International Journal of Scientific Research in Engineering and Management (IJSREM)
Volume: 07 Issue: 07 | July - 2023 SJIF Rating: 8.176 ISSN: 2582-3930

A CLEVER DEVELOPMENT OF LACTOBACILLUS SP. IN ALGAL FILTRATE AS AN EFFECTIVE NATURAL DEVELOPMENT MEDIUM

V.Babu,
Department of Microbiology, Valliammai Women's College of Arts and Science,
Arakandanallur, Villupuram (DT), Tamilnadu. India

ABSTRACT:

Algae form a vast group of eukaryotic, autotrophs which do photosynthesis in both marine and freshwater ecosystems. Microalgae in particular are the most primitive plant species to have appeared on Earth. Our study reveals that the growth of lactic acid bacteria was enhanced in the presence of *spirulina*. Apart from increasing the growth of lactic acid bacteria, spirullina also has abundant sources of essential amino acids and proteins. So the regular intake of *Spirulina* will not only improve the intestinal lactic acid bacteria but also inhibit the growth of harmful human pathogenic finally leading to the improved intestinal absorption. The abundance of bioactive components in S.platensis is of great importance from a nutritional point of view because it provides a new opportunity for the use of *Spirulina* as a perfect Neutraceutical supplement. Nowadays, when the dairy industry is supplementing milk with minerals, vitamins and antioxidants, it would be of interest to consider the possibility of adding *Spirulina* biomass, as a natural product, to fermented milk to induce a faster production of LAB and increase the number of viable cells in the product and in the gut.

Keywords: *Spirulina*, LAB, Neutraceutical, autotrophs, dairy.

INTRODUCTION.

Algae form a vast group of eukaryotic, autotrophs which do photosynthesis in both marine and freshwater ecosystems. Microalgae in particular are the most primitive plant species to have appeared on Earth. They make up the most of the mass of organisms called phytoplankton that are found near the surface of water and they are the base of the food chain in aquatic ecosystems. Furthermore they are the primary fixer of CO2 in the atmosphere by carrying out 80% of the world's photosynthesis. (John Sheehan 1998) The

growth cycle of algae in an outdoor environment as it is encountered in the natural environment follows a cycle in which the bacteria and the algae interact together. Although they are generally free-living, some live in symbiotic association with a variety of other organisms [Richmond, 2008]. The biodiversity in this group is astounding, with estimates going from the 200, 000 up to several million species [Norton et al.,1996]. *Spirulina* is one of the oldest life forms on Earth. In fact, this blue-green microalgae is partly responsible for producing the oxygen in the planet's atmosphere that billions of years ago allowed the planet's originating life forms to develop. *Spirulina* is the world's first super food, and one ofthe most nutrient-rich foods on Earth [Anagnostidis and Komarek, 1985]. Lactobacillus, (genus Lactobacillus), any of a group of rod-shaped, gram- positive, non-spore- forming bacteria of the family Lactobacillaceae. Similar to other genera in the family, Lactobacillus are characterized by their ability to produce lacticacidas a by-product of glucose metabolism. Lactobacillus are commensal inhabitants of animal and human gastrointestinal tracts, as well as the human mouth and the vagina. Commercial preparations of lactobacilli are used as probiotics to restore normal flora after the imbalance created by antibiotic therapy. To cultivate lactobacillus in *spirulina* filtrate and analyze the growth efficiency.

Methodology

GROWTH DATA OF *SPIRULINA*

The dry weight was measured after desiccation on pre-weighed filters with a porosity of 0.7 µm (Sartorius Stedim, Göttingen, Germany): 10 mL of *Spirulina* cultures were filtered and rinsed twice with the same volume of ultrapure water. Theprefilters were maintained at 80 ◦C overnight in a ventilated oven. Optical density was measured at 660 nm (OD660) in order to reduce the influence of the pigments absorption (mainly chlorophylls and phycocyanin) with an Epoch 2 Micro plate Spectrophotometer from BioTek Instruments, Inc. (Winooski, VT, USA). Productivities and nutrient consumption rates were calculated using the following formula, with t being the time and A_t being the dry weight or the nutrient concentration.

$$Rate\ of\ A_{t2\rightarrow t1} = \frac{A_{t2\rightarrow t1}}{t_2 - t_1}$$

BIOMASS ANALYSIS OF *SPIRULINA*

Iron content was measured using inductively-coupled plasma (ICP) coupled with atomic emission spectrometry (AES) using an ICP-AES Vista MPX (Agilent Technologies, Santa Clara, CA, USA). Fifty

milliliters of *Spirulina* culture were centrifuged at 4750 rpm, 4 ◦C for 10 min. The iron content in the supernatant was measured. Then, the pellet was washed twice with 15 mL of a 10 mM EDTA solution. The resulting 2 × 15 mL (separated by centrifugation) was analyzed for its iron content from which the adsorbed iron content of the biomass could be deducted. Finally, the washed pellet was hydrolyzed for 24 h at 80 ◦C in 1 mL of 70% nitric acid and then diluted to 6 mL with ultrapure water. The hydrolysate was analyzed forits iron content from which the internalized iron content of the biomass could be deducted. The iron concentrations were determined using standard curve obtained by analyzing ICP grade iron standard solutions. The C/N ratio was determined using an organic elemental analyzer. [Florian *et.al.*,2017]

CHEMICAL COMPOSITION ANALYSIS OF FILTRATE

The presence of protein in the filtrate (optical density) was measured using Systronics UV Visible double beam spectrophotometer: 2202 (bandwidth – 2.0 nm). The amount of elements such as Ca, Cu, Fe, K, Mg, Mn, Na, Mo, Co and Zn(elements in Zarrouk's medium) was identified using ICP-OES of Perkin Elmer Optima 5300 DV. Furthermore, the presence of protein functional groups and metal ions present in the filtrate was analysed using FT-IR spectrum one: FT-IR Spectrometer (Perkin Elmer ACCEPTED MANUSCRIPT 11 Spectrum), Scan Range: MIR 450-4000 cm-1, Resolution: 1.0 cm-1. The Total Organic Carbon (TOC) analysis was determined using a Shimadzu TOC analyzer (model 5050A) for degraded water.

INOCULATION OF LACTOBACILLUS

The strains were sub-cultured twice in MRS broth containing 1 g/L cysteine- hydrochloride in an anaerobic workstation (Whitley DG250, Don Whitley Scientific Limited, West Yorkshire, UK) at 37 °C for 12–24 h under an atmosphere of 80% N2, 10% CO2 and 10% H2. The bacteria were used as seed inoculums for the subsequent fermentation. The above seed inoculums solution (1% v/v) was inoculated into Erlenmeyer flasks (250 mL volume) containing 200 mL MRS broth (containing cysteine hydrochloride), followed by incubation in batch culture at 37 °C in an anaerobic workstation (Whitley DG250, Don Whitley Scientific Limited, West Yorkshire, UK). Samples of 10 mL were taken after every 2 h and centrifuged at 5000g for 5 min to get the cell free supernatant (CFS). The CFS was stored at −20 °C for determination of the total acid and residual glucose contents in the fermentation broth.

GROWTH ACTIVITY OF LACTOBACILLUS

The inoculated culture sample is retrieved at regular time intervals and corresponding growth curve is populated by characterizing it in UV spectrophotometer at 590nm.

ANALYSIS OF LACTIC ACIDCONCENTRATION

Analysis of lactic acid content was the principle of acid-base titration according to [AOAC 1995]. Two or three drops of 1% phenolphthalein (PP) C20H14O4 indicator was mixed with the samples and was titrated with 0.05 N NaOH solution until the color was pink. The total titrated solution was the total lactic acid in thesample.

The formula was as follows:

$$Crude\ Lactic\ acid\ (\%) = \frac{(VT_S - Vt_0\ \times N\ \times MW\ \times Df}{V\ sample\ \times 1000} \times 100\ \%$$

VTs = titrant sample volume (mL)

MW = relative molecular of lactic acids 90.0 (g/mol) Vto = blank (mL)

Df = Dilution factorN = normality of the titrant (NaOH 0.05 N) V

Sample = sample volume

BIOMASS ANALYSIS OFLACTOBACILLUS

The final dry weights of the cells are measured by harvesting the supernatant/filtrate free cells.

RESULTS AND DISCUSSION.

Nutrients affect the metabolism and growth rate of microalgae and the composition of the final product. Carbon (C) is the principal element in the media composition for the cultivation of microalgae. Nutrients need to be supplied in balance with what the specifications of the final products are. Nitrogen supplied in the form of nitrates (NO3 -), nitrites (NO2 -), ammonia (NH4 +) or urea are important for the synthesis of amino acids for proteins and growth of microalgae. The minimum temperature that allows for growth of *Spirulina* is around 18°C and the culture is reported to deteriorate below 12°C. Significant biomass losses occurring at night due to dark respiration have been reported (Tomitani et al., 2006) and can be reduced by lowering the temperature of the culture at night (Vetayasuporn,2004). *Spirulina* cells are sensitive to pH changes and require monitoring and control for optimal growth. *Spirulina* thrives at an optimum pH range between 9-10.5 (Belkin&Boussiba 1971). 18 At pH higher than the optimum range, micro algal growth can be inhibited (tab-1).

Table 1. Daily *Spirulina* growth monitor data

Culture Day	Temp (^{0}C)	pH	Optical density (OD)	Dry Weight *Spirulina* (g/L)	*Spirulina* Condition
0	29	8.95	0.364	0.311	Filamentous, healthy, light blue green colour
1	29	9.5	0.447	0.526	Filamentous, healthy, light blue green colour
2	30	9.7	0.520	0.611	Filamentous, healthy, light blue green colour
3	28	9.9	0.593	0.689	Filamentous, healthy, light blue green colour
4	30	10	0.658	0.762	Filamentous, healthy, light blue green colour
5	29	10.2	0.721	0.789	Filamentous, healthy, light blue green colour
6	30	10.5	0.861	0.815	Some filaments are good, some are broken, light blue green colour
7	29	10.6	0.890	0.892	Some filaments are good, some are broken, light blue green colour
8	29	10.8	0.932	0.985	Some filaments are good, some are broken, light blue green colour

BIOMASS ANALYSIS OF *SPIRULINA*

Spirulina is a source of highly available iron with a 6.5 times more available iron than beef meat. Increasing iron content in *Spirulina* could lead to a very beneficial increase of its nutritional value.Modified ZM contains iron in the form of FeSO4,6H2O (2 mgFe/L) stabilized with EDTA (50 mg/L). The opportunity to use commercial formulations of Fe-EDTA was tested in *Spirulina* cultivation experiments using Fe-EDTA from Plantin and Akzo Nobel at two different concentrations (3 and 10 mgFe/L) in duplicates. Figures 6 and 7 show the growth curves in OD880 for these experiments. Concentration of 3 mgFe/L of Fe-EDTA did not impact significantly the growth of *Spirulina*. However, at

10 mg Fe/L, *Spirulina* growth was slightly inhibited, especially after 10 days.

Nevertheless, the *Spirulina* iron content was significantly increased when Fe-EDTA concentration in the medium was increased from 3 to 10 mg Fe/L for the Plantin solution (Table 1). Iron mass balances were not always coherent with large disparities over the duplicates.

However, the *Spirulina* iron content (internalized iron) showed three consistent behaviors. First, Fe-EDTA increased the iron content. Then, higher Fe-EDTA concentrations in the medium increased the iron content. And, Fe-EDTA solution from Plantin led to higher iron content in comparison to the solution from Akzo-Nobel at 10 mg Fe/L.

This difference could be explained by Fe-EDTA counter-ion which is NH4+ for the Plantin solution and Na+ for the Akzo-Nobel solution. Further studies should be conducted on the counter-ion effect on iron accumulation, as well as on the chelating agent (EDDHA, DTPA for example). The results were tabulated in table 1.

SPIRULINA BIOMASS NUTRITIONAL COMPOSITION:

Spirulina has high quality protein content (59–65 percent). The composition of commercial *spirulina* powder is 60 percent protein, 20 percent carbohydrate, 5 percent fats, 7 percent minerals, and 3–6 percent moisture, making it a low-fat, low calorie, cholesterol-free source of protein. All the essential minerals are available in *spirulina* which contributes about 7 percent. The physiochemical characteristics of *Spirulina* are tabulated in Table 2.

S.No	Components	Percentage %
1.	Crude Protein	61
2.	Moisture	9
3.	Ash Content	11
4.	Phycocynin	2

Table 2. Physiochemical Characteristics of *Spirulina*

Chemical Composition Analysis of Filtrate

In the present study, *S. platensis*was homogenized and then centrifuged. Table 3 presents a multi-elemental composition of *S. platensis* products filtrate and homogenate and commercial biostimulant of plant growth. It is worth noting that macroelements-K, Mg, P, S occurred in larger quantities in a

homogenate than in filtrate, whereas microelements-Cu, Fe, Ni and Zn in an algal filtrate. The content of all studied elements was much higher in *Spirulina* products than in the commercial biostimulant. Therefore, microalga products not only improve plant growth and development but also enhance the mineral composition of cultivated plants.

Table 3. Multi-elemental composition of *Spirulinaplatensis* products

Element/wavelength (nm)		*Spirulina* filtrate	*Spirulina* homogenate
Al	308.215	197.2 ± 29.6	756.1 ± 113.4
Ca	315.887	3042 ± 608	3141 ± 628
Cr	267.716	< 0.3	3.236 ± 0.485
Cu	324.754	3.364 ± 0.505	3.947 ± 0.592
Fe	259.940	353.6 ± 53.0	1464 ± 293
K	766.491	38,687 ± 7737	9641 ± 1928
Mg	285.213	4588 ± 918	2701 ± 540
Mn	257.61	74.40 ± 11.16	46.64 ± 7.00
Na	588.995	13,375 ± 2675	3753 ± 751
Ni	231.604	2.289 ± 0.343	8.096 ± 1.214
P	213.618	18,687 ± 3737	7336 ± 1467
S	181.972	7234 ± 1447	6789 ± 1358
Si	251.611	154.0 ± 23.1	119.1 ± 17.9
Zn	213.857	18.74 ± 2.81	19.42 ± 2.91

GROWTH ACTIVITY OF LACTOBACILLUS

Lactic acid bacteria growth promotion was evaluated when they were incubated with *S.platensis* dry biomass. The increase in growth percentage was 45.63 % after 5 hrs and 63.93% after 10 hrs. When 1mg/ml *S.platensis* was added with *L. casei*. In the same concentration growth was promoted in L. acidophilus was 42.92 % in 5 hrs and 85.84 % in 10 hrs *S.thermophilus* was promoted 27.32% and 45.63% after 5 hrs and 10 hrs of incubation respectively. Maximum growth was promoted at 10 mg/ml concentration of *S.platensis* up to 10 hrs. 145.90%, 171.67% and 185.84% growth was observed in *L.acidophilus.*

Table 4: Growth kinetics of lactic acid bacteria (cfu/ml) added with *S. platensis* filtrate

Concentration of *Spirulina* (mg/ml)	Lactobacillus acidophilus		
	0 hr.	5 hr.	10 hr.
05	2.3±0.7	4.36±0.6^{ns}	5.33±0.3*
10	2.3±0.7	6.33±0.3**	6.66±0.25**
Control	2.3±0.3	3.0±0.2^{ns}	4.0±0.3^{ns}

ANALYSIS OF LACTIC ACID CONCENTRATION

Concentration of lactic acid producing using varying range of *spirulina* filtrate as supplement was determined and results were tabulated in the table 5. Accordingly the sample with higher content of *spirulina* supplement showed the higher level of production of lactic acid.

Concentration of *Spirulina* (mg/ml)	Lactic acid (g/l)	Lactic acid yield (%)	Sugar utilization (%)
10	90.0	96.7	93.0
05	41.0	95.3	43.0
Control	14.0	93.3	15.0

Table 5: Lactic acid production using *spirulina*

BIOMASS ANALYSIS OF LACTOBACILLUS

Both cfu/ml and the dry weight of the biomass were calculated for the samples grown with *spirulina* supplement. From the table 6, it was clearly evident that the sample with higher *spirulina* content showed higher growth in comparison with the rest of the samples. The control without any *spirulina* content showed a nominal level of growth proving that *Spirulina* increased the growth of the lactic acid bacteria.

Spirulina (mg/ml)	Viable cells (c.f.u/ml)	Initial Dry Weight (g/L)	Dry Weight (g/L)
01	0.8 · 105	1.35	1.2
05	1.3 · 103	2.35	2.1
10	1.6 · 102	3.2	2.9
Control	5.6 · 102	0.97	0.82

Table 6. Final dry weights and productivity

CONCLUSION.

Our research shows that the presence of *spirulina* can promote the growth of lactic acid bacteria. *Spirulina* can not only promote the growth of lactic acid bacteria, but also contains a variety of essential amino acids and proteins. Therefore, regular intake of *spirulina* not only improves lactic acid bacteria in the gut, but also inhibits the growth of harmful human pathogens, ultimately improving intestinal absorption. From a nutritional point of view, the richness of bioactive components in *Spirulina* platensis is very important as it opens up new opportunities for the use of *Spirulina* as a perfect health food supplement. Today, as the dairy industry adds minerals, vitamins, and antioxidants to milk, consider adding *spirulina* biomass to fermented milk as a natural product to induce faster production of lactic acid bacteria and increase the number of viable cells in the product and in the gut.

Acknowledgement.

The authors extend their sincere thanks to The Chairman, Valliammai Women's College of Arts and Science, Villupuram. For providing the facilities, recognizing our research work and that successful completion of the work.

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