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Isolation, identification and evaluation of *Spirulina platensis* for its effect on seed germination of groundnut (*Arachis hypogaea* L.), Wolaita Sodo, Southern Ethiopia

Krishna Moorthy Sivalingam

Associate Professor, Department of Biology, College of Natural and Computational Sciences, Wolaita Sodo University, Wolaita Sodo, Ethiopia. Email: skrish.microbiologist@gmail.com

Abstract:

Spirulina can play an important role in human and animal nutrition, environmental protection through wastewater recycling, energy conservation and plant Biofertilizer. The present study was mainly focused on the isolation, identification and evaluation of Spirulina platensis for its effect on seed germination potential groundnut *Arachis hypogaea* L. In this present study totally 50 samples were randomly collected from different water source such as stagnant water, spring water and river in and around Wolaita Sodo University. After the isolation of cyanobacterium with spiral structure, further identified as *Spirulina platensis* based on the morphometric characters using microscopic observation. The characteristic features of the isolate *S. platensis* were evaluated by inoculating the isolate in to five different media such as BG11, BG11 modified, Bold Basal Media (BBM), Zarrouk's media, Conway media (CM) and F/2 media; at different pH (pH-6, pH-6.5, pH-7, pH-7.5, pH-8, pH-8.5, pH-9, pH-9.5, pH-10) and at different temperature such as 16^0C and 25^0C to find out the optimum conditions to maximize massive production of *Spirulina platensis*. The liquid form of *Spirulina platensis* was used to determine the plant growth promoting efficiency using *Arachis hypogaea* L. (groundnut) by seed germination experiment. The *Spirulina platensis* showed excellent growth in Zarrouk's medium with pH value of 8.0 and at 25^0C temperature. The cyanobacterium *Spirulina platensis* at 5% (high) concentration level showed significant effects in the aspects of morphological parameters such as seed germination percentage, radicle length, coleoptile length and epicotyls length and biochemical parameters, like protein and carbohydrates when compared to control. Based on finding of present study results, the Spirulina platensis is possible to mass cultivate by using Zarrouk's medium at pH-8.0 and at 25^0C. The non-heterocystous cyanobacterial isolate *Spirulina platensis* can be used as bio-stimulant to improve the seed germination status of *Arachis hypogaea* L. seed.

Keywords: Cyanobacteria, *Spirulina platensis*, Seed germination, Groundnut, Zarrouk's Medium

Introduction

Ethiopia is one of the most populated country in Africa with a population of 100.6 million UNICEF (2019). Even though Ethiopia has faster growing economy in the African continent, it still remains poorest, (World Bank, 2017). The agriculture sector contributing more towards to the poverty reduction. Agriculture sector is the mainstay or pillar of the Ethiopian's economy and therefore this sector regulates the growth of all the other sectors and subsequently the whole national economy of Ethiopia. Crop production alone taking 60% of the agriculture sector's total output whereas livestock 27% and others taking 13%. While the agriculture sector is an important part of the Ethiopian economy, particularly in rural, suburban and urban zones, where 55 percent of the women and 83 percent men are working in agriculture especially involved in-crop cultivations (CSA and ICF, 2016, and World Bank, 2017).

Fertilizers usage has progressed vigorously from about 3,500 tons consumption level during 1970s seasons (NFSAP, 2007) and further the consumption level increased up to 450,000 tons in 2008 cropping season in Ethiopia (World Bank, 2008). Several study reports carried out at different locations of Ethiopia indicated that the indiscriminate applications of chemical fertilizers have adverse effects on soil health which leads to unsustainable yield (Yasin, 2015).

Microbial fertilizers or biofertilizers are one of the most essential components of organic agriculture, play an important role in the plant nutrition, sustainable soil fertility with eco-friendly way and cost effective. Different kinds of microorganisms such as bacteria, fungi and algae can be utilized for the production of biofertilizers (Lucy et al., 2004 and Vessey, 2003). Among these various kinds of microorganisms, algae especially cyanobacteria placed in the first place. Cyanobacteria or Blue green alga" are gram negative, nitrogen fixing, oxygenic, photosynthetic prokaryotic, aquatic microorganisms with wide range of diversities (Olsen, 2006). They are highly adaptable to the environment and can be found in soil, rocks, and most water bodies, ranging from hot springs to the cold water of Antarctic lakes and low-nutrient freshwater environments. As part of the aquatic environment ecology, cyanobacteria play an important role in the ecosystem maintenance. Photosynthesis of bacteria provides oxygen, while nitrogen-fixing cyanobacteria provide atmospheric nitrogen for another organism (Beck *et al*., 2012).

Cyanobacteria are one of the major modules of the potential sources of nitrogen fixation and convert it into a bio available ammonia form as well as plant growth regulators required for plant growth. These organisms have a unique potential to enhance productivity in a variety of agricultural and ecological situations and they play an important role in building up soil fertility, consequently increasing the yield (Parry *et al.,* 2011). Cyanobacteria play a vital role in build-up and maintenance of soil fertility, consequently increasing growth and yield as a natural biofertilizer (Song *et al.,* 2005). Hence the present study mainly focused on the isolation and identification of

cyanobacterium *Spirulina platensis* and its germination effects on using *Arachis hypogaea* L.(groundnut) as an experimental crop.

Material and Methods

Sample source and sample collection

For the isolation of cyanobacterial cultures, total of 50 samples of fresh water and high wet soil which having appropriate cyanobacterial colonial growth was collected randomly by using phytoplankton net with mesh size 20 µ from different locations of in and around Wolaita Sodo University. All the above said samples were collected in clean plastic bottles or glass container with screw cap number and were brought to the Wolaita Sodo Biology Department Post Graduate Microbiology laboratory for isolation and purification of the Blue green algae.

Isolation and purification of *Spirulina platensis*

The water sample were inoculated after serial dilution into the BG11 agar medium for isolation of *Spirulina* and incubated in a growth chamber with light 1500lux of 16:8h of light and dark. The purity of the culture was ensured by repeated inoculation by streaking techniques. The BG11medium were prepared in sterilized distilled water and the initial pH was adjusted to 7.2 (Allen and Stinier, 1967; Castenholz, 1992).

Identification of Spirulina platensis

Identification of cyanobacterium *Spirulina platensis* were done microscopically based on morphological observation, the length and the width of the vegetative cells also the width of the sheath, type of spores, presence or absence of hormogonia, presence or absence of spores and its position, presence of akinetes and its type, the nature of cell type of coil and helix- shape, presence or absence of gas vacuoles, as well as pigment colour were taken in consideration according to Desikachary, (1959), Iyengar and Desikachary (1981), Cronberg and Komárek (2004), Komárek (2005), Koma´rek and Hauer (2013), Khare *et al.* (2014), Komárek *et al.,* 2014.

Characterization of Spirulina platensis

The characteristic features of the isolate *S. platensis* were carried out by inoculating in to five different media such as BG11, BG11 modified, Bold Basal Media (BBM), Zarrouk's media, Conway media (CM) and F/2 media; at different pH (pH-6, pH-6.5, pH-7, pH-7.5, pH-8, pH-8.5, pH-9, pH-9.5, pH-10) and at different temperature such as 16^0C and 25^0C to find out the optimum conditions to achieve massive production. The growth of *Spirulina platensis* in different media, different pH and different temperature was determined by biomass and chlorophyll *a* content. Biomass concentration (gl-1) was calculated by measuring dry weight. For dry weight measurement homogenous suspensions of known quantity of *Spirulina* sample were filtered through screen-printing paper and oven dried at 75oC for 4 to 6hours. The dried filter paper containing Spirulina biomass were cooled and weighed. The difference between the initial and final weight were taken as the dry weight of Spirulina biomass. The dry weights were expressed in terms of g/l (Jai Prakash and Tiwari, 2010). Chlorophyll *a* was estimated by the Mackinney method (Mackinney, 1941). The mass cultivation of *Spirulina platensis* was carried based on the results obtained from the characterization of the isolate using 1L capacity conical flasks.

Pre-treatment/ Seed Germination Experiment using plate method

The *Arachis hypogaea* L. seeds were collected from local market. Seeds were surface sterilized with 70% ethanol or 0.1 % $HgCl_2$ for 3 min. 10 viable seeds in each plat was tested for each cyanobacterial aqueous extract (non-nitrogen fixers). Seeds, without cyanobacterial extract were served as control. Each Petri dish contain ten surface sterilized seeds was placed on filter paper and moistened with 10 ml of the aqueous extract of cyanobacterial isolates in different concentrations like 1% (1gm/100ml), 2% (2gm/100ml), 3% (3gm/100ml), 4% (4gm/100ml) and 5% (5gm/100ml). Petri-dishes containing seeds with 100 ml of distilled water served as a control. The growth parameters including germination percentage, radicle length and coleoptile length were recorded on the 2 days interval up to 8 days after incubating seed at 28^0C (Pitchai *et al.,* 2010; Krishna Moorthy and Kibrom Delfe. 2019). The Biochemical parameters such as Carbohydrate (Yemm and Willis, 1954) and Protein (Lowry *et al.,* 1951) were also analysed by using standard protocol at 2 days interval.

Data and Statistical Analysis

The measurements of growth and biochemical parameters were subjected to one-way analysis of variance (ANOVA) technique (Origin pro software package 7.0) and mean separations were adjusted by the Multiple Comparison test. Means were compared by using Fisher's LSD test at $p<0.05$ level of significance. All the data included in the figures were presented in mean and standard error (±) of mean of three replicates per treatment and repeated three times.

Results

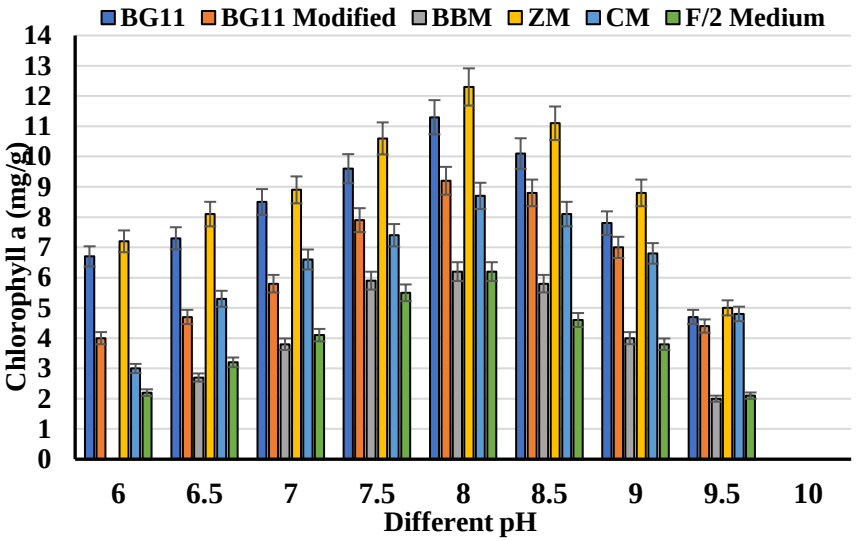
Isolation and identification of cyanobacteria

The isolated cyanobacterial culture was identified as *Spirulina platensis* based on the following morphological microscopic characteristics features. The isolated cyanobacterium appeared as a coiled thread of

cells (trichomes) and cells were unicellular with motile. Individual trichomes were essentially comprised of single cells that spiral down its entire length. Species are differentiated in part based on the tightness of the spiral. Like many blue-greens, *Spirulina* species extruded mucilage which flows along the length of the thread. This mucilage creates movement which was observed microscopically.

Characterization of isolated cyanobacterium Spirulina

The effect of different media at different pH on the chlorophyll a content of the isolated cyanobacterium is clearly displayed in the Fig.1. The maximum enhancement in the chlorophyll a content (12.3±0.2mg/g) was observed in the *Spirulina* culture inoculated with Zarrouk's medium followed by BG11 medium and BG11 modified medium with respective value of 11.31mg/g and 9.5 mg/g chlorophyll a content at pH-8. The content was decreased when the pH of the media decreased. The isolated cyanobacterial growth was absent in all the media such as BG11, BG11 modified, Bold Basal Media (BBM), Zarrouk's media, Conway media (CM) and F/2 media at pH 10 (Fig.1.).



*Data are the means of three replicates and Error bars represent the standard errors of the means

Figure 1: Effect of different media at different pH on the growth of *Spirulina platensis* using chlorophyll *a* content

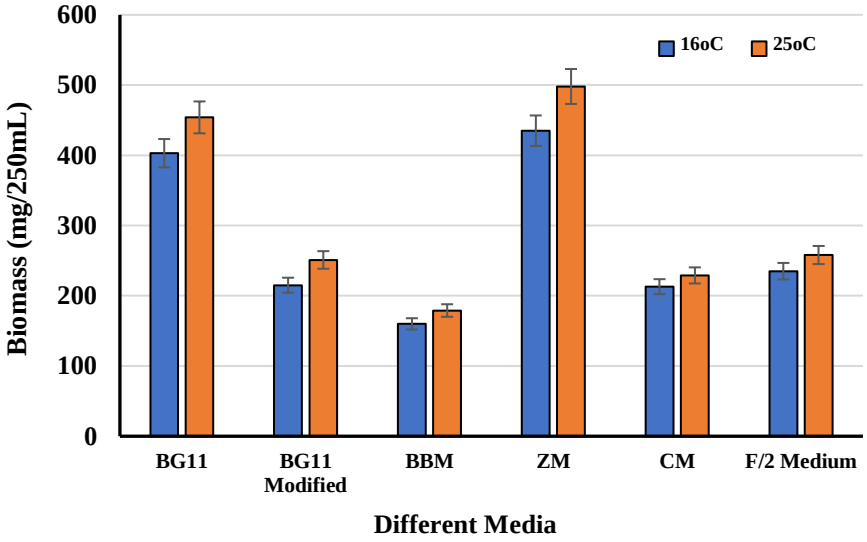
The biomass production was started to increase from the pH value of 6 and reached maximum at the pH value of 8.0. Similarly, the biomass level of *S. platensis* was found to decreased after the pH value of 8 and reached 0 at pH-10 in all the media. The maximum biomass (329±12mg/250mL) of *Spirulina* was found to be in the BG11 followed by BG11 medium, Conway medium, BG11 modified medium, F/2 medium and Bold Basal Medium with respective biomass values of 300 ± 10mg/250mL, 250 ± 11mg/250mL (Table.1).

Table 1: Effect of different media on the biomass production of *Spirulina platensis* at different pH under laboratory condition on 25th day

pH	Biomass Production Using Different Media (mg/250mL)					
	BG 11	BG 11 modified	Bold Basal Media (BBM)	Zarrouk's medium	Conway medium	F/2 Medium
6	177 ± 14	60 ± 18	15 ± 10	97 ± 13	78 ± 16	60 ± 12
6.5	193 ± 11	105 ± 20	45 ± 15	214 ± 10	152 ± 12	99 ± 15
7.0	205 ± 9	154 ± 16	91 ± 17	235 ± 19	191 ± 20	127 ± 16
7.5	245 ± 13	210± 12	132 ± 11	280 ± 14	215 ± 14	171 ± 10
8.0	300 ± 10*	245 ± 14*	163 ± 16*	329 ± 12*	250 ± 11*	193 ± 13*
8.5	268 ± 16	223 ± 19	120 ± 20	293 ± 16	221 ± 17	144 ± 19
9.0	207 ± 15	185 ± 10	101 ± 12	232 ± 19	195 ± 15	118 ± 11
9.5	84 ± 12	65 ± 13	41± 17	90 ± 15	52 ± 10	65 ± 14
10.0	--	--	--	--	--	--

Values are the mean of three replicates ± SEM.
* - Indicates significantly higher results over all ($p<0.05$)
-- - No Growth

Temperature is one of the important factors for cyanobacterial biomass production. Here in this present study two different temperatures (16^{0}c and 25^{0}C) were used to determine the optimum temperature range for the maximum biomass production. The isolated cyanobacterium *Spirulina platensis* inoculated in Zarrouk's medium as well as in BG11 medium reached maximum amount of biomass with respective values of 498±22mg and 454±15mg at 25^{0}C when compared to all other media at 25^{0}C as well as at16^{0}C (Fig. 2).



*Data are the means of three replicates and Error bars represent the standard errors of the means

Figure 2: Effect of different temperature on the biomass production of *Spirulina platensis* under laboratory condition on 25th day

Effect of Spirulina platensis on seed germination experiment by plate method using Arachis hypogaea L. as an experimental crop

Morphological parameters

The results in the Fig.3. shows that the seed germination percentage increased progressively throughout the period in all plates inoculated with different concentrations of cyanobacterial culture extracts of *S. platensis*. All concentrations of aqueous extracts (1%, 2%, 3%, 4% and 5%) of *S. platensis* showed significantly higher level of seed germination percentage when compared to control (Only distilled water). The seeds treated with only distilled water showed only 60% of germination even on 8th day of incubation. The cyanobacterial aqueous extracts 5% concentration showed 100% of seed germination on 8th day of incubation while the other concentrations like 1%, 2%, 3%, 4% showed only 60%, 80%, 80% 90% percentage of seed germination respectively.

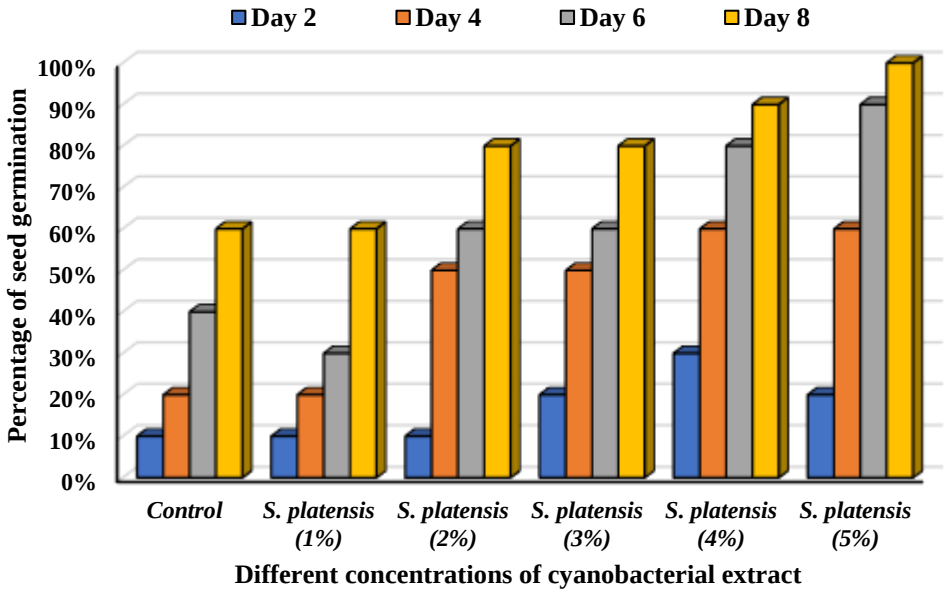


Figure 3: Effect of different concentrations of aqueous extract of *Spirulina platensis* on percentage of seed germination of *Arachis hypogaea* L. Under seed germination experiment (8th day)

The changes in the radicle length are showed in the Fig. 4. The cyanobacterial culture of *Spirulina platensis* in all the five concentrations of aqueous extracts (1%, 2%, 3% 4% and 5%) showed significantly higher results when compared to control. The highest radicle length was observed in the plates inoculated with *S. platensis* at 5% concentration level followed by 4%, 3%, 2% and 1% concentration level of aqueous extracts. The very least level of radical length was observed in the plates treated by distilled water that is control.

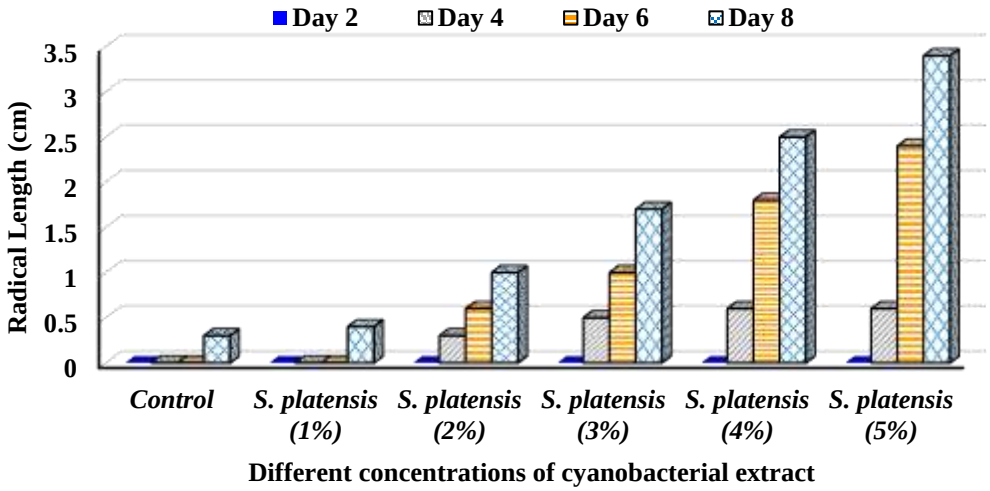


Figure 4: Effect of different concentrations of aqueous extract of *Spirulina platensis* on radical length of *Arachis hypogaea* L. under seed germination experiment (8th day)

The changes in the coleoptile length of the *Arachis hypogaea* L. seed by different concentrations of aqueous extracts (1%, 2%, 3%, 4% and 5%) of *Spirulina platensis* cyanobacterial culture are presented in the Fig.5. The very least coleoptile length of *A. hypogaea* L. seed was observed in the control when compared to different concentrations of cyanobacterial treatment even at 2nd, 4th, 6th and 8th day of incubation. The cyanobacterial isolates *S. platensis* showed maximum coleoptile length at 5% concentration of aqueous extracts when compared to *S. platensis* with rest of the other concentrations such as 4%, 3%, 2% and 1%.

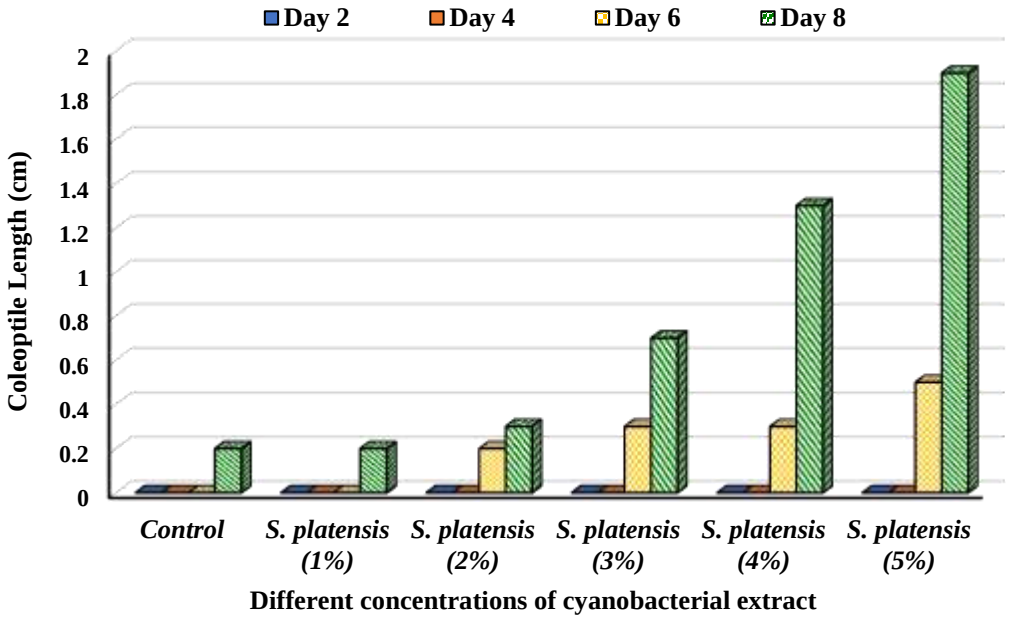


Figure 5: Effect of different concentrations of aqueous extract of Spirulina platensis on coleoptile length of Arachis hypogaea L. under seed germination experiment (8th day)

Biochemical parameters

Protein is one of the reserve foods in the *Arachis hypogaea* L. All the stored forms of reserved foods are hydrolysed during the germination process. Hence, the study about protein changes in the seed germination experiments is more important. Here in this present study, seeds from all the experiments were analysed properly

and presented in the forms of figures in this result section. Fig.6 showed clearly about the changes of protein content of all the treatments including control from 0th day to 8th day. The protein contents of *A. Hypogaea* seeds in the control treatment were not decreased in high level from 0th day to 8thday. The cyanobacterium *Spirulina platensis* in five different concentrations (1%, 2%, 3%, 4% and 5%) showed significant changes in the protein content when compared to control. The maximum level of protein reduction was observed in the seeds treated with *S. platensis* at 5% level of concentration when compared all other concentrations and control.

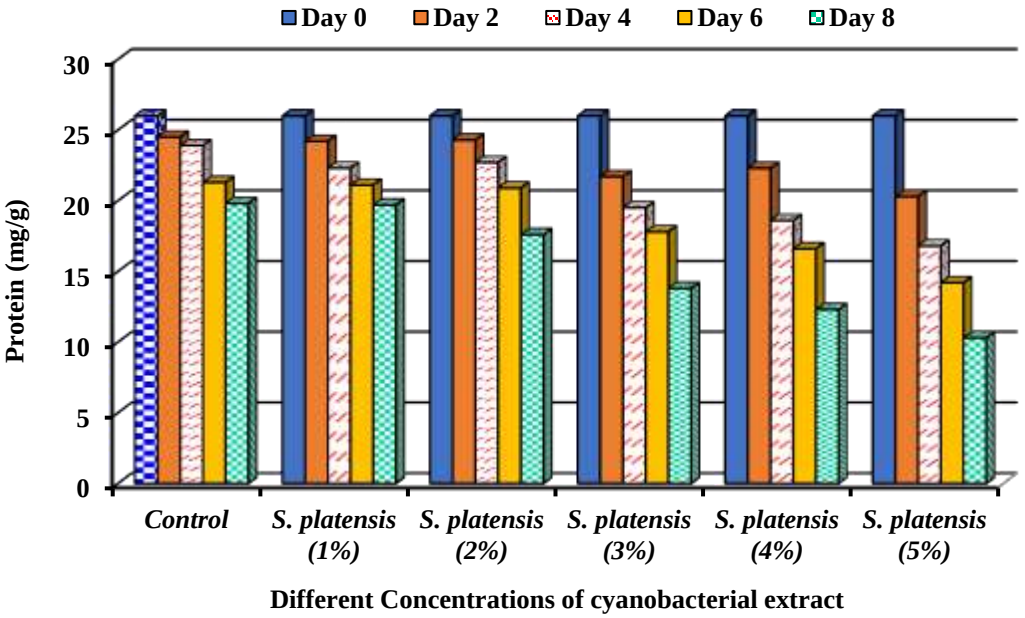


Figure 6: Effect of different concentrations of aqueous extract of *Spirulina platensis* on Protein content of *Arachis hypogaea* L. under seed germination experiment (8th day)

Carbohydrates content of all the treatments were analysed properly and displayed in the form of Graph (Fig. 7). The changes in the carbohydrate content of control were significantly lesser than all other treatments in all the concentrations even at the 8th day incubation. The maximum amount of carbohydrate reduction was observed in the treatment of *Spirulina platensis* at 5% level of concentrations which is significantly higher than control and all other concentrations.

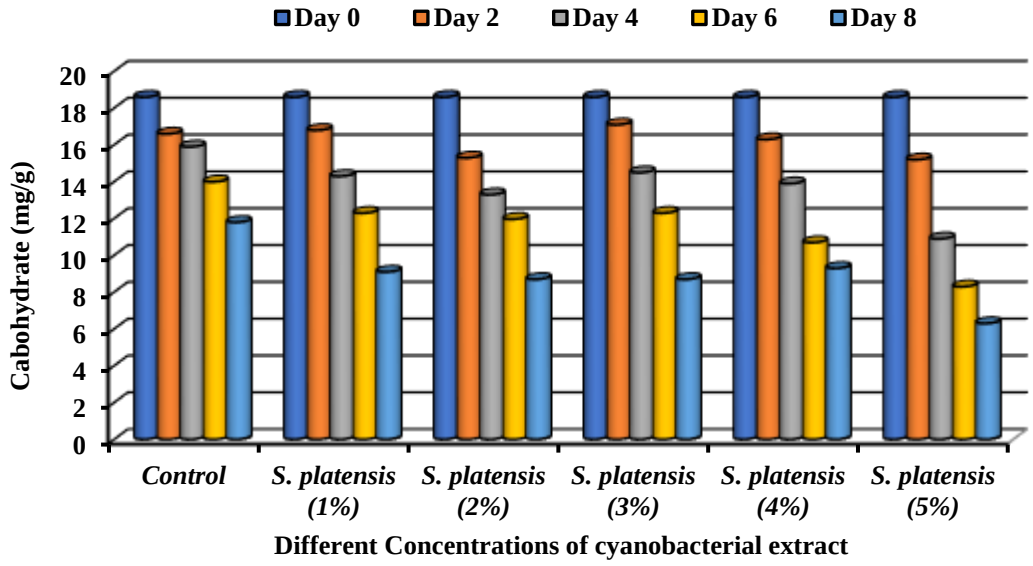


Figure 7: Effect of different concentrations of aqueous extract of *Spirulina platensis* on Carbohydrate content of *Arachis hypogaea* L. under seed germination experiment (8th day)

Discussion

Isolation and identification

From all the collected samples, total of 3 samples were positive with *Spirulina* and was isolated, identified based on the morphometric characteristic's features such as morphology of cells and filaments, shape of the terminal cells, presence or absence of sheaths, gas vacuoles, motile hormogonia, nitrogen-fixing heterocyst's and resting akinetes/spores using microscope. The cyanobacterial identification process of current study is highly supported by Gomont, (1892), Komárek and Anagnosti-dis (1998 & 2005). Similarly, Stanier *et al.* (1978); Castenholz and Waterbury, (1989) used morphological, biochemical, genetic, physiological and ecological characteristics for the identification of cyanobacterial identifications. The morphology of cyanobacteria is diverse, and species are sometimes difficult to identify because of a high phenotypic plasticity. They may be unicellular or may have cells arranged in colonies. Some cyanobacteria form filaments with none, false or true branches (Graham and Wilcox, 2000). The heterocyst's and non-heterocyst's cyanobacterial isolates were identified based on the morphological characters using microscope in 10x, 40x magnifications by Mayur Gahlout (2017); Krishna Moorthy and Kibrom Delfe (2019) and Krishna Moorthy *et al*., 2019.

Characterization of isolated cyanobacterium Spirulina platensis

Six different media such as such as BG11, BG11 modified, Bold Basal Media (BBM), Zarrouk's media, Conway media (CM) and F/2 media were used to evaluate the isolated *Spirulina platensis* at different pH and different temperature. This experiment is too important to find out the best media with optimum pH and temperature to cultivate mass under laboratory. The isolated *Spirulina platensis* showed maximum growth in Zarrouk's media at pH-8.0 when compared to BG11, F/2 medium and sea water medium. Similarly, Jai Prakash et al. (2010) investigated the effect of six different media [Zarrouk media, Rao's media, CFTRI media, OFERR media, Bangladesh Medium No. 3 and Revised Medium 6.] on the growth of *Spirulina maxima.* The highest biomass production, highest protein content and chlorophyll *a* content was found to be in the Zarrouk's medium when compared to other media. Similar to the present study, the *Spirulina maxima* yielded highest biomass and more chlorophyll *a* in the Zarrouk's medium at alkaline pH (Jai Prakash and Amit Tiwari, 2010). Similar several other studies have also been done by Kim et al. (2007), that support Zarrouk's medium is the best medium for production of *Spirulina platensis*

Determination of Growth promoting efficiency of cyanobacterial isolates on Arachis hypogeal using seed germination experiment by plate method

Morphological parameters

All the concentrations of cyanobacterial aqueous extracts such as 1%, 2%, 3% 4% and 5% showed significant response in the percentage (%) of seed germination in all different concentrations of aqueous extracts when compared to control. The reason for this great response is naturally cyanobacteria having the potential to release the plant growth hormones like auxins, cytokinins and gibberellins. These plant growth hormones directly involved in the seed germination and increased the percentage of seed germination. This result was highly supported by Osman *et al.* (2010), who reported that the cyanobacteria play a major role in the in the seed germination by secreting phytohormones like auxins, cytokinins and gibberellins. The present study results are well supported by Mayur et al. (2017) who reported that the cyanobacterial isolates *Rivularia* spp., *Nostoc* spp., *Oscillatoria* spp., *Closterium* spp., *Gloeothece* spp., *Anabaena* spp., *Aphanocapsa* spp. and *Gloeocapsa* spp. showed positive effects on the seed germination rate of wheat. Similarly, Krishna Moorthy & Gibrome (2019) who reported that the seed inoculated with cyanobacterial isolates such as *Pseudanabaena* sp., *Phormidium* sp., *Geitlerinema* sp., *Lyngbya* sp., *Spirulina* sp. and *Calothrix* sp., (heterocystous) reached maximum of 100% in the 6th day of incubation at respective concentrations of 1%, 2%, 2%, 3%, 2%, and 3% of aqueous extracts.

The *Arachis hypogaea* L. seeds treated with cyanobacterial isolates *Spirulina platensis* at 5% level of concentration showed significantly higher in radicle length and coleoptile length than that of control and all other cyanobacterial aquatic extract concentrations on 8thday of incubation. All the concentrations of cyanobacterial isolate *Spirulina platensis* such as 1%, 2%, 3%, 4% and 5% showed significantly higher results in case of radicle and coleoptile when compared control where seeds were treated with only distilled water. The reason for this great response in the radicle and coleoptile length is naturally cyanobacteria having the potential to release the plant growth hormones like auxins, cytokinins and gibberellins. Present study was supported by Gayathri *et al.* (2017) who reported that the cyanobacterial isolates *Scytonema bohneri* MBDU 104 (80%), *Aphanothece stagnina* MBDU 803 (66.6%), *Calothrix* sp. MBDU 901(66.6%), *Nostoc microscopicum* MBDU 102 (56.6%) and *Dolichospermum spiroides* MBDU 903 (70%) showed comparatively increasing responses (radicle, plumule and total seedling responses) than control (53.3%). The cyanobacterial isolate *Geitlerinema* sp. AK-3 at 2% concentration showed highest radicle length, coleoptile length and epicotyl length when compared to control and all the other cyanobacterial isolates such as *Pseudanabaena* spp. AK-1 and *Lyngbya* spp. AK-2 (Krishna Moorthy *et al.,* 2019).

Biochemical parameters

The protein and carbohydrate contents of *Arachis hypogaea* L. seeds in the control treatment was not decreased in high level from 0th day to 8th day. The maximum level of protein and carbohydrate reduction was observed in the seeds treated with *Spirulina platensis* at 5%level of concentration followed by *S. platensis* at 4% and 3% concentration. Protein and carbohydrate are the reserve food materials in the *Arachis hypogaea* L. seeds. On seed hydration, the seeds containing protein and carbohydrates acted as energy sources. So, during the seed germination all these protein and carbohydrate based reserved food materials may be hydrolyzed by hydrolytic enzymes and converted in to simple available form for embryo uptake. So, during the seed starts to germinate, the protein and carbohydrates level will be reduced automatically.

This result was highly supported by Bewley and Black (1985). Mayer and Poljakoff – Mayber (1989); Salisbury and Ross (1991) and Shutov and Vaintraub (1987) who are all reported that the seed received hydration, separate intercellular bodies of seed stored carbohydrates, proteins, lipid and phosphate act as energy source and carbon skeleton. Seed imbibition triggered many metabolic processes such as activation or freshly synthesis of hydrolytic enzymes which resulted in hydrolysis of stored starch, lipid, protein hemicelluloses, polyphosphates and other storage materials into simple available form for embryo uptake. The Blue green algal (*Cylindrospermum muscicola* and *Anabaena oryzae*) extract influences the activity of hydrolytic enzymes such as amylase, protease and amino transferase activities were progressively increased the utilization of reserve food materials in the seed (Haroun and Hussein, 2003). Similar to the present study results, the research done by Krishna Moorthy et al. (2019), who showed the protein and carbohydrate contents of *Phaseolus vulgaris* L seeds were decreased by the treatment of different concentrations of cyanobacterial aqueous extracts.

Conclusions

Based on the findings of isolation, identification and characterization studies, the *Spirulina platensis* was very rare in the aquatic samples collected from in and around Wolaita Sodo University, Wolaita Sodo, Ethiopia, and the isolated *Spirulina platensis* can be cultured mass level by using Zarrouk's medium and BG11 medium in alkaline pH-8.0 at 25^0C. The seed germination study results directed that the cyanobacterium *Spirulina platensis* showed more active in the seed germination in aqueous extracts formulation at different concentrations. Based on the overall seed germination experiment results, it has concluded that the cyanobacterium *S. platensis*at 5% aqueous extract concentration can be used as effective liquid biofertilizers for the pretreatment of *Arachis hypogaea* L. seeds. Further the present study has to be established in Pot and Field level to conform the growth promoting efficiency of *S. Platensis* and then only the complete research will be fulfilled.

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