Assiut Univ. J. of Botany
44(2), p-p37:52 (2015)

LIPOPEROXIDATION, PHENOLIC COMPOUNDS, ENDOPHENOL OXIDASE ENZYME AND PROTEIN METABOLISM IN KIRCHNERELLA LUNARIS CULTURES GROWN UNDER SALINITY

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Received: 7/7/2015 **Accepted:** 9/9/2015

This work was conducted to study the effect of salinity treatments on growth and metabolism of the green micro-alga *Kirchneriella lunaris* (Selenastraceae, Chlorophyta) isolated from sewage water treatment plant at El-Kola , Sohag district, Egypt. The levels of salinity used were 00, 50, 100, 150, 200, 250 and 300 mM. NaCl. The test organism was left to grow for 10 days under the various salinity levels. Dry mass and photosynthetic pigments were increased at the level of 50 mM NaCl,. These values were decreased at the higher concentrations of NaC l in the culture media. Phenolic compounds content was reduced at 50 mM NaCl, and then increased significantly with increasing the dose of sodium chloride. Malondialdehyde (MDA) contents were elevated at all salinity concentrations. Salinity increased the intensity and the number of isoenzyme forms of indophenol oxidase (IPOX) which represents an increase in enzyme expression and activity. All concentrations of salt used stimulated the production of proteins (soluble and insoluble and total. Salinity induced the synthesis of newly protein bands. The higher doses of sodium chloride produced a large number of polypeptides bands. The concentrations of sodium chloride from 200 to 300 mM were accompanied by the synthesis of polypeptide bands with high molecular weight.

Key words: Lipoperoxidation, Phenolic compound, Endophenol oxidase, Protein metabolism, *Kirchneriella lunaris*, Salinity.

INTRODUCTION

The salinity-induced growth reduction may be attributed to the accumulation of reactive oxygen species (ROS) [1]. ROS's are partially reduced forms of atmospheric oxygen, which are produced in vital processes such as photorespiration, photosynthesis and respiration [2]. Evidence suggests that membranes are the primary sites of salinity injury to cells and organelles because ROS can react with unsaturated fatty acids to cause peroxidation of essential membrane lipids in plasmalemma or intracellular organelles [3]. Some authors working on some salinized algae, recorded that the values of some growth criteria (Cell number, dry weight and photosynthetic pigments) were generally lowered, when compared with those of control cultures [4, 5, 6, 7].

In the halotolerant green alga Dunaliella tertiolecta, salt stress causes an increase in the intracellular lipid content and glycerol pool [8, 9], and leads to changes in the activity of antioxidative enzymes [10]. An increase in the expression levels of antioxidative enzymes was also detected in the fresh water alga Chlamydomonas reinhardtii upon NaCl stress [11].

Phenolic compounds and flavonoids are among the most influential and widely distributed secondary products in the plant kingdom. Many of them play an important physiological and ecological roles, being involved in resistance to different types of stress[12,13]. NaCl stress induced disturbances of the metabolic process leading to an increase in phenolic compounds [13] and oxidative enzymes [14].

Malondialdehyde (MDA) is the main decomposition product of polyunsaturated fatty acids in biomembranes was known to show greater accumulation under salt stress [15, 16, 17, 18]. Determining the MDA content and hence, the extent of membrane lipid peroxidation, has often been used as a tool to assess the degree of plant sensitivity to oxidative damage [19].Peroxidation of plasmalemma leads to the leakage of cellular contents, rapid desiccation and cell death. Intracellular membrane damage can affect respiratory activity in mitochondria, causing break down of pigment leading to the loss of the carbon fixing ability in chloroplasts [20].

Indophenol oxidase (IPOX) represents an important part in machinery which is responsible for detoxification of the reactive oxygen species [21]. Phenol oxidases use phenolic compounds to detoxify reactive oxygen species [22]. Indophenol oxidase expression and activity was found to increase under salinity stress [23].

MATERIAL AND METHODS

I- A- Normal and salt stress algal cultures

The alga *Kirchneriella lunaris* (Selenastraceae, Chlorophyta) was grown in BG-11 medium according to [24] under the conditions of fluorescent illumination (50 μE/m^2/s) and room temperature (25±2 ^oC). Filtered dry air was let to bubble in the culture vessels to provide carbon dioxide and to prevent settling of algal cells.

In this experiment, the alga was grown in BG-11 medium containing different concentrations of NaCl (50, 100, 150, 200, 250 and 300 mM.) for 10 days at the same conditions mentioned above. Each treatment was made in three replicates. At the end of incubation period, the algal cells were harvested and used for growth and metabolic determinations.

1-B-Determination of growth parameters

1-B-1- Cell number

The cell count of control and treated cultures was measured by Hemacytometer, 0.1 mm deep, having improved Naubauer ruling (A.O. Spencer "Bright fine"). The count was expressed as cells / ml algal suspension.

1-B-2-Dry mass

Dry mass was determined according to [25] by filtering Culture aliquots (50 mL) through Whatman GF/C filters. The filters were dried and weighed.

1-B- 3- Photosynthetic pigments extraction

Chlorophyll a, b and Caroteniod were extracted in 100% acetone at 65°C and their contents were determined spectrophotometrically (SPEKOL 11, CARL ZEISS, JENA, GERMANY) according to [26].

II-A-Biochemical determinations

11-A-1- Estimation of phenolic compounds

Phenolic compounds content was determined according to [27] using 0.1 gm of fresh tissue. Algal samples were homogenized with a plastic pestle in an Eppendorf tube containing 1 ml. phosphate buffer o.1 M pH= 7.0. The homogenate was centrifuged in an Eppendorf microcentrifuge at 12800 for 10 min. Aliquots of 50 µ L were added to a reaction mixture containing 3% of sodium carbonate and o.3 M Folin reagent in a final volume of 1 ml. The reaction mixture was incubated for 2 hr. at room temperature and the absorbance at 765 nm. Total phenolic compounds were expressed as nanoequivalents of gallic acid using a caliberation curve prepared with 10-50 µ M Gallic acid.

11-A-2-Estimation of lipid peroxidation (MDA) content.

Taken 500 mg of algal cells, and homogenized in 10 ml of 5% trichloroacetic acid (TCA), the homogenate was centrifuged at 15,000 g For 10 min. A level of lipid peroxidation was measured in terms of malondialdehyde (MDA) content using the method of [28] .To 2.0 ml aliquot of the supernatant, 4.0 ml of 0.5% thiobarbituric acid (TBA) in 20% TCA was added. The mixture was heated at 95 °C for 30 min. and then quickly cooled in an ice bath and centrifuged at 10,000 g for 10 min., the absorbance of supernatant was recorded at 532 nm. The value for non-specific

absorption at 600 nm was subtracted. The MDA content was calculated using its absorption coefficient of 155 n mol^{-1} cm^{-1} and expressed as nmol (MDA) g-1 fresh weight.

11-A-3- Protein content estimation.

Protein content was determined according to [29]. The alga of 10 mL of algal suspension was extracted in distilled-water (soluble protein) and in NaOH (total protein) for 2 h at 90°C. The extract was centrifuged and the supernatants were pooled. The water-soluble protein was estimated by the Folin-phenol reagents and measured spectrophotometrically (SPEKOL 11, CARL ZEISS, JENA, GERMANY.) Bovine serum albumin was used as a standard.

II1-A- Gene expression studies:

111-A-1- studies of Isoenzyme expression:

Numerous publications and reports on the applications of electrophoresis in the fields of isoenzyme detection were utilized in this work **[30, 31].**

111-A-2- Detection of proteins by SDS-PAGE:

The algal suspension containing 10^9cells/ml. was centrifuged at 4000 rpm for 5 min. The pellet was used for the extraction of protein by the method cited by [32] using an extraction buffer as recommended by [33]. The method of SDS vertical polyacrylamide gel electrophoresis (SDS- PAGE) was used as described by **[**32] for the determination of protein electrophoretic pattern. The gels were subjected to the staining solution for 1-2 h., followed by distaining solution overnight .There-after, the distained gels were photographed while wet.

RESULTS

The effect of NaCl treatments on cell number of *Kirchneriella lunaris* was given in table (1). The data revealed that, the cell number decreased considerably with the rise of salinity levels reaching the lowest value (23.105% that of control) at the highest level used (300 mM NaCl). Dry mass of *Kirchneriella lunaris* given in table (2) demonstrated that, the values of dry mass increased significantly at the level of 50 mM NaC l (120%) , then they slightly and gradually decreased up to the level of 300 mM NaCl (59%). At this level the percent decrease in dry mass yield was about 40% in comparison with the control. The lower salinity 50 mM NaCl stimulated the concentration of photosynthetically active pigments (chl. a, chl. b, and carotenoids) (table 3). The percent increase in chl. a, chl. b, and carotenoids was 6.887 %, 14.164 % and 3.968 % respectively as compared

with control, then significant decrease occurred up to the level of 300 mM NaCl..

Table (1): Effect of salinity treatments on cell number (cell/ ml algal suspension) percentage of control of *Kirchneriella lunaris* cultures.

	NaCl(mM)	Cell number	% of control
control	0.0	277x 10^4	100.0
NaCl conc.	50	226x 10^4*	81.6
	100	188x 10^4**	67.8
	150	152x 10^4**	54.9
	200	118x 10^4**	42.6
	250	97x 10^4**	35.0
	300	64x 10^4**	23.11
LSD	5%	36.775x 10^4	
	1%	54.408x 10^4	

*** Significant differences** ****Highly significant differences**

Table (2): Effect of salinity treatments on the dry mass (µg ml $^{-1}$ algal suspension) and percentage of control of *Kirchneriella lunaris* cultures.

	NaCl (mM)	Dry mass	% of control
control	0.0	184.6	100.0
NaCl conc.	50	222.0**	120.2
	100	176.0**	95.3
	150	169.0**	91.6
	200	146.0**	79.1
	250	131.3**	71.1
	300	109.3**	59.2
LSD	5%	17.0	
	1%	23.6	

****Highly significant differences as compared with control.**

At this level, the percent decrease of chl. a, chl. b and carotenoides was 67.951%, 83.470% and 63.828% respectively as compared with the control values.

Table (3): Effect of salinity treatments on photosynthetic pigments (mg g^{-1} dry weight) of *Kirchneriella lunaris* cultures.

	NaCl (mM)	Chl.a	%	Chl.b	%	Car.	%	Total	%
control	0.0	8.175	100.00	3.170	100.00	6.55	100.00	17.90	100.0
NaCl conc.	50	8.738	106.89	3.62*	114.16	6.81	103.97	19.21	107.1
	100	7.72	94.40	3.41*	107.60	6.71	102.46	17.84	99.7
	150	6.56*	80.28	2.91*	91.80	6.02	91.82	15.49	86.6
	200	4.21**	51.55	1.68**	52.97	3.70**	56.53	9.60 **	53.6
	250	3.11**	38.08	0.659**	20.47	3.23**	49.33	7.0 0**	39.1
	300	2.62**	32.04	0.52**	16.53	2.370**	36.17	5.51**	30.8
LSD	5%	1.21		0.07		1.733		4.08	
	1%	1.68		0.93		2.41		5.67	

*** Significant differences** ****Highly significant differences**

The concentration of phenolic compounds reduced at the level of 50 mM NaCl, then a sharp and sudden increase in these contents was exhibited by further increase in the concentration of NaCl in the culture media. At this level the percent increase was 133.33% over those of control (table.4).

Table(4) : Effect of salinity treatments on Phenolic compounds content (mg g^{-1} dry weight) of *Kirchneriella lunaris* cultures

	NaCl(mM)	Phenolic compounds	% 0f control
control	0.0	0.63	100.00
NaCl conc.	50	0.58	92.06
	100	0.65	103.17
	150	0.77	122.22
	200	0.98	155.55
	250	1.07	169.84
	300	1.47	233.33
LSD	5%	3.564	
	1%	4.946	

*** Significant differences** ****Highly significant differences**

Table (5): Effect of salinity treatments on MDA content (mg g^{-1} dry weight) of *Kirchneriella lunaris* cultures.

	NaCl (mM)	MDA	%
control	0.0	47.66	100.00
NaCl conc.	50	51.69	108.5
	100	62.21**	130.5
	150	75.77**	159.0
	200	96.45**	202.4
	250	134.8**	282.9
	300	158.6**	332.7
LSD	5%	7.814	
	1%	10.844	

****Highly significant differences as compared with control**

The data in table 5 demonstrated that, there is a marked and progressive increase in MDA content as the salinity level increase. At the dose of 300 mM NaCl the percent increase was 232.7 % over those of control values.

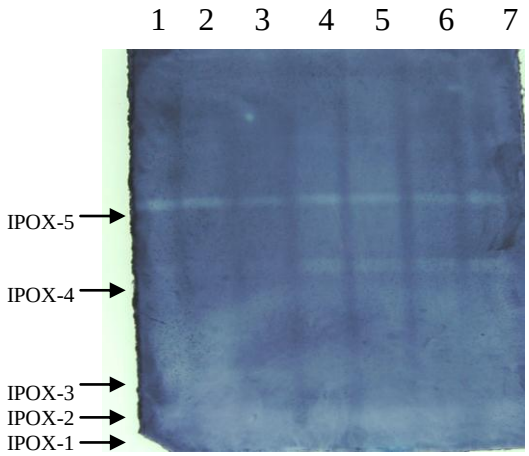

Fig. (1): Native gel electrophoresis of IPOX isoenzymes during culturing of *Kircheneriella lunaris* under the influence of different concentrations of NaCl: 50 mM(lane 2), 100 mM(lan 3), 150 mM (lane 4), 200 mM (lane 5), 250 mM (lane 6), 300 mM (lane 7) in comparison to those cultured on NaCl free medium (lane 1).

Indophenol oxidase (IPOX) expression under the influence of salinity stress was given in figure 1 .One indophenol oxidase isoenzyme form (IPOX-3) was detected only in lanes 1 (control), 2 (50 mM NaCl) and 3 (100 mM NaCl) but disappeared with the other lanes (4-7) from 150 to 300 mM NaCl). (IPOX-4) was detected in all concentrations but the density was increased from 150 to 300 mM.

The total, soluble and insoluble proteins were increased with the increasing of NaCl concentrations 238%, 249% and 224% of control (Table 6.)

Table (6): Effect of salinity treatments on protein contents [soluble, insoluble and total] (mg g^{-1} dry weight) of *Kirchneriella lunaris* cultures.

	NaCl (mM)	Soluble	%	Insoluble	%	Total	%
control	0.0	33.43	100.00	28.41	100.00	61.84	100.00
NaCl conc.	50	37.68	112.71	29.04	102.22	66.72	107.89
	100	50.82**	151.99	34.66	122.01	85.48*	138.22
	150	53.81**	160.93	35.14	123.11	88.99**	143.91
	200	62.05**	185.60	47.47**	167.11	112.53**	181.96
	250	69.54**	207.99	54.61**	192.23	124.15**	200.75
	300	83.511**	249.79	63.76**	224.42	147.27**	238.14
LSD	5%	11.73		11.36		18.57	
	1%	16.28		15.76		25.77	

*** Significant differences **Highly significant differences as compared with control**

Electrophoretic changes in protein patterns of *Kirchneriella lunaris* grown under salinity stress are summarized in Tables (7) and illustrated in Fig.(2). The number and the molecular weight of protein bands varied according to the dose of NaCl. The lower number of polypeptides bands was accompanied by the lower concentrations of NaCl. The high doses of the salt produced a large number of polypeptides bands. The high salinity levels (from 200 to 300 mM) accompanied by the synthesis of the high molecular weight polypeptide. Several polypeptides band were apparently suppressed whereas others were induced. The increase in molecular weight of polypeptide seemed to be increased by salinity stress up to 200 mM NaCl i.e. the highest molecular weight was 51, 53, 55, 94 and 318 at the level of control, 50, 100, 150 and 200 mM respectively. The appearance of polypeptides with M. wt. 318, 288, 258 and 228 occurred only at the level of 200 mM NaCl (Table7).

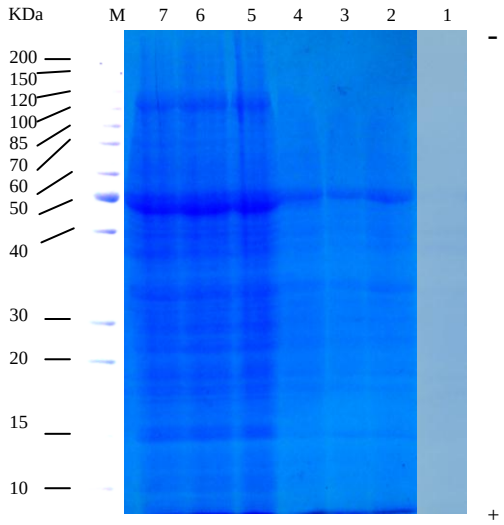

Fig.2: Coommasseel blue-stained SDS polyacrylamide gel of polypeptides of *Kirchneriella lunaris* of 6 different lines that were cultured under salinity stress. Lane M is protein marker, Lane 1 represents the control.

Table (7): Molecular weights of new induced and inhibited proteins detected in *Kirchneriella lunaris* treated with salinity. Data were obtained by Total Lab version 1.10 electrophoresis data system program

NaCl conc. (mM)	New protein bands induced under salinity stress	New protein bands inhibited under salinity stress
50	53, 48, 40, 37, 31, 28, 18, 15.	51, 46, 43, 30, 17.
100	55, 47, 40, 34, 31, 26, 25, 22, 18, 15.	46, 38, 33, 30, 17.
15o	112, 100, 94, 88, 81, 73, 67, 64, 62, 58, 55, 48, 45, 42, 40, 39, 37, 36, 34, 27, 24, 22, 18, 15.	46, 43, 41, 38, 17.
200	318, 288, 258, 228, 193, 165, 136, 119, 111, 102, 95, 87, 83, 76, 68, 66, 64, 62, 59, 48, 45, 40, 39, 37, 36, 34, 31, 28, 23, 20, 18, 15.	46.
250	132, 116, 106, 93, 86, 78, 70, 67, 63, 59, 54, 45, 42, 40, 39, 37, 36, 32, 31, 29, 27, 26, 25, 24, 21, 20, 18, 15.	51, 46, 43.
300	176, 144, 118, 103, 95, 87, 79, 72, 66, 64, 61, 59, 55, 50, 45, 42, 40, 39, 37, 36, 34, 32, 27, 25, 23, 21, 18, 15.	51, 46, 43,

DISCUSSION

The salinity induced disturbances in growth and it reflected a decrease in productivity [34, 35]. The obtained results in the present work revealed that, *Kirchneriella lunaris* tolerated salt stress up to 150 mM NaCl, thereafter, there is a gradual reduction in growth with increasing salt levels. This in accordance with [36, 37]. This reduction in growth might be due to

toxicity of the ions or due to low osmotic potential as well as decrease in wall extensibility [38, 39].

The promotion in the photosynthetically active pigment was recorded at the lowest salinity level while; highest decrease was recorded at the level of 300mM NaCl. Such reduction in photosynthetic pigment due to the higher dose of NaCl was recorded by other authors [37, 4]. This reduction in the photosynthetic active pigment may attribute to a salt mediated degradation associated with a slower pigment synthesis [40] and the destruction of chlorophyll pigments and the instability of the pigment protein complex [41].

Salinity induced production of Phenolic compounds in *Kirchneriella lunaris*. The accumulation was much more detected at the level of 300 mM. At this level the percent increase was 237.052% over those of control. Sodium chloride stress induced disturbances of the metabolic process leading to an increase in phenolic compounds [42, 13] and oxidative enzymes [43, 14].

The higher malondialdehyde (MDA) content in *Kirchneriella lunaris* indicating that, the great damaging in this alga consequently confirmed the break down in the salt tolerance of *Kircheneriella lunaris*. This agreed with the observation recorded by other authors [44,45,4]. MDA has been associated with damage provoked by a variety of environmental stresses. Poly-unsaturated fatty acids (PUFA) are the main membrane lipid components susceptible to peroxidation and damage [46].

An increase in the products of lipid peroxidation may suggest the reduced ability of the test organisms to scavenge free radical efficiently [45]. The increase in MDA content resulted in an increase in permeability of plasma membrane or less membrane integrity [47, 48].

Isoenzyme as well as biochemical tools are closer to the genetic information; consequently, they could be used to study genetic variation. In addition, the detected differences in banding pattern are more often qualitative (presence or absence of bands) than quantitative (staining intensity of bands) thereby making easier an accurate distinction between genotypes [49].

In this study, one indophenol oxidase isoenzyme form (IPOX-3) was detected only at low concentrations but disappeared in high concentrations (from 150 to 300 mM NaCl). IPOX-4 was detected in all concentrations but the density was increased from 150 to 300 mM NaCl. An increase in the staining intensity and the number of isoenzyme forms of indophenol oxidase represents an increase in enzyme expression and activity [23].Under salinity stress, increasing the expression of IPOX may prevent

the accumulation of a toxic concentration of H_20_2 [50]. Phenol oxidases use phenolic compounds to detoxify reactive oxygen species [22].

Salinity induced the synthesis of newly produced protein bands in SDS profile of *Kirchnerella lunaris* grown under salt stress. The increase in molecular weight of polypeptide seemed to be increased by salinity stress especially up to 200mM NaCl . Appearance of four polypeptides with molecular weight 318, 288, 258, and 228 kDa were found only at the level of 200 mM NaCl,.

This was also reported by other investigators in some salt stressed algae [51,52]. These extra bands of soluble protein which induced in the salt affected *Kirchneriella* (with different degrees of salinity tolerance) suggested that, these bands play an important role in the mechanism of salinity tolerance. This results in accordance with [53]. [54] reported that, the osmotin which induced under salinity stress providing the osmotic adjustment to the cells either by facilitating the accumulation of solutes or by providing certain metabolic alterations in the cells, which may be helpful in osmotic adjustment.

CONCLUSION

Low salinity (50 mM NaCl) increase dry mass and photosynthetic pigments of *Kirchneriella lunaris* cultures. Phenolic compounds content was decreased at (50 mM NaCl) and increased as sodium dose increased. All salinity concentrations elevated Malondialdehyde in salinized cells. The intensity and the number of isoenzyme forms of indophenol oxidase were increased at all salinity levels. Salinity induced the production of proteins and synthesis of newly protein bands. The high concentrations of salt produced a large number of new polypeptides with high molecular weight. The alga can tolerate high salinity through change of some metabolites.

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اكسدة اليبيدات، المركبات الفينولية ، وإنزيم مؤكسد الفينولات وايض البروتينات فى مزارع طحلب *كرشنيللا ليونا*رز النامية تحت تأثير الملوحة

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في هذا البحث تم عزل طحلب *كرشنيللا ليونا*رزمن محطة معالجة مياه الصرف الصحي في الكولا منطقة سوهاج- مصر . ولقد تم استخدام هذا الطحلب لدراسة تأثير التركيزات المختلفة من كلوريد الصوديوم على نمو الطحلب ،كمية الأصباغ التمثيلية (كلوروفيل أ ، كلوروفيل ب ،والكاروتينيدات)،أكسدة الليبيدات ومحتوى المركبات الفينولية ونشاط انزيم مؤكسد الفينولات وأيض البروتينات (ذائبة ، وغير ذائبة وكلية بالإضافة إلي الطرز البروتينية).

لقد تم تنمية هذا الطحلب في وسط غذائي يحتوي علي تركيزات مختلفة من كلوريد الصوديوم (٥٠-١٠٠-١٥٠-٢٠٠-٢٥٠-٣٠٠ مللي مول) ولقد سبب تركيز ٥٠ ملليمول في زيادة النمو ممثلة فى الوزن الجاف من الخلايا إلي١٢٠% ومحتوي الأصباغ التمثيلية إلي١٠٧ % من العينة الغير معامله). بالإضافة إلي خفض نسبة محتوي المركبات الفينولية الى نسبة (٩٢%،من العينة الغير معامله).

- لقد انخفض الوزن الجاف ومحتوى الأصباغ التمثيلية بزيادة تركيزات الملح فى المزارع المعالجة بنسبة٥٩% و ٣٠% على التوالى.

لقد ازداد نشاط الإنزيم مؤكسد الفينولات ومحتوي المواد الفينولية بنسبة ٢٣٣% بزيادة تركيز كلوريد الصوديوم فى المزارع المعالجة ومن ناحية أخري أدي زيادة تركيز كلوريد االصوديوم الي زيادة أكسدة الليبيدات وزيادة محتوي المالونداى الديهيد بنسبة ٣٣٢%.

لقد ازداد محتوى البروتينات الذائبة والغير ذائبة والكلية بنسبة ٢٢٤,٢٤٩، ٢٣٨ % من العينة الغير معامله عند اعلى تركيز للملوحة فى الوسط.

لقد أدي زيادة تركيز كلوريد الصوديوم خصوصاً تركيز (٢٠٠مللي مول) إلي ظهور عدد من البيبتيدات الجديدة وزيادة كثافتها وكذللك ظهورعدد من البيبتيدات ذات الاوزان الجزيئية العالية مثل ٣١٨، ٢٥٨، ٢٢٨ مما يزيد من مقاومة الخلية للاجهاد.