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DEVELOPMENT OF MULTI STRESS TOLERANT *SPIRULINA PLATENSIS* STRAIN USING CHEMICAL MUTAGEN

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Abstract

Spirulina spp., a promising source of protein, phycocyanin and carotenoidis commercially grown inopen ponds and raceways photo-autotrophically. However, the economic exploitation in an open systemseems to have been limited because of lack of multiple stress-tolerant strains. In order to have an improved production ofprotein, lipid, chlorophyll-a, total phycobiliprotein and total carotenoid, the multi stress tolerant strains of *S. platensis* were developed from wild type of strains Sp2 (isolated from temple pond) treated with the chemical mutagen (Ethyl Methane Sulfonate).The *S. platensis* strains Sp2 treated with various concentrations of EMS (25, 50, 75 and 100 µl) at different periods (10, 20, 30, min) and survival rate were estimated. The mutant of *S. platensis* Sp2 strains(^{M}Sp2) recorded significantly increased higher amount of biomass, protein, lipid, chlorophyll-a, total phycobiliprotein and total carotenoid than the wild *S. platensis* Sp2 strain (^{W}Sp2) at all concentration of NaCl with 40°c followed by 45 and 50°c.Among the three strains (^{M}Sp2-I, ^{M}Sp2-II and ^{W}Sp2), ^{M}Sp2-II strain recorded highest growth in all combination of salinity with higher temperature followed by ^{M}Sp2-I and wild strain ^{W}Sp2. The mutant *S. platensis* ^{M}Sp2-II showed, higher biomass (0.316 mg ml^{-1}), protein t (0.196 mg ml^{-1}), total phycobiliprotein (40.45 mg ml^{-1}) and total carotenoid (10.17 mg ml^{-1})

Keywords: Phycobiliprotein, carotenoid, mutagen (Ethyl Methane Sulfonate), NaCl stress, *Spirulina platensis*, strain improvement.

Introduction

Spirulina is a multicellular and filamentous blue-green alga that has gained considerable popularity in the health food industry and increasingly as a protein and vitamin supplement to aquaculture diets. It grows in water, can be harvested and processed easily and has very high macro and micronutrient contents. It has long been used as a dietary supplement by people living close to the alkaline lakes where it is naturally found, for instance those living adjacent to Lake Chad in the Kanem region have very low levels of malnutrition. This traditional food, known as dihe, was re-discovered in Chad by a European scientific mission and is now widely cultured throughout the world (Jana *et al.*, 2014).

Strain improvement is an essential part of process developmentfor improved production of microbial products,chemical mutagenesis is a popular method frequently used toimprove the industrially important micro-organisms. Chemical mutagens at sub-lethal dose are used to produce randommutants that require desired screening. Salinity inhibits almost every process of cyanobacteria including, photosynthesis, cellular homeostasis and osmoticpressure of cell (Bhargava and Srivastava, 2013). However, in response to salinity, cyanobacteria, either accumulate compatible solutes, and/oralter their metabolic pathways, leading to either enhancementor induction of biologically active compounds (Shalaby*et al*., 2010). In *Spirulina* spp., photosynthetic oxygen evolution, respirationand growth have been shown to be inhibited by salt stress(Vonshak, 1988). Such a decrease can be associated with the state-2 transitionand a decrease in Photosystem II (PSII) activity (Sudhir and Murthy, 2004).

Salt stress has also been reported to inhibit Photosystem I(PSI) activity (Allakhverdiev *et. al.,* 2000). However, phycocyanin production isknown to be stimulated in response to salt in *S. platensis* and *S. maxima* (Pawar and Puranik, 2014). Considering that stress tolerant cultureshave more advantages in the development of open culturesystems for industrial production of metabolites (Pawar and Puranik, 2014), an attempthas been made to isolate an improved and stable NaCl and temperaturetolerant mutant of *S. platensis*from wild strainusing chemical mutagen.

Materials and Methods

Strain improvement of *Spirulina platensis* by chemical mutation (Ruengjit chatchawalya *et al.*, 2002)

The multi stress tolerant strains of *S. platensis* were developed from wild type of strains Sp2 (isolated from temple pond) treated with the chemical mutagen. Ethyl Methane Sulfonate (EMS) solution was used as chemical mutagenic agent. EMS ($CH_2SO_2OC_2H_5$) is a colorless liquid with a peppermint odor. It was obtained from HI-MEDIA

laboratory, Mumbai. It has a dosimetry/ half-life period of 30 hours.

The wild type (Sp2) *S. platensis* strains were grown at 35**°C** in Zarrouk's medium at pH 9.5 in light chamber. The cells were collected by centrifugation at 1000 rpm for 10 mins. The cells were suspended in 30mM potassium phosphate buffer (pH 7.0). For mutation, 2.5 ml of wild type strains cell suspension was treated with various concentrations (25, 50, 75 and 100 µl) of EMS and the suspension was vortexed and incubated at dark place at different time duration *viz*., 10, 20 and 30 min separately. After incubation period the EMS was inactivated by the addition of 5% Sodium thiosulfate. Survival count of *S. platensis* Sp2, strains (wild type) treated with various concentrations of EMS at different time duration.

The mutated algal cells were collected by centrifugation at 1000rpm for 10 min and transferred to test tubes containing potassium phosphate buffer (pH 7.0) the treated algal cell cultures were thoroughly washed twice with distilled water and incubated in the dark place overnight prior to plating. The plates were, incubated in light chamber at room temperature for 12 days. After 12 days of incubation the individual mutant algal colonies were observed. The maximum total viable count was observed at random and these colonies were differentiated into healthy green colonies (Dark green color filaments) and unhealthy pale green colonies (pale yellowish green filaments, due to loss of pigmentation).

Selection of multi-stress (*i.e*. higher salinity and high temperature) tolerant strains from mutant *S. platensis* colonies

The mutant healthy colonies from Sp2 (1 ml of 0.075 OD at 560 nm) standard inoculums containing 1 x 10^4cells per ml were inoculatedto Zarrouk's solid medium and incubated at various temperature (40, 45 and 50°C) in BOD incubator fitted with fluorescent light. After 12 days of incubation, healthy green colonies of multi-stress (salinity and temperature) tolerant mutant strains were selected based on their growth at such conditions.

Morphological characterization of multi-stress tolerant mutant strains of *S.platensis*

The four healthy dark green color mutant colonies were selected and characterized based on their colony morphological characters on solid medium. (*i.e.,* macroscopic view of strains) and microscopic observations like shape of the filaments, size of the filaments, length of spirals, direction of the helix and average number of spirals.Based on the morphological characters two healthy mutant colonies were selected and designated as ^{M}Sp2-I, ^{M}Sp2-II from Sp2 strain and used for further studies.

Effect of high salinity and higher temperatures on growth of wild and mutant strains of *S. platensis* - Sp2

The Zarrouk's broth was prepared with 0.3 M sodium chloride in 1000 mleach of 10 two-liter Erlenmeyer conical flasks separately. The standard inoculums (50ml) of wild type WSp2 and two mutant strains (MSp2-I and MSp2-II) were inoculated and maintained at higher temperatures (40, 45 and 50°C) in BOD incubator for 30 days. After 30days, the growth parameters such as biomass, protein, chlorophyll- a, lipid, total phycobiliprotein and total carotenoid content were estimated by various methods described earlier (Table-1)

Statistical analysis

The experimental results were statistically analyzed as suggested by Gomez and Gomez (1984). For the significant results, critical difference was worked out at 5 per cent probability level.

Experimental Results

Survival of *S. platensis* (wild type) Sp2 strains treated with various concentrations of EMS of different period (minutes)

The *S. platensis* strains Sp2 treated with various concentrations of EMS (25, 50, 75 and 100 µl) at different periods (10, 20, 30, min) and survival rate were estimated and results are presented in Table- 2. The survival rate of healthy mutant strains of *S. platensis* Sp2 was decreased with increasing level of EMS concentration (25 to 100 µl) and increasing the treatment period. The viable mutant colonies were higher in Sp2 strain. In contrast, the unhealthy cell populations increased with increasing level of EMS concentration and increasing the treatment period (10 to 20 min). The dark green color filaments refer healthy colonies, while pale yellowish green color filaments refer unhealthy colonies.

Morphological characterization of multi-stress tolerant mutant strains of *S. platensis*

The morphological characters of mutant strains like macroscopic view and microscopic observations like, shape of the filament, size of the filaments, length of spirals, direction of the helix and average number of spirals were observed and compared with their respective wild type strain by macroscopic view and microscopic observation at low and high-power compound microscope and results are showed in Table. (3).The mutant strains were designated as ^{M}Sp2-I, ^{M}Sp2-II from ^{W}Sp2 strain.

The morphological characters of mutant *S. platensis* ^{M}Sp2-I^{M}Sp2-II strains were compared with their wild strain *S. platensis* ^{M}Sp2. *S. platensis* ^{M}Sp2-I exhibited macroscopically dark green with bigger colonies, spiral shape with tightly coiled filaments, size of the filaments is too short, length of spirals (0.2 – 0.8 mm), right hand direction of helix and average numbers of spirals (2-5). The mutant *S. platensis* ^{M}Sp2-II strain showed macroscopically dark green with bigger colonies, spiral shape with loosely coiled filaments, size of the filaments is very long, length of spirals (0.5-10.0 mm) right hand direction of helix and average numbers of spirals (4-10).

Effect of high salinity and higher temperature on growth comparison between wild and mutant types of *S. platensis* Sp2

The effect of high salinity (0.3 M, NaCl) and higher temperature (40, 45 & 50°C) on growth of both wild and mutant type of *S. platensis* Sp2 was studied and results are represented in Table-4. The mutant strains of *S. platensis* Sp2 strains recorded significantly increased higher amount of biomass, protein, lipid, chlorophyll-a, total phycobiliprotein and total carotenoid than the wild *S. platensis* Sp2 strains at 0.3 M concentration of NaCl with 40°C followed by 45 and 50°C.

Among the three strains, ^{M}Sp2-II strains recorded highest growth in all combination of salinity with higher temperature followed by ^{M}Sp2-I and wild strain ^{W}Sp2. The mutant *S. platensis* ^{M}Sp2-ll showed, higher biomass (0.316 mg ml^{-1}), protein t (0.196 mg ml^{-1}),total phycobiliprotein (40.45 mg ml^{-1}) and total carotenoid (10.17 mg ml^{-1}) followed by ^{M}Sp2-I at 40°C and 0.3M NaCl. Whereas the lower amount of biomass (0.273 mg ml^{-1}), protein (0.179 mg ml^{-1}), lipid (26.98 mg ml^{-1}), chlorophyll-a (25.61 mg ml^{-1}) total phycobiliproteins (38.18 mg ml^{-1}) and total carotenoid (8.87 mg ml^{-1}) were recorded in wild type of *S. platensis* Sp2 at 0.5M NaCl and 50°C). Among the two mutant strains, ^{M}Sp2-II *S. platensis* strain exhibited the best growth with multi stress tolerant ability and selected for further studies.

Discussion

Strain improvement of *S. platensis* Sp2 by chemical mutagenesis

Lin *et al*, (2010) have developed the two thermal-tolerant mutants of *chlorella sp*. MT-7 and MT-15. In indoor cultivation, specific growth rate of the mutants were higher than wild type *Chlorella sp.* The carbon dioxide fixation rate of both microalgae mutants was also significantly higher than that of wild type. In outdoor closed cultivation, where the temperature of culture broth was $41+1^{0}$C, the specific growth rate of mutant strain MT-15 was 0.238 d-1 during the 8-day cultivation. Whereas, the growth of wild type was inhibited the outdoor cultivation.

The mutant cells of *S. platensis* after chemical mutagenesis (NTG treatment) exhibited approximately three – fold higher tolerance to metronidazole and DCMU (3, 4 – dichloropheynl-1, 1-dimethylurea) as compared with wild-type cells (Singh and Singh, 1997). Ruenjitchatchawalya*et al.* (2002) have reported the mutant of *Spirulina* (Arthrospira) *platensis* strain 122, obtained by mutagenesis with Ethyl Methane Sulfonate, was partially defective in the production of g-linolenic acid. However, when compared with the wild form, the mutant 122 strainalmost lost its capacity to grow at low temperature, although at optimal temperature growth was unaffected. Similarly, the present study reported that, the development of multi-stress (high salinity and higher temperature) tolerant mutant strains of *S. platensis* from wild *S. platensis* strains Sp2 by using chemical mutation *i.e.,* Ethyl Methane Sulphonate (EMS).

Morphological characterization of multi-stress tolerant mutant strains of *S. platensis* Sp2

Lin *et al.,* (2010) have reported that the two thermal tolerant mutant strains of *Chlorella sp*. were developed by treating chlorella sp., (1 x 10^{7} cells) with 100 mM Ethyl Methane Sulfonate (EMS) for 1 h, and plated with approximately 1 x10^{3} cells on agar plates and were incubated at 40°C. The bigger colonies were selected for outdoor cultivation.Similarly, in the present study the morphological characters of mutant S. *platensis* strains were observed by macroscopic view and microscopic observations like shape of the filament, size of the filaments, length of spirals, direction of the helix, andaverage number spiral and multi-stress (high salinity with higher salinity) tolerant ability. Based on the morphological characterization, two mutant strains were isolated and designated as ^{M}Sp2-I, ^{M}Sp2-II strains from ^{W}Sp2 strain.

Effect of high salinity and higher temperature on growth comparison between wild and mutant types of *S. platensis* Sp2

Temperature represents one of the major biological limitations for biomass production of *Spirulina.* To enhance yield of biomass it seems important to maintain the culture temperature as close as possible to the optimum. It is an important environmental factor, which affects all metabolic activities and also affects nutrient availability and uptake as well as other physical properties of cells aqueous environment (Rafiqul *et al*., 2003a). The algae failed to adapt to the higher salinity environment as more than 1.00 M NaCl (Zeng and Vonshak, 1998). The salt stress caused a decrease in dry weight, chlorophyll-a content as well as certain xanthophylls (neoxanthin and violaxanthin) while b-carotene production was stimulated especially at higher salt concentrations (Shalaby*et al*., 2010).

Abd el-Baky*et al.,* (2008) have reported that some microalgae species have well known ability to accumulate higher amount of caroteniods, phycocynin and a – tocopherol when grown under stress conditions. It seems that, the *S. maxima* algal growth was negatively associated with enhancement of carotenoids biosynthesis. The chlorophyll and phycocyanin content of the mutant *S. platensis* 122 was about 20% higher than that of the wild type. Lin *et al*., (2010) have observed the thermal tolerant mutant strain *Chlorella sp.* (MT – 7 and MT – 15) tolerated high temperature 40°C and obtained lipid content was 12.4 and 11.8% and the biomass obtained was 0.9 g L^{-1} and 1.2 g L^{-1}first day and third day respectively. The carbon dioxide fixation rate of both microalgae mutants was also significantly higher than that of wild type.

Similarly, the present study revealed that, the mutant type of *S. platensis* Sp2 strains recorded significantly increased higher biomass, protein, lipid, chlorophyll-a, total phycobiliprotein and total carotenoid than the wild *S. platensis* Sp2 strain at higher sodium chloride (0.3 M) concentration and higher temperature (40°C).

Conclusion

In the present study, a temperature and NaCl-tolerant mutant of *S. platensis*was isolated by EMSmutagenesis and selection for NaCl tolerance. the mutant type of *S. platensis* Sp2 strains recorded significantly increased higher biomass,

protein, lipid, chlorophyll-a, total phycobiliprotein and total carotenoid than the wild *S. platensis* Sp2 strain at higher sodium chloride (0.3 M) concentration and higher temperature (40°C). This result indicated that, the wild *S. platensis* Sp2 strains were unable to tolerate the high salinity with higher temperature in Zrrouk's medium.Among the two mutant strains, ^{M}Sp2-II *S. platensis* strains exhibited the best growth with multi stress tolerant ability and selected for further studies like mass production of Spirulina in waste water as well as pure water.

Table 1: Methodology adapted for estimation of biomass, chlorophyll, protein, lipid, total carbohydrates and phycocyanin content of *S. platensis*

S. No.	Parameters estimated	Methodology adapted
1.	Biomass	Pandey *et al.* (2010)
2.	Chlorophyll content	MC Kinney (1941)
3.	Protein content	Lowry *et al.* (1951)
4.	Lipid content	Foich and Lees (1957)
5.	Phycocyanin content	Horvath *et al*., (2013)

Table 2: Survival of *S. platensis* (wild type) Sp2, strain treated with various concentrations of EMS at various time duration

EMS Conc.(μ1)	Survival count colonies (X 10^3) Sp2					
	10 mins		20 mins		30mins	
	H.C.	U.C.	H.C	U.C	H.C.	U.C.
25	48	25	36	29	24	30
50	35	32	28	30	15	27
75	25	40	21	20	18	40
100	16	45	19	45	15	43

H.C. Healthy (Dark green filaments) colonies, U.C. – Unhealthy (pale yellowish green filaments colonies initial population = 1 x 10^4 E.M.S. (Ethyl Methyl Sulfonate).

Table 3: Morphological characteristics comparison between wild type and mutant type of *S. platensis* strains

Name of the strain	Type of the strains	Macroscopic view of strain	Microscopic observation				
			Shape of the filament	Size of the filaments	Length of spirals	Directions of helix	Average numbers of spirals
	^{w}Sp2	Dark green with Lawn	Spiral shape with loosely coiled	Long type		Right hand	3-12
Sp2	^{M}Sp2-I	Dark green with bigger colonies	Spiral shape with tightly coiled	Too short type	0.2 – 0.8	Right hand	2 – 5
	^{M}Sp2-II	Dark green with bigger colonies	Spiral shape with loosely coiled	Very long type	0.5 – 10.0	Right hand	5-16

^{w}S – Wild type of *S. platensis*; ^{M}S – Mutant type of *S. platensis.*

Table 4: Effect of high salinity (NaCl, 0.3M) and high temperatures on growth of wild and mutant strains of *S. platensis* – Sp2

Strains	Different Temp. (^{0}C)	Saline (NaCl) Conc. (0.3M)					
		Biomass (mg ml-^{1})	Protein (mg ml-^{1})	Lipid (µg ml ^{1})	Chl-a (µg ml $^{-1}$)	Total Phycobili proteins (µg ml-^{1})	Total Carotenoid (µg ml^{-1})
^{w}Sp2	40	0.277	0.153	27.14	27.35	36.60	8.43
	45	0.232	0.135	23.61	21.96	35.96	7.78
	50	0.192	0.102	18.37	16.95	30.25	5.65
Mean		0.232	0.129	23.06	21.13	34.64	7.13
SEd		**0.013**	**0.009**	**1.771**	**2.056**	**1.765**	**0.587**
CD (p = 0.05)		**0.027**	**0.018**	**3.543**	**4.118**	**3.531**	**1.175**
^{M}Sp2-I	40	0.273	0.179	26.98	25.61	38.18	8.87
	45	0.246	0.142	22.90	20.45	36.95	7.63
	50	0.194	0.118	18.64	15.80	31.60	5.90
Mean		0.241	0.140	22.26	20.43	35.74	7.45
SEd		**0.186**	**0.012**	**1.602**	**2.292**	**1.410**	**0.573**
CD (p = 0.05)		**0.372**	**0.025**	**3.205**	**4.587**	**2.821**	**1.147**
^{M}Sp2-II	40	0.316	0.196	26.82	25.65	40.45	10.17
	45	0.265	0.159	23.40	22.55	38.48	8.65
	50	0.204	0.115	18.25	15.87	33.09	7.78
Mean		0.275	0.150	22.95	21.13	36.49	08.61
SEd		**0.026**	**0.018**	**2.058**	**1.770**	**1.205**	**0.757**
CD (p=0.05)		**0.043**	**0.038**	**4.117**	**3.541**	**2.411**	**1.516**

^{W}S – Wild type of *S. platensis*; ^{M}S – Mutant type of *S. platensis*

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