IJPSR (2023), Volume 14, Issue 9 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 09 January 2023; received in revised form, 24 March 2023; accepted, 25 April 2023; published 01 September 2023

DESIGN OF A NOVEL PHOTOBIOREACTOR FOR THE OPTIMIZATION OF GROWTH PARAMETERS OF *SCENEDESMUS DIMORPHUS* AND UTILIZATION OF DAIRY WASTE FOR BIOENERGY CONVERSION

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Keywords:

Scenedesmus dimorphus, Photobioreactor, Optimization, Dairy waste, Nutraceuticals, Bioenergy source

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ABSTRACT: *Scenedesmus dimorphus* isone of the widely exploited microalgae in several arenas of science, such as in food, nutraceuticals, agriculture, and pharmaceutical industries due to its high nutritional content and bioactivities. The present study designed a novel, cost-effective, eco-friendly, easy-to-operate and rectangular photobioreactor using glass material. Photobioreactor was provided with monochromatic LED light of 3000, 5000, 10,000 and 15,000 lux, agitation, and aeration facility. The effect of inoculum concentration, pH, temperature, and light intensity on the growth of *S. dimorphus* was evaluated in a novel photobioreactor. Finally, to convert dairy waste into valuable bioresources, dairy waste's effect on microalgae growth was investigated in an optimized condition using a novel photobioreactor. The optimum inoculum concentration to obtain the highest growth of *S. dimorphus* was 1-2x10^7 cells/ml in proteose peptone medium (pH 6.8) at 20ºC with photoperiodicity of Dark/Light (D/L) 12/12h and 12,000 lux.In similar experimental conditions, *S. dimorphus* revealed maximum growth at pH 7.0 and the optimum light intensity was 15,000lux.Growth temperature optimization in optimum conditions with D/L 12/12h indicated significant growth at 20ºC. The dairy waste at 50% resulted in the highest growth of *S. dimorphus*in optimum conditions. The growth of *S. dimorphus* was increased with increased incubation days indicating the highest growth on the 28th day. Therefore, it is inferred that optimum physical parameters and nutrient-rich dairy waste play a significant role in enhancing the growth of *S. dimorphus,* which is extensively used in various fields of science.

INTRODUCTION: As a consequence of the Global population explosion in the 1950s, such as poverty, famine, and migration, food production remarkably increased after that to fulfill the need of the growing population [1]. Though we have become self-sufficient regarding food needs, lack of nutrients in our daily life is a big concern.

QUICK RESPONSE CODE	DOI: 10.13040/IJPSR.0975-8232.14(9).4432-39
	This article can be accessed online on www.ijpsr.com

DOI link: http://doi.org/10.13040/IJPSR.0975-8232.14(9).4432-39

Nutrients are integral to our food and are pivotal in maintaining health. Food supplies nutrients for normal growth and is a reservoir of medicines to keep our bodies healthy. Microalgae, an under-exploited crop used in the human diet for thousands of years, has several advantages in terms of productivity, efficiency, and nutritional value in contrast to plants.

They don't compete with resources and land as the plants require, and the microalgae's protein level is almost equivalent to sources like soybeans, meat, egg, and milk. The protein content of microalgae is estimated as 4 to 15 tons/ hector/year versus 0.6 to 1.2, 1 to 2 and 1.1 tons/ hector/ year of soybean, pulse legumes, and wheat, respectively [2]. It is generally accepted that microalgae have huge potential to produce nutraceuticals. But, many exciting and important biochemicals are yet to be discovered from these microscopic plants. More recently, the microalgal species number for food supplements has increased greatly and their applications in the pharmaceutical industry are becoming more widespread [3].

Microalgae like *Spirulina, Chlorella, Haematococcus pluvialis* and *Nannochlorpsis sp.* are abundant sources of bio-products, including carbohydrates, proteins, polyunsaturated fatty acids, dietary fibers, carotenoids, and bioactive compounds with a wide range of health benefits [4]. Eicosapentaenoic acid and docosahexaenoic acid are mysterious molecules that reduce the risk of arrhythmia, stroke, rheumatoid arthritis, and high blood pressure [5]. Food additive, Omega-3-fatty acid can be co-preventive or co-therapeutic and imparts health benefits as a natural medicine in certain major diseases. Higher plants are the major supplier of carotenoids in the human diet. However, increased demand for natural food additives has paved the way for alternative sources of carotenoids [6].

Microalgae biomass production has two cultivated methods, one open type and another closed type, also known as photobioreactor. In open type, conventional methods are used to cultivate microalgae [7]. This system is less expensive, easy to clean, and may be used in non-agricultural lands. In this type, limitation factors like poor biomass efficiency, uncleanness, partial microalgal strains, and a requirement for lands are more [8]. A photobioreactors overcome the problem of open type and essential parameters present at bioreactor volume, artificial optimum light for the organism's growth, airflow, and agitator. A photobioreactor is a container that can be easily opened and closed, or semi-closed and is made of transparent and waterproof materials in which microalgae cultivation is carried out [9]. In the present investigation, a novel photobioreactor was designed for the growth of microalgae, *S. dimorphus*. The growth parameters such as inoculum, pH, temperature and light intensity required for the growth of *S. dimorphus* were optimized to achieve maximum biomass production. Further, dairy waste is used to enhance biomass production of *S. dimorphus* and convert it into a valuable energy source.

MATERIALS AND METHODS:

Collection and Culturing of *S. dimorphus*: The Algal strain *S. dimorphus*(UTEX 1237) was procured from the Culture Collection of Algae, University of Texas Austin, USA, in July 2021. The original culture slant on receiving was subcultured on the same day in a sterileproteose peptone agar medium and in a broth medium. Both media were incubated at 20^oC in a Biochemical Oxygen Demand (BOD) incubator at an L/D (diurnal) ratio of 12/12 for 4 weeks at 3200 lux intensity of the light source. Cells of *S. dimorphus* were preserved by cryopreservation until their usage. Followed by the incubation, growths of the colonies were noticed on both proteose peptone agar medium as well as in broth medium. Colonies were taken on a clean glass side and a thin smear was prepared in sterile saline. Cells of *S. dimorphus* were observed under a compound microscope with 1000x magnification and morphology was compared with the available algal database (algabase.org).

Designing of a Novel Photobioreactor for the cultivation of *S. dimorphus*: Laboratory scale photobioreactor of 2.5liter capacity was designed for culturing *S. dimorphus* using glass material for the walls and lid of the bioreactor. A rectangular shape photobioreactor with dimensions of 15.5cm length, 10cm width, and 11cm height was constructed. The top of the bioreactor was closed with a glass lid having dimensions of 20.5 cm and 13cm in length and width, respectively. A light source of different intensities was provided at the top of the lid of the photobioreactor. A small pump was used to agitate the culture and continue to avoid the settling and clumping of algal cell mass. The air pump was connected with a two-foot-long main silicon pipe, which was further bifurcated into four outlets. Each outlet was inserted into four bioreactor tanks to ensure equal pumping and agitation. Each bioreactor tank was equipped with a LED light source of different intensities such as 3000, 5000, 10,000 and 15,000, to provide optimum light intensity. Intermixing of light of each adjacent bioreactor tank was avoided by placing carton blocks between tanks and keeping all LED bulbs facingin one direction so the light of one tank can't reach the algal growth of the neighboring tank.

Optimization of Growth Parameters of *S. dimorphus* in a Photobioreactor: The effects of inoculum, light intensity, pH, and temperature on the growth of *S. dimorphus* were evaluated in a photobioreactor. Proteose peptone medium (Peptone – 2%, $NaNO_3$ - 25g, $CaCl_2.2H_2O$ – 2.5g, $MgSO^4 \bullet 7H^2O$-7.5g, K_2HPO_4 -7.5g KH_2PO_4-7.5g - 17.5g, NaCl- 2.5g, and pH 6.8) was prepared in one liter of distilled water and the medium was sterilized by autoclaving at 121°C, 15lb pressure for 15 - 20minutes. The sterile medium was equally added in into all five bioreactor tanks of the photobioreactor. Initially, to optimize inoculum, each bioreactor tank was inoculated with 1-2x10^4, 1-2x10^5, 1-2x10^6, 1-2x 10^7 and 1-2x10^8 of *S. dimorphus*. The photobioreactor was provided with LED light of 12,000 lux and incubated at 20°C with D/L 12/12 hrs duration for 28 days.

The optimum pH for ideal growth of microalgae was determined by inoculating the medium with optimum inoculum and adjusting it for pH 5.0, 6.0, 7.0, 8.0, and 9.0 and incubating at 20ºC and 12,000 lux for 28 days with D/L 12/12 hrs duration. The medium was adjusted for optimum pH, inoculated with optimum inoculum, and provided with LED light with the light intensity of 3000, 5000, 10,000, and 15000 lux to find optimum light intensity for the growth of *S. dimorphus*. The bioreactor was incubated at 20°C with D/L 12/12hrs for 28days. Finally, the growth temperature was optimized by maintaining the photobioreactor at 20°C, 22°C, 24°C, 26°C, and 28°C and adjusting the medium with optimum pH. The medium was inoculated with optimum inoculum, provided with optimum light, and incubated for 28 days with D/L 12/12 hours. On every 7th day of incubation, 1ml of growth medium was withdrawn from the bioreactor and cells/ml were determined under the microscope using a hemocytometer for 28 days.

Study of the Effect of Different Concentrations of Dairy Waste on the Growth of *S. dimorphus*: The dairy waste collection was done from Bengaluru dairy with prior permission directly from the plant discharge pipeline using the sterile container. The sample was immediately brought to the laboratory and it was sterilized before incorporating into the growth medium. In proteose peptone medium, the dairy waste was mixed at 25%, 50%, and 75%, and *S. dimorphus* was grown in 100% of the dairy waste. Each bioreactor tank containing proteose peptone medium was inoculated with 50 x 10^6 cells/ml of seed culture of *S. dimorphus*. The photobioreactor was incubated at an optimum temperature of 20°C, in 15,000 lux light for 28 days with D/L 12/12 hrs. Once on every 7th day, one ml of growth medium from each tank was withdrawn and cells of *S. dimorphus*/ml were determined under the microscope using a hemocytometer for 28 days. The concentration of dairy waste that resulted in the growth of the highest cells in 1ml of the withdrawn sample was taken as optimum dairy waste for the growth of *S. dimorphus.*

RESULTS:

Cultivation and Strain Confirmation of *S. dimorphus*: *S. dimorphus*procured from the algal culture collection center was successfully grown in proteose peptone agar medium and broth medium. The smear of microalgae colonies of *S. dimorphus* observed under the microscope and the morphology of the cell, when compared with the algal resource database, revealed long pointed tips on both sides of the cells. The color of the cells was green, and a majority of the cells were arranged in groups though a few were also present in single as shown in **Fig. 1**. Phenotypic characteristics of microscopic cells such as color, shape, length, and arrangement have resembled the morphology of microalgae *S. dimorphus.* The general characteristics of the eukaryotic microalgae cells observed in the current study were comparable to the available scientific publication [10].

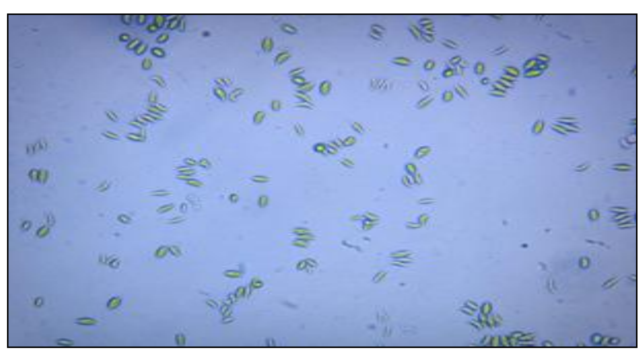

FIG. 1: MORPHOLOGY *S. DIMORPHOUS* (UTEX 1237) CELLS OBSERVED UNDER 1000X MAGNIFICATION

Biological applications of *S. dimorphus* are immense, and their rich nutrients and bioactive compounds made them extensively used as nutraceuticals and food supplements. As a nutraceuticals, *S. dimorphus* helps in repairing bone marrow damage and Omega-3 polyunsaturated fatty acids present in *S. dimorphus* play a significant role in preventing and treating diabetes, hypertension, cancer, autoimmune and heart diseases [11].

Novel Photobioreactor Design for *S. dimorphus* Cultivation: A novel photobioreactor with different monochromatic light intensities ranging from 3000 to 15000 lux was constructed successfully as shown in **Fig. 2**. A photobioreactor of 2.5 litre capacity was rectangular with size 15.5Lx10Wx11H centimeters and was constructed using glass material. The photobioreactor was provided with a small pump for continuous culture agitation to avoid clumping and settling of algal biomass and ensure proper aeration. Aeration in the photobioreactor was further enhanced by pumping air through a silicon pipe of 2 feet in length which is further diverged into 4 outlets. Each bioreactor tank of the photobioreactor has received each outlet to maintain a similar level of agitation and pressure.

As photobioreactor walls and lids were made of glass material, it is cost-effective, easy to visible, and monitor the growth of cells. It provides sufficient space for the growth of algal cells. As the shape is rectangular, the monochromatic light can reach all locations of the photobioreactor, as light is an important factor in algae growth. It is environmentally friendly, easy to clean and operate, and also equipped with good aeration and agitation system that ensures uniform oxygen supply throughout the bioreactor.

Open ponds and photobioreactors (closed ponds) are widely used for culturing microalgae. The better-controlled cultivation system, higher surface-to-value ratio, and efficient nutrient usage made the photobioreactor an excellent method for higher biomass productivity of *S. dimorphus* compared to economically viable open pond methods [12]. *S. dimorphus* cultured in the flask with propylene carbonate for CO_2 consumption enhanced 63% biomass production and reduced by 71% CO_2 when cultivated in an air-lift photobioreactor with polyethylene glycol dimethyl ether [13]. However, very few studies show the effect of monochromatic LED light of different intensities on the growth of *S. dimorphus.*

FIG. 2: A LABORATORY-SCALE PHOTOBIOREACTOR DESIGNED FOR THE CULTIVATION OF *S. DIMORPHUS*

Determination of Optimum Growth Parameters for the Growth of *S. dimorphus*: Several physical parameters like the amount of the cells inoculated, medium pH, light intensity and temperature that influence the growth of microalgae were determined in the current study. Medium inoculate with different concentrations of cells of *S. dimorphus* and incubated at 20ºC with the light intensity of 12,000 lux in 12/12 hours D/L for the 0^{th} to the 28 days showed increased cell growth with increased inoculum concentration till 1-2x10^7 cells/ml which further declined as indicated in **Table 1**. Microalgae growth also increased from 0^{th} day to 28^{th} day of incubation. The highest cell biomass was detected in the medium inoculated with 1-2x10^7 cells/ml; hence, it is considered the optimum inoculum concentration.

The decrease in the cell biomass in a medium inoculated with 1-2 $x10^8$ cells/ml may be due to early exhaustion of nutrients, and results are shown in **Fig. 3.**

TABLE 1: DETERMINATION OF OPTIMUM INOCULUM CONCENTRATION OF THE GROWTH OF MICROALGAE

Incubation (Days)	Growth of *S. dimorphus* ($x10^6$ cells/ml)				
	1-2$x10^4$	1-2$x10^5$	1-2$x10^6$	1-2$x10^7$	1-2$x10^8$
0th	0.156±0.006	0.161±0.004	1.54±0.05	1.65±0.03	1.71±0.06
7th	0.167±0.004	0.219±0.007	1.80±0.04	2.43±0.04	1.86±0.04
14th	0.219±0.004	0.288±0.005	2.28±0.05	3.35±0.05	2.27±0.03
21st	0.250±0.004	0.347±0.005	2.69±0.05	3.91±0.05	2.63±0.06
28th	0.293±0.005	0.382±0.003	3.27±0.04	4.23±0.04	3.17±0.04

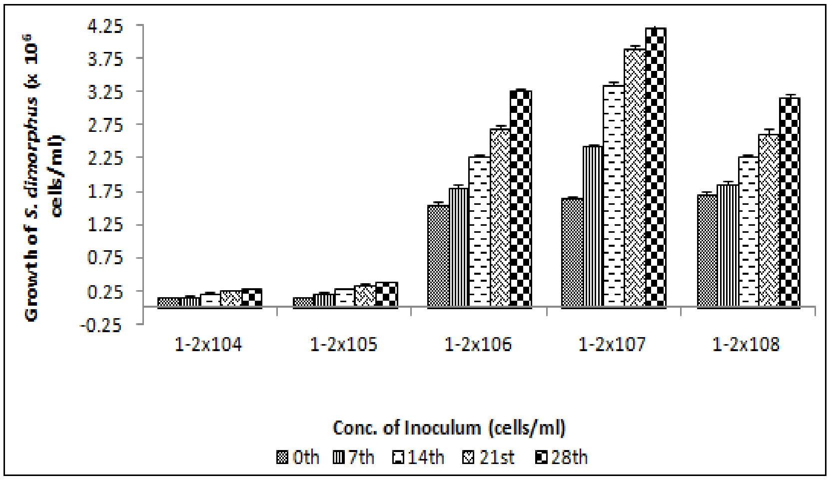

FIG. 3: OPTIMIZATION OF INOCULUM CONCENTRATION FOR THE GROWTH OF *S. DIMORPHUS*

The study of medium pH's effect on the growth of *S. dimorphus* revealed increased growth of microalgae in acidic and neutral pH when a medium was inoculated with optimum inoculum and incubated for 0^{th} to 28 days at 20ºC with 12,000 lux light intensity in D/L 12/12 hours. The growth of microalgae cells decreased in alkaline conditions. The biomass of *S. dimorphus* noticed in the growth medium of pH of 7.0 on the 7^{th}, 14^{th}, 21^{st}, and 28^{th} days of incubation was 28^3x10^7, 354 $x10^7$, 416 $x10^7$, and 480 $x10^{7,}$ respectively. Microalgae cell counts are observed in increased order with increased incubation time in all pH ranges as indicated in **Fig. 4**.

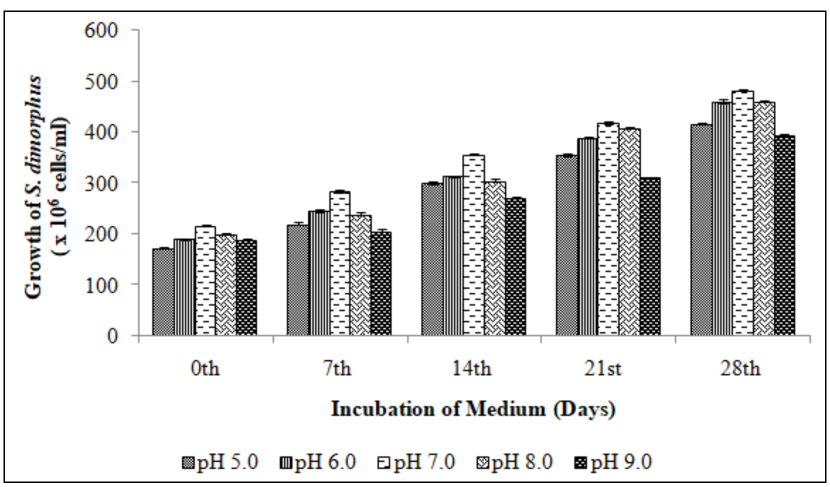

FIG. 4: DETERMINATION OF OPTIMUM PH FOR THE BIOMASS PRODUCTION OF *S. DIMORPHUS*

The light intensity optimized using optimum inoculum concentration, pH, and incubation at 20ºC with photoperiodicity of D/L 12/12 hours for 0^{th} to 28^{th} days indicated increased cells/ml in each tank provided with the intensity of light of 3,000 to 15,000 lux. The amount of *S. dimorphus* cells/ml on the 7th day of incubation at 20° at 3000, 5000, 10,000 and 15,000 lux were found to be 5.87x10^6, 6.42x10^6, 7.99 x10^6 and 8.42 x10^6 respectively. An increase in the cell number of *S. dimorphus* was observed with an increase in the light intensity and number of incubation days as shown in **Fig. 5** and **6.** Therefore, the optimum light intensity and the number of incubation days to attain the highest growth *S. dimorphus* were found to be 15,000 lux and 28th day. Thus, it is inferred that the biomass of *S. dimorphus* was directly influenced by the light intensity and number of incubation days. The study was supported by the reports that propose low light intensity reduces the growth of the *S. dimorphus* which can be enhanced by increasing the light intensity [14, 15].

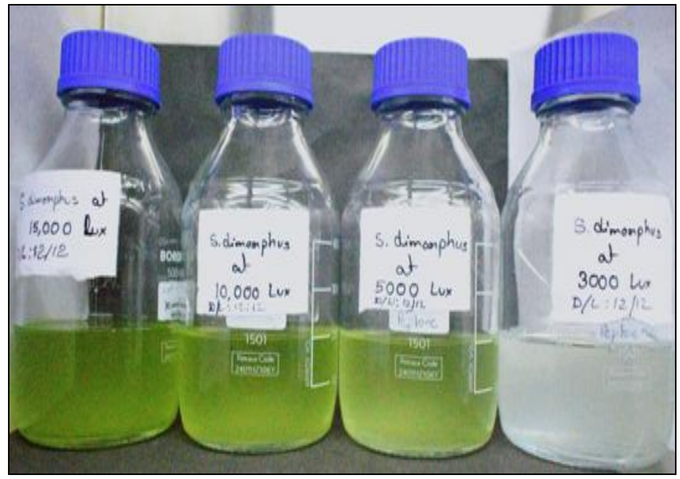

FIG. 5: GLASS BOTTLES CONTAINING *S. DIMORPHUS*GROWN AT THE DIFFERENT LIGHT INTENSITY

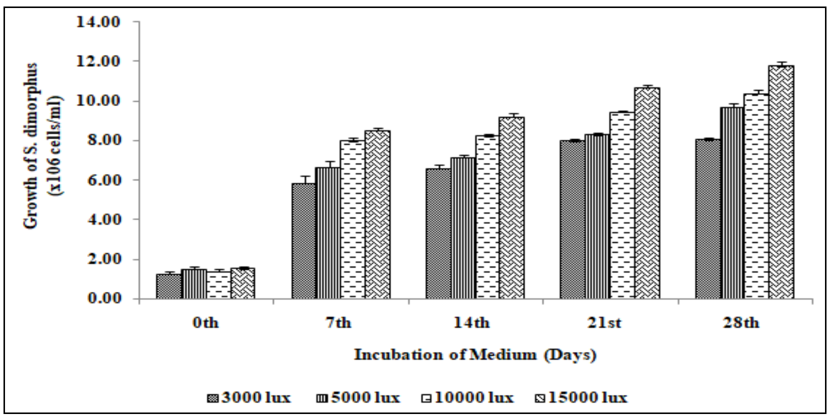

FIG. 6: BAR GRAPH REPRESENTATION OF THE GROWTH OF *S. DIMORPHUS* AT DIFFERENT LIGHT INTENSITIES AND INCUBATION DAYS

Temperature is an essential physical factor for the growth of all living beings. Temperature also plays a significant role in enhancing the growth of microalgae. Optimization of growth temperature of *S. dimorphus* at different temperatures *i.e.*, 20 to 28ºC using all above optimum conditions such as inoculum, pH, and light intensity indicated highest growth of *S. dimorphus* at 20°C with photo-periodicity of D/L 12/12 hours in contrast to other temperature ranges. While an increasing in the growth of microalgae was noticed from the 0th to 28th days, a significant increase in *S. dimorphus* was observed at 20ºC. Surprisingly, enhancement of each successive 2ºC resulted in a decrease in the growth of microalgae and results are indicated in **Fig. 7**.

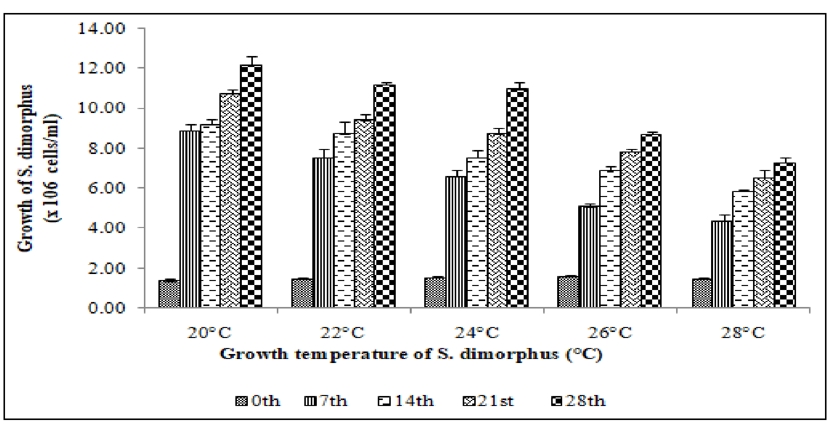

FIG. 7: EVALUATION OF DIFFERENT TEMPERATURES FOR THE GROWTH OF *S. DIMORPHUS*

Effect of Dairy Waste on the Growth of *S. dimorphus*: The effort has been made to utilize dairy waste to convert it into the biomass of *S. dimorphus* which is widely used in several industrial applications especially in neutra-cueticals. The study was conducted at 20°C with pH 7.0, inoculum concentration of 1×10^7 cells/ml, photoperiodicity of D/L 12/12 hours, and light intensity of 15,000 lux. The growth of the microalgae was monitored from the 0^{th} day to 28 days by withdrawing 1ml of sample and counting algal cells/ ml. The result indicated increased growth of *S. dimorphus* at 25% and 50% of dairy waste, decreasing at 75% and 100%. The number of cells counted on the 7^{th} day of incubation at 25% and 50% of dairy waste was found to be 6.99 $\times 10^6$ and 8.33 $\times 10^6$ cells/ml, respectively. The cell count of *S. dimorphus* was found highest in 50% of dairy waste *i.e.*, 13.49$\times 10^6$, 16.98 $\times 10^6$ and 18.75$\times 10^6$ cells/ml on the 14^{th}, 21^{st} and 28^{th} day of incubation respectively and at 75% and 100% of dairy waste the growth of the *S. dimorphus* was declined. Hence, the optimum dairy waste that supports the maximum growth of *S. dimorphus* was determined as 50% as shown in **Fig. 8**. As the growth of cells was increased from the 0^{th} to the 28^{th} day, the optimum incubation period was found to be 28 days.

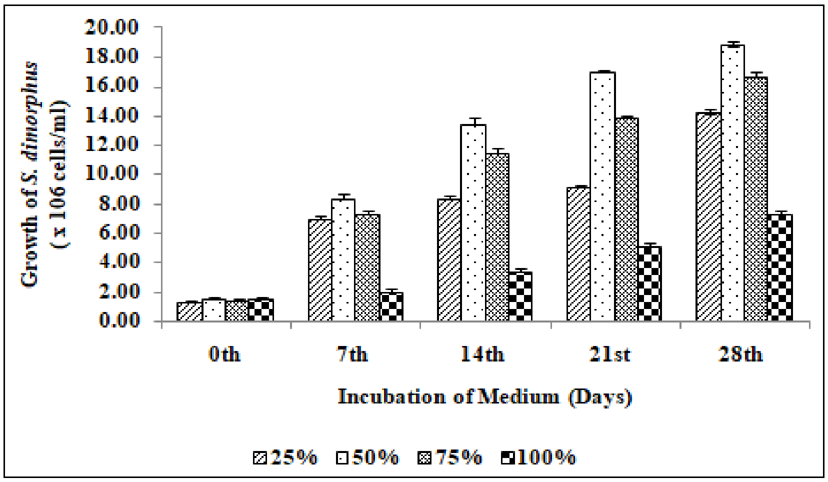

FIG. 8: OPTIMIZATION OF DIFFERENT PERCENT OF DAIRY WASTE FOR THE GROWTH OF *S. DIMORPHUS*

DISCUSSION: Extensive applications of microalgae in several industrial sectors posed immense demand for their biomass. Further, many biocomponents such as proteins, pigments, biopolymers, vitamins, polysaccharides, lipids, and other products these organisms produce require large amounts of microalgae biomass [16]. These biomolecules produced from microalgae have shown great therapeutic potential in medicine and human health. The bioactivities of these molecules include antimicrobial, anticancer, anticoagulant, and anti-inflammatory activity. They are also frequently used in the management of cardiovascular health, enhancing the immune system, cholesterol reduction, antiulcer, and healing wounds [17]. Hence, there is a huge demand for microalgae biomass to meet the requirement of various industrial applications.

On the other hand, handfuls of cultivation media yield significant quantities of algal biomass. Moreover, the existing media take long to achieve the intended amount of microalgae biomass. Hence, in the present research, optimization of physical growth parameters such as inoculum concentration, pH, temperature, and light intensity revealed substantial microalgae growth in a novel photobioreactor. As mentioned earlier, enhancing the growth of *S. dimorphus* through the optimization of growth parameters plays an important role in its better exploitation for various applications. For instance, *S. dimorphus*is expansively used in biodiesel production as its cell contains 90% of diesel and polysaccharides *S. dimorphus* are proven to have an anti-skin aging property in an animal model [18, 19]. Therefore, the present study is necessary for the production of *S. dimorphus* for above mentioned applications. Furthermore, for the first time, dairy waste exploited for the production of algal biomass is introduced in the current investigation. Microalgal biomass enhancement in the present study helps in a novel drug development process and other important scientific applications using cheap and nutrient-rich dairy waste.

CONCLUSION: The photobioreactor designed in the current study is distinctive, cost-effective, eco-friendly, and operated with wide light intensity. It offered wide space for microalgae growth under effective agitation and aeration facility. Optimum conditions like inoculum concentration, pH, light intensity, and temperature were crucial in enhancing the growth of *S. dimorphus*, which is exploited widely in several industries.

ACKNOWLEDGEMENTS: The Author would like to thank Dr. Lingayya Hiremath for his valuable guidance in the current investigation. The Authors also thank Mr. Veeresh Nandikolmath of Stroma Biotechnologies Private Limited for his technical support.

CONFLICTS OF INTEREST: Authors declare no conflict of interest

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How to cite this article:
Narasanagi M and Hiremath L: Design of a novel photobioreactor for the optimization of growth parameters of *Scenedesmus dimorphus* and utilization of dairy waste for bioenergy conversion. Int J Pharm Sci & Res 2023; 14(9): 4432-39. doi: 10.13040/IJPSR.0975-8232.14(9).4432-39.