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Evaluation of *Spirulina platensis* as microbial inoculants to enhanced protein levels in *Amaranthus gangeticus*

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The demand for increase in food production for increasing population with adequate bioavailable nutrients has become a challenge for the agriculturists, nutritionists, biotechnologists to meet the requirements of mankind. The microbial biofertilizers are applied in the form of seaweed liquid extracts, microbial inoculants, biostimulators and biofortification agents. All these categories of microbial biofertilizers are involved in the enhancement of plant nutrient uptake and result in increase of vitamin and nutrient contents in plants producing high yields. *Spirulina platensis* is a blue green alga of cyanobacterial member and it is rich in protein. In the present study, *Spirulina platensis* is used as a biofortification agent to enhance leaf protein levels in crops such as *Amaranthus gangeticus*. Different experimental methods were followed including soaking seeds in different concentrations of *Spirulina* (5 to 30 g); soaking seeds in *Spirulina* at different time intervals (1 h – overnight); *Spirulina* in combination with biofertilizers, chemical fertilizer, organic fertilizer and Vermicompost in various proportions (25:75; 50:50; 75:25). The protein content of the yield was estimated and the study results indicated that there was significant increase in protein with biofortification of *S. platensis.*

Key words: *Spirulina platensis, Amaranthus gangeticus,* protein, dietary supplements and biofortification.

INTRODUCTION

Spirulina platensis also called as *Arthrospira* is a microscopic and filamentous cyanobacterium (Blue green algae) that has a long history of use as food. Its name derives from the spiral or helical nature of its filaments (Becker, 1993). *S. platensis* has been used as food for centuries by different populations and only rediscovered in recent years. It grows naturally in the alkaline waters of lakes in warm regions. Measuring about 0.1 mm across, it generally takes the form of tiny green filaments coiled in spirals of varying tightness and number, depending on

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the strain (Abdulquader et al., 2000). *S.platensis* is cultivated worldwide, used as a dietary supplement as well as whole food and is available in the forms of cakes, tablets, powder. It is also used as a food supplement in the aqua culture, aquarium and poultry industries (Vonshak, 1997).

S. plantensis is rich in protein (65 to 71%) and it is a complete protein with all essential amino acids. The protein of *Spirulina* can be compared to that of legumes. It has about 7% of lipids by weight. It is rich in gamma-linolenic acid (GLA), alpha-linolenic acid (ALA), linolenic acid (LA), stearidonic acid (SDA), ecicosapentaenoic acid (EPA), decosahexaenoic acid (DHA) and arachidonic acid (AA). It also possesses vitamins like B1, B2, B3, B6, B9, vitamin C, D, A and E. It is a good source of minerals like Potassium, Calcium, Chromium, Copper, Iron, Magnesium, Manganese, Phosphorous, Selenium, Sodium and Zinc. *Spirulina* contains pigments like β-carotene, Zeaxanthin, Chlorophyll, Xanthophylls, Echinenone, Myxoxanthophyll, Canthaxanthin, Diatoxanthin, 3`-hydroxyechinenone, β-cryptoxanthin and Oscillaxanthin which are phytochemicals and antioxidants (FAO, 1981).

It possesses phycobiliproteins like C-phycocyanin and Allophycocyanin (Vonshak, 1997) which are phytonutrients and antioxidants that combat cancers effectively. *Spirulina* is a natural super food, because it does not contain any preservatives, cultivated with no use of pesticides and the important thing is that it is naturally green in color and no artificial colors are added to it (Henrikson, 1994).

In the current scenario, the diet and nutrition around the world has lot of effect on wellbeing of humans. The crash diets, insufficient nutrient uptake and even the junk food culture have become order of the day and leading to health hazards. When the low nutritious foods lacking in essential trace elements loaded to human digestive system, enough quantity and quality nutrients cannot be assimilated. These conditions would lead to over eating resulting in Obesity. To this problem *Spirulina* would be a good solution with complete wholesome nutrients allowing the body to absorb (Switzer, 1982).

The increased utility of the chemical fertilizers, the cost of organic fertilizers has necessitated the production of *Spirulina* in bulk quantities to replace minerals in soil in required quantity. Organic farming methods using vermicompost and either of the two methods mentioned above have not given satisfactory results over the years (Madan et al., 1988). Hence, use of appropriate combination of fertilizers is the suggestible practice in Agriculture in order to reduce cost and get good yields (Kollerstrom and Staudenmaier 2001).

Amaranthus gangeticus is grown as leafy vegetable, and ornamentals. *A. gangeticus* shows a wide variety of morphological diversity among and even within certain species. Although the family is distinctive, the genus has few distinguishing characters among the 70 species included (Juan et al., 2007). *A. gangeticus* leaves are good sources of dietary minerals including calcium, iron, magnesium, phosphorous, zinc, copper and manganese (Tucker, 1986). Vegetables, especially leafy vegetables are important in the diet as they are micro-nutrient dense foods, rich in carotene, and minerals such as calcium, iron and Phosphorous (Ali and Tsou, 2001). In this context the present study aimed to evaluate the effect of *S. platensis* as a cost effective microbial inoculant to increase the protein content in *A. gangeticus* which is edible green leafy vegetable.

MATERIALS AND METHODS

Culturing of *Spirulina*

Spirulina culture was obtained from department of Microbiology, Andhra University Visakhapatnam, Andhra Pradesh, India. To obtain a good amount of growth, *Spirulina* was cultured in Zarrouk's medium. The medium was prepared as per composition and inoculated with *Spirulina*. Incubation was carried out up to 21 days. After 21 days the media along with culture is filtered using a Whatman filter paper No:1. The culture thus obtained as a residue on the filter paper, was sun dried for two days and weighed.

The experimental design followed for the present study was randomized block design and all measures were taken care of to reduce the measurement error. Experimental design with various methods and combinations were given in Table 1.

Estimation of protein level in *A. gangeticus*

The protein content in *Spirulina* and also in the yield on dry basis was analyzed by Kjeldahl method Pelican Kelplus – KES 12 INL (Ravi et al., 2010).

Molecular studies

The protein content of the leaf yield of *Amaranthus* plants were analyzed for both experimental and control set-ups. The set-up which has shown the highest protein in the yield was further subjected to molecular analysis by SDS–PAGE.

MALDI–MS procedure

The bands obtained from SDS-PAGE were extracted and digested for further analysis by MALDI-MS (Rauser et al., 2010). MALDI is expanded as Matrix Assisted Laser Desorption/ Ionization and by using this process the peaks were blasted.

Statistical analysis

The data obtained from the present study is processed and analysis was carried out with SPSS package and MINI tab (Version 16).

RESULTS AND DISCUSSION

Protein in diet is an important nutrient constitute and

Table 1. Field experimental set-ups.

S/N	Name of Set up	Variations						
1.	Time period Soaking (5 g of *Spirulina* in 100 ml of sterile water)	1 h	2 h	3 h	4 h	5 h	Over night	C
2.	Seed soaking in different concentrations (In 100 ml sterile water)	5 g	10 g	15 g	20 g	25 g	30 g	C
3.	*Spirulina*+Biofertilizer (S:B)	25:75	50:50	75:25	-	-	-	C
4.	*Spirulina*+Vermicompost (S:B)	25:75	50:50	75:25	-	-	-	C
5.	*Spirulina*+Organicmatter (S:B)	25:75	50:50	75:25	-	-	-	C
6.	*Spirulina*+Chemical fertilizer (S:B)	25:75	50:50	75:25	-	-	-	C
7.	Spray method (g/L)	25/5	50/5	75/5	100/5	-	-	C

*C: Control.

proteins are known as the building blocks. Sufficient protein levels are required to carry out the metabolic functions of body cells. Protein malnutrition is a major problem faced by the developing countries ((Kaniszewki and Elkner, 1990; Sainju et al., 2000).

Protein can be obtained in diet through plant and animal origins. The animal protein is highly expensive and hence dependence on plant protein has become inevitable (Butt and Rizwana, 2010). The quality and the quantity of protein either conventional or fabricated have to meet the protein requirements and improve the status both in the plants as well as human population (Maheswarulu, 2011).

Table 2 show the protein content (g/100 mg) of *Amaranthus* yield grown with *Spirulina* as microbial inoculant in various concentrations and combinations along with biofertilizer, vermicompost, organic manure, chemical fertilizer and spray method. The results were represented as mean ± standard deviation. The results of percent increase when compared with reference value were also shown in graph (Figure 1). It is evident from the tables that the protein content has been increased in *Amaranthus* in various treatments when compared with reference value of 4.98 g/100 g according to National Institute of Nutrition (NIN), Hyderabad, India.

In set up – I where the seeds were soaked for increasing time intervals that is, from 1 to 5 h and overnight, the highest protein content of leaf yield was noted in 4 h soaked sample. The highest protein in set up – II, III, IV, V, VI, and VII were observed for 20 g, 25:75, 75:25, 50:50, and 25 g/5 L concentrations and combinations respectively. In set up-I due to less surface area of the seeds the penetration of *S. platensis* hydrolysate into the seed was maximum up to 4 h. The fluctuations in this set up – I has to be subjected to further studies. In set up – II as the concentration of *S. platensis* increased there was an increase in protein till 15 g and after there was a decrease, because of saturation point.

In set up – III 25:75 ratio showed the highest protein which indicates that biofertilizers is playing a major role. As the ratio of *Spirulina* is increased the percent protein decreased, because of the synergistic action of biofertilizers. In the combination of *S. platensis* vs vermicompost highest protein percent was observed in 75:25 ratio which indicates that as the ratio of *Spirulina* increased the percent protein increased. In set ups – IV to VI equal effect of *Spirulina* and combination of fertilizers can observed (Table 2).

In the last set up that is the spray method more protein level can be observed with less concentration of *Spirulina* that is, 25 g/5 L. This effect may be attributed to the effective absorption of nutrients from *Spirulina* into the leaf directly.

Seeds of *Amaranthus*, when soaked in *Spirulina*, there was a positive increase that is, as the concentration of *S. platensis* increased, protein content of leaf yield was found to be increased. The same trend was observed for *Spirulina* + Biofertilizer, *Spirulina* + Chemical fertilizer and with spray method. However, same trend was observed in *Spirulina* + vermicompost treatment. The positive results shown from the present study has been supported by the earlier studies done by Shozeb and Aruna (2013) that is, high protein content was observed in urad (black gram) plants grown in soil treated with biofertilizer + chemical fertilizer than the plants grown in soil treated with biofertilizer alone. However, the quality of the protein has to be checked by analyzing the amino acid composition. However, the increments in leaf protein of *Amaranthus* need the bioavailability studies.

The protein in the *Amaranthus* yield was estimated by using SDS-PAGE. The results of the

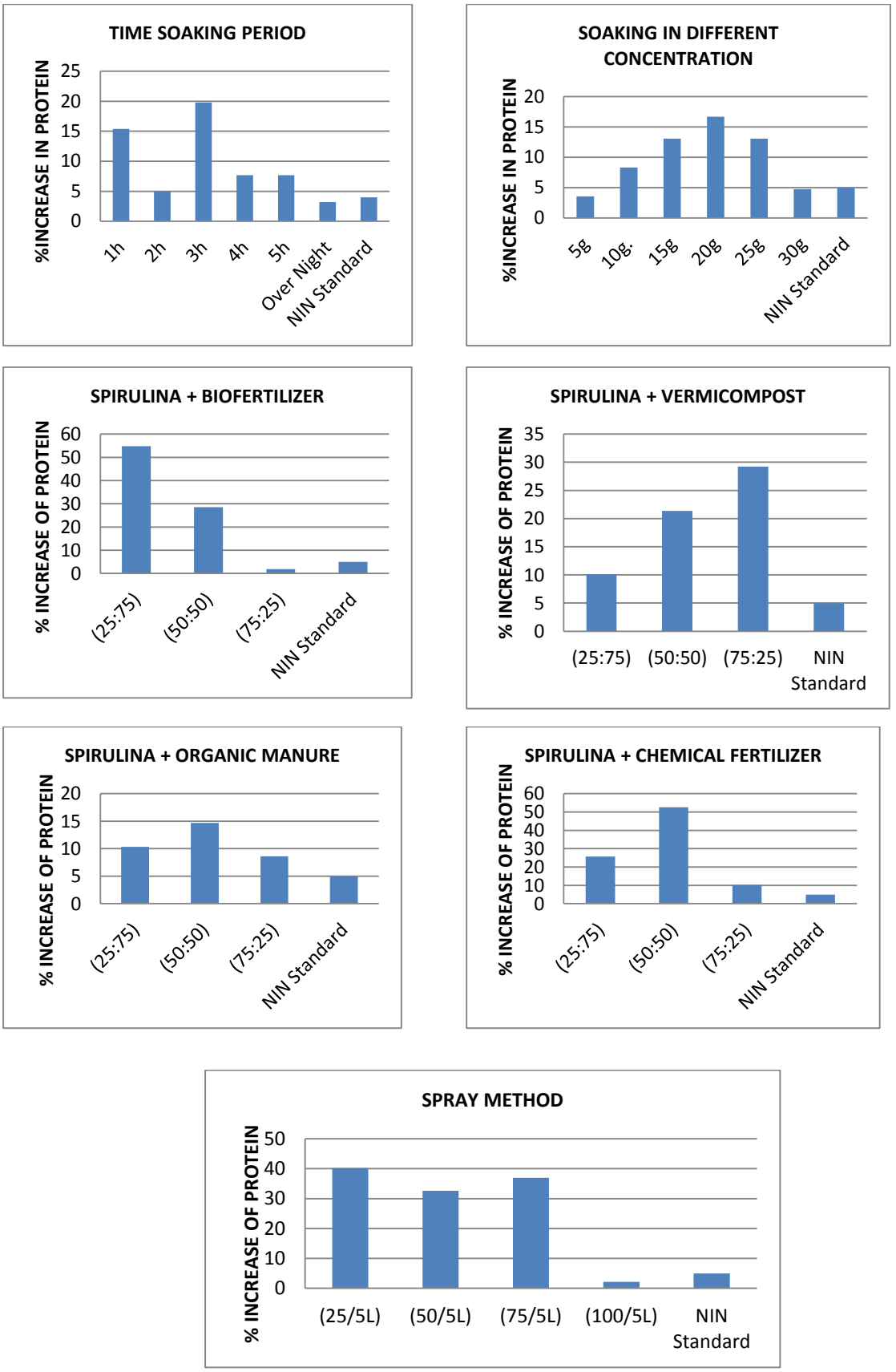

Figure 1. Percent increase of protein in different set ups as compared to reference standard.

Table 2. Protein content of the leaf yield of *Amaranthus* treated with *Spirulina*.

S/N	Treatments	Protein%
	SET-I Time period soaking	
1	1 h	7.7±0.00
2	2 h	3.2±0.00
3	3 h	7.3±7.07
4	4 h	**9.8±0.00**
5	5 h	8.4±7.07
6	Overnight	9.4±0.00
7	Control	9.1±0.00
8	NIN standard	4.98
	SET-II Soaking in different concentration	
1	5 g	8.7±0.00
2	10 g.	9.1±0.00
3	15 g	**9.5±0.00**
4	20 g	7.0±0.00
5	25 g	7.3±0.00
6	30 g	8.0±0.00
7	Control	8.4±7.07
8	NIN Standard	4.98
	SET-III Biofertilizer (S:B*)	
1	(25:75)	**13.0±0.00**
2	(50:50)	10.8±0.00
3	(75:25)	9.4±0.00
4	Control	8.4±0.00
5	NIN Standard	4.98
	SET-IV Vermicompost (S:V*)	
1	(25:75)	8.0±0.00
2	(50:50)	10.8±0.00
3	(75:25)	**11.5±0.00**
4	Control	8.9±0.00
5	NIN Standard	4.98
	SET-V Organic manure (S:O*)	
1	(25:75)	12.8±0.00
2	(50:50)	**13.3±0.00**
3	(75:25)	12.6±0.00
4	Control	11.6±0.02
5	NIN Standard	4.98
	SET – VI Chemical fertilizer (S:C*)	
1	(25:75)	9.8±0.00
2	(50:50)	**11.9±0.00**
3	(75:25)	7.0±0.00
4	Control	7.8±0.00
5	NIN Standard	4.98
	SET-VII Spray method (S/W*)	
1	(25/5 L)	**12.9±0.00**
2	(50/5 L)	12.2±0.00
3	(75/5 L)	12.6±0.00
4	(100/5 L)	9.4±0.00
5	Control	9.2±0.00
6	NIN Standard	4.98

Table 3. Band appearance on gel (in kda): Interpreted from semi log graph of mol wt vs rm.

M	C	AO	AV	AB	AC
	110	110	110	110	110
97.4					
	92	92	92	92	92
	72	72	**72**	72	72
	70	70	70	70	70
66					
	55	55	55	55	55
	45	45	45	45	45
43					
	34	34	34	34	34
29					
	14.5	14.5	14.5	14.5	14.5
	12.5	12.5	12.5	12.5	12.5

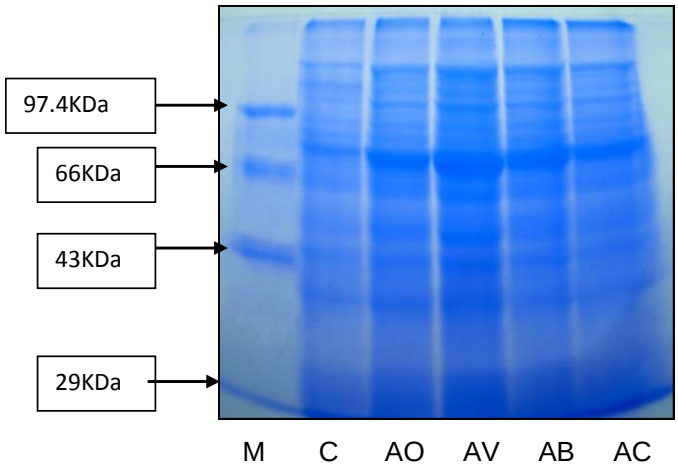

Figure 2. SDS PAGE gel showing protein bands of yield in different experimental set ups. M -Molecular weight marker (Bovine serum albumin (BSA); AO –*Amaranthus* treated with experimental set up containing *Spirulina* + organic manure in 50:50 proportions; AV-*Amaranthus* treated with experimental set up containing *Spirulina* + Vermicompost in 50:50 proportions; AB -*Amaranthus* treated with experimental set up containing *Spirulina* + Biofertilizer in 50:50 proportions; AC -*Amaranthus* treated with experimental set up with *Spirulina* + Chemical fertilizer in 50:50 proportions; C-*Amaranthus* control without adding any type of *Spirulina.*

SDS-PAGE were obtained in the form of protein bands (Table 3, Figure 2).

The protein was run on SDS PAGE has shown clearly marked bands after electrophoresis. The bands shown in the Figure 2 exhibit the highest molecular weight protein and were indicated in Table 3.

All the samples run have shown distinct bands. The bands of all samples were found prominently between 97.4 KDa regions to 66 KDa region when compared with marker protein. Among all the samples the thick band was observed for the sample AV. The molecular weight of this band was 92 KDa. After AV the order of band thickness was observed in AB with molecular weight -72 KDa, AO has molecular weight of 70 KDa and AC has molecular weight of 69 KDa respectively. All the samples run exhibited increased protein expression when compared with control. The expression levels were concluded on the basis of thickness of bands as well as intensity of stain taken (Figure 2).

The protein bands isolated by SDS-PAGE were further subjected to analysis to determine the molecular weight of proteins by MALDI. The MALDI technique results were obtained in the form of spectra with intensity shown on y-axis and the mass/charge ratio of proteins (m/z values) taken on x-axis (Figure 3a, b and c) and Table 4.

The peptide peaks obtained in MALDI spectra has shown the similarity with the *Spirulina* Phycocyanin α and β subunits with regards to molecular weight. This may be due to the supplementation of *Spirulina* to plants in combinations with other methods of fertilization (Wang et al., 2013). In the MALDI-MS spectra for sample containing *Spirulina* + Vermicompost (AV) the peptides were found to have molecular weight >10,000 Da (15103.5, 13239.0 Da) and had shown similarity with an unidentified protein CAB 69331 with molecular weight 18066 Da and another protein-peptidyl prolyl isomerase of *Escherichia coli K*-12 strain having the molecular weight 20, 418 Da (Thammasorn et al., 2009). The MALDI-MS mass spectra for the sample containing *Spirulina* + Biofertilizer (AB), the theoretical mass of the peptide was estimated as 10437.8 Da which has similarity with an *Enolase* protein from *E. coli* AAA 24486 with molecular weight 12523 Da. The control sample has the MALDI-MS spectra with molecular weight 10398.4 Da has been found to be similar with Plant protein- Albumin (nsLTP1) having molecular weight of 9748.29 Da (Del et al., 2003) was clearly shown in Table 4. As *Spirulina* and *E. coli* both are prokaryotic organisms, the protein expressed in the experimental samples might be similar with the protein found in *E. coli*.

Conclusion

S. platensis treated plants have shown increase in protein content when compared to the control and reference value. From the present study analysis, among all the different variations and combinations of *S. platensis* treated plants, the effect was best observed in the plants that were treated with soaking the seeds in different concentrations, and in the combinations of *S. platensis* with Biofertilizer and Vermicompost. At the end it is concluded that *S. platensis* which is a blue green algae can be helpful in agriculture as a biofortification agent when compared with chemical fertilizer as an enhancer of plant growth in terms of protein content.

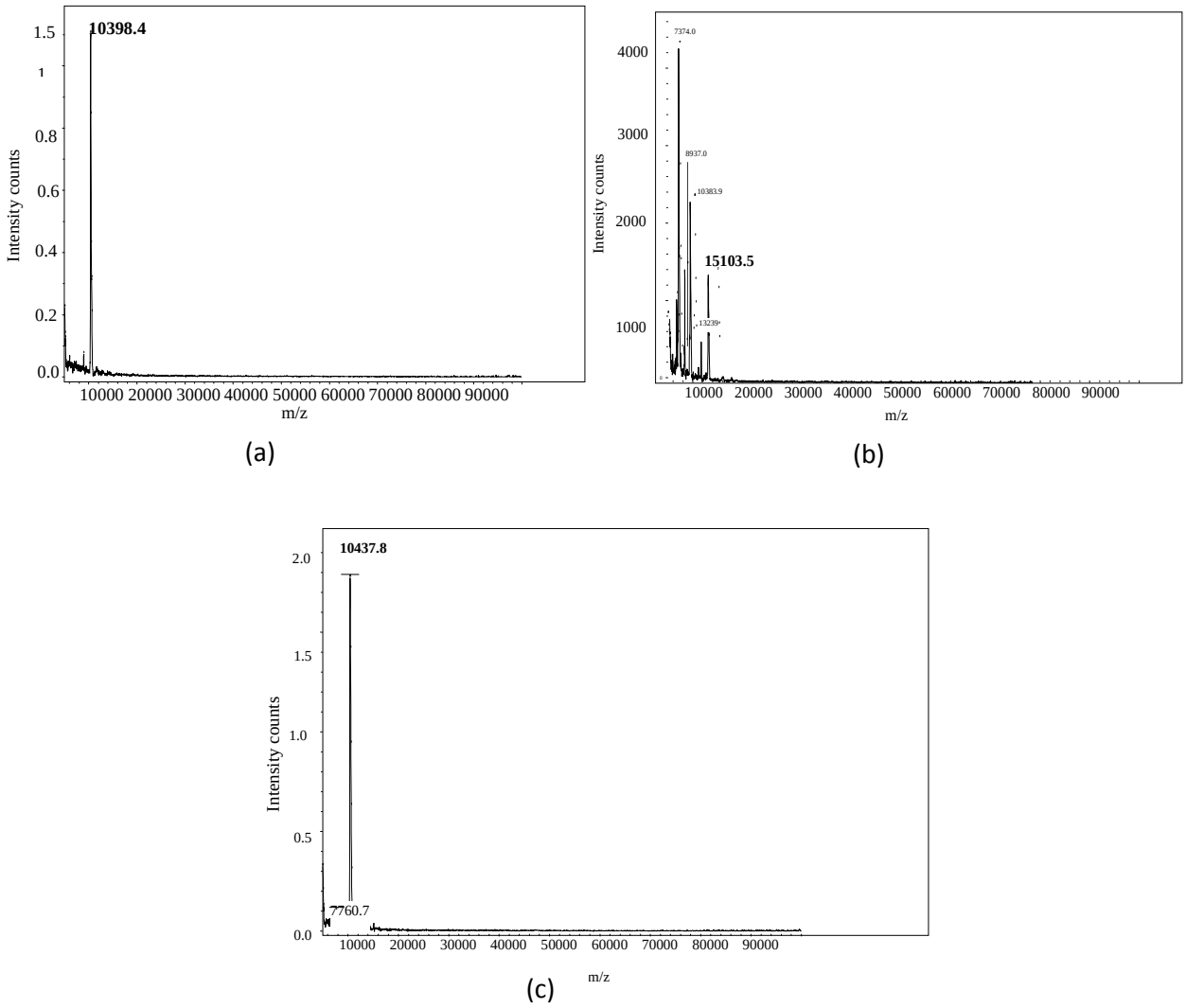

Figure 3. (a) MALDI-MS spectrum of *Amaranthus* sample control, **(b)** MALDI-MS spectrum of *Amaranthus* sample with experimental treatment containing *Spirulina* + vermicompost, **(c)** MALDI-MS spectrum of *Amaranthus* sample with experimental treatment containing *Spirulina* + biofertilizer.

Table 4. Matched sequence of protein fragment from experimental samples.

CAB69331	SEQUENCE 1 FROM PATENT WO9845454 (fragment) – unidentified	18066
AAA24486	ECOPYRG NID: - *Escherichia coli*	12523
nsLTP1	Plant protein- Albumin	9748.29

Conflict of Interests

The authors have not declared any conflict of interests.

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