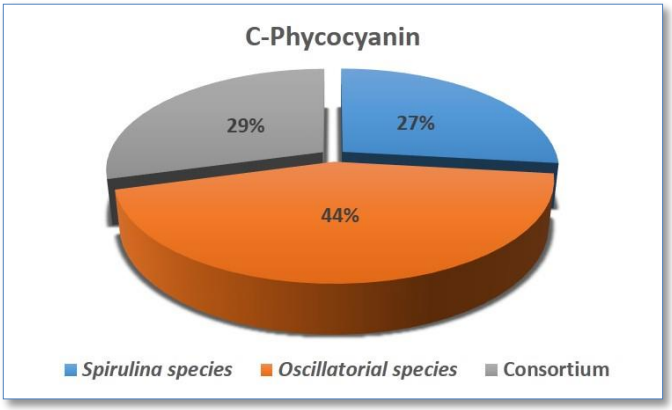
Extraction of high value compound from cyanobacterial biomass

The amount of biomass production after dairy effluent treatment by *spirulina*, *oscillatoria* species and its consortium was rise upto 99.00%, 99.80% and 99.26%, respectively. [Table-5] & [Fig-4] shows that the extraction of phycobiliproteins (C-PC and PE) and its quantitative measurement, the amount produced by *spirulina*, *oscillatoria* species and its consortium.

[Fig-5] shows the absorption spectrum pattern of the crude extract from the *oscillatoria* and spirulina species contain mainly C-PC (with absorption maxima at 620 nm). Among *spirulina*, *oscillatoria* species and its consortium, *oscillatoria* species contain maximum amount i.e. 0.431 mg/L of C-PC, while *spirulina* species and its consortium contain 0.263 mg/L, and 0.287 mg/L, respectively. In case of PE, *oscillatoria* species contain maximum amount i.e. 0.165 mg/L, while *spirulina* species and its consortium contain 0.064 mg/L and 0.109 mg/L, respectively. Based on the results *oscillatoria* species contain maximum quantity of C-PC compare to *spirulina* species and its consortium, similarly same results also in case of PE. [11] extract the phycobiliproteins from three different cyanobacterial species with almost similar amount of phycobiliproteins, our result is at par with this.

Table-5 *Quantitative measurement of antioxidant phycobiliproteins by UV-Visible spectrophotometer in four cyanobacterial species and its consortium*

Sr. No.	Name of phycobiliproteins	*Spirulina* species mg/L	*Oscillatoria* species mg/L	Consortium species mg/L
1	C-phycocyanin (C-PC)	0.263	0.431	0.287
2	Phycoerythrin (PE)	0.064	0.165	0.109



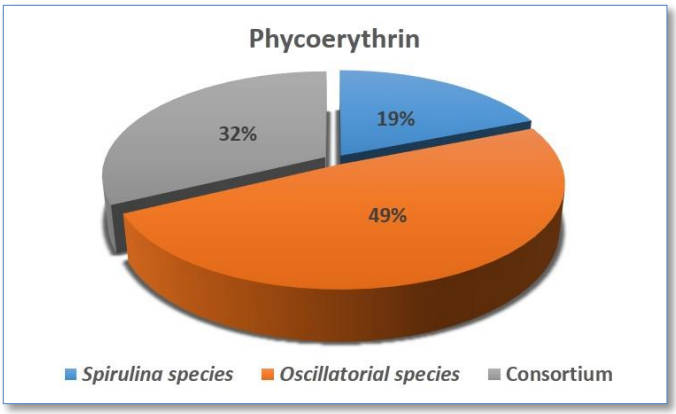


Fig-4 Phycobiliproteins production from biomass of *spirulina, oscillatoria* species and it consortium

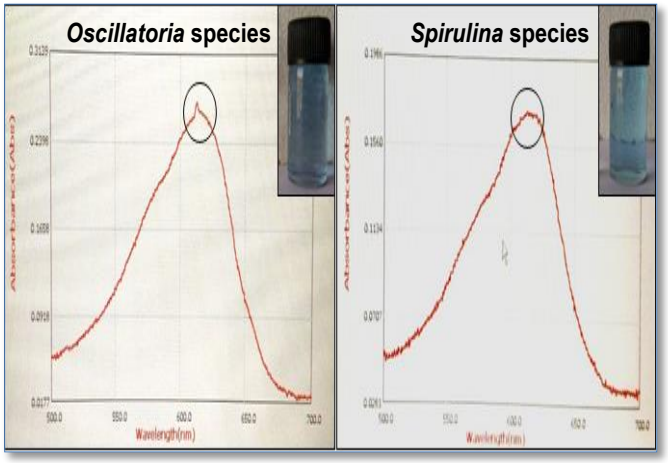


Fig-5 Absorbance spectrum of phycobiliproteins extraction from oscillatoria and spirulina species

Extraction of lipid from cyanobacterial biomass

The lipid content from different isolated species of cyanobacteria i.e. *spirulina*, *oscillatoria* species and its consortium shown in [Fig-6]. The lipid content from *oscillatoria* species was about 125.65 mg/l^{-1} which was greater than that from *spirulina* species and its consortium. [Table-6] shows the *oscillatoria* species has highest lipid productivity *i.e.*, 6.98 mg/l^{-1}/d^{-1} then *spirulina* species and its consortium. Lee, *et al*., (2010) reported that the average lipid content extracted from microalgae by Bligh and Dyer's method [17]. Our result is affirmation to this.

Table-6 *Lipid extraction from spirulina, oscillatoria species and its consortium*

Sr. No.	Item	*Spirulina* species mg/L	*Oscillatoria* species mg/L	Consortium species mg/L
1	Incubation days	18	18	18
2	Biomass productivity (mg/l^{-1} d^{-1})	843	831	845
3	Dry weight (g/l)	1.0	1.0	1.0
4	Lipid content (mg/l^{-1})	97.31	125.65	119.91
5	Average lipid productivity (mg/l^{-1} d^{-1})	5.40	6.98	6.66

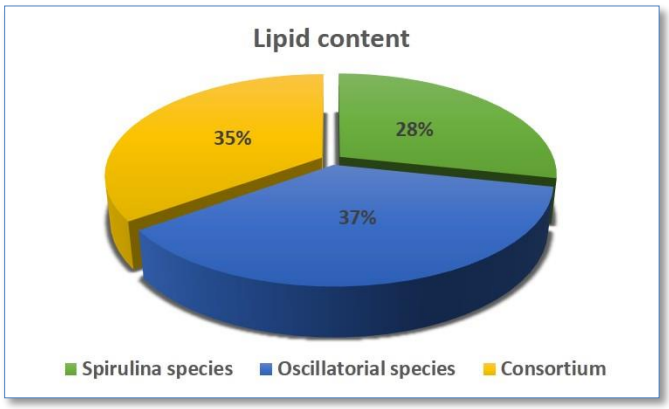


Fig-6 Lipid extraction from *spirulina, oscillatoria* species and its consortium

Conclusion

In this study, the isolation of cyanobacteria from collected dairy effluent samples was done and it was treated by isolated cyanobacterial species that pools nutrient removal and extraction of high value compound from generated cyanobacterial biomass. High value compounds *i.e.*, C-PC extracted from *spirulina*, *oscillatoria* species and its consortium was found to be 0.263 mg/L, 0.431 mg/L and 0.287 mg/L, respectively. This study shows the capability of cyanobacterial species for the removal of nutrients present in dairy effluent to produce high value compounds and can remove 92% COD as well as 87% overall nutrient of dairy effluent very efficiently.

Application of research

The application of this research cyanobacterial based low-cost dairy effluent treatment simultaneously biomass was produced and from this cyanobacterial biomass extract of high value compounds such as antioxidant compounds, pigments, nutraceuticals like omega 3, carotenoids, astaxanthin which also beneficiary for dairy industries.

Research Category: Dairy Microbiology, Cyanobacterial based dairy effluent treatment, High value compounds

Abbreviations:

DO: Dissolve Oxygen
BOD: Biochemical Oxygen Demand
COD: Chemical Oxygen Demand

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University: Kamdhenu University, Amreli, 365 601, Gujarat, India
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Author Contributions: All author equally contributed

Author statement: All authors read, reviewed, agree and approved the final manuscript

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