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Selection of *Arthrospira platensis* strains with productivity in brackish water with high boron levels for commercial production in the Lluta Valley

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Abstract Adaptation and selection of *Arthrospira platensis* strains, for cultivation in brackish water with excess boron (B) in the Lluta Valley can become an interesting alternative that would allow to extend these cultures to areas that possess the environmental conditions, but that lack the fresh water needed to do it. Strains TX98 and P88 were evaluated in laboratory conditions with three different media of brackish water and with the white medium, the Zarrouk modified medium (MZM). The growing media with brackish water with a B concentration present in the Lluta River of 20 mgL^{-1} (B20) and medium with 30 mgL^{-1} (B30), and 10 mg L^{-1} of B (B10). The effect of the different media on the growing parameters with a culture temperature of 25 ± 1ºC in the three treatments, strains TX98 and P88 triplicate, *Arthrospira platensis*, showed tolerance. It was statistically determined that in the growth, the two strains, the three treatments and in the interrelation of both there were significant differences ($p < 0.05$). The TX98 strain reached a concentration of 1.139 g L^{-1} (dry weight) in brackish water with medium B20. Therefore, the highest rate of specific growth (μmax) was obtained with the TX98 strain grown in the brackish medium B30 and the lowest duplication time (0.597 days). Cells grown in brackish water with B had a slightly biochemically modified composition with the white, in relation to the protein content, in the cases in which there are differences in the B content, specifically B30 treatment. For the culture with brackish water from the Lluta River, the TX98 strain is recommended with 10 mg of B using a laboratory to pilot scale.

Keywords: brackish water, boron, growth medium, microalgae, *Spirulina* (*Arthrospira)*

INTRODUCTION

The hydrographic basin of the Lluta River is located in the Region of Arica-Parinacota, Chile and it extends between 18º-18º 30'S and 70º 20'-69º 22'' W. The agricultural area of the basin corresponds to 7,606 hectares and it accounts for 37% of the total land (Cade-Idepe, 2004). The remaining area is not cultivated due to the poor quality of the irrigation water and the poor soil drainage capacity. In addition, the high salinity and the presence of B in the Lluta River, determines the type of crops in the valley, which correspond to those adapted to these conditions, such as corn and alfalfa (Bastías et al. 2004; Cade-Idepe, 2004). In 1996, the General Water Direction (GWD) (1996) published the B values between the 4 mgL^{-1} (Caracarani Est. GWD in summer and spring) to 27 mgL^{-1} (Est GWD Lluta River in Panamerican - fall) in the hydrographic report.

To diversify agriculture or to allow investments in the area of microalgae industrial aquaculture, using the water of the Lluta River basin, requires the presence of species of wide adaptation. The

cyanobacteria are a group of photosynthetic microorganisms with specific morphological and physiological characteristics that allow them to adapt to a wide range of environmental conditions (Liotenberg et al. 1996). *Arthrospira sp.* (*Spirulina*) is a filamentous cyanobacterium undifferentiated, that inhabits alkaline lakes, grown for human consumption because of its interesting nutritional content. The *Arthrospira sp*. genus is characterized by a high content of proteins, vitamins, minerals and polyunsaturated fatty acids (Jassby, 1988) properties that make of it an extraordinary alternative as food source (Plaza et al. 2008).

Large-scale *Spirulina* cultivation is limited from the economical point of view, due to the high cost of the nutrients needed to prepare the artificial culture medium for it to grow (Kebede, 1997). Adaptation of *Spirulina* culture to the Lluta Valley water can become an interesting alternative, not only because of the lower cost of the preparation media, but also because this would allow its cultivation in areas that have the required environmental conditions, that is (high levels of radiation and moderate temperatures), but that where the lack of enough fresh water constitutes a limiting factor to the development of these systems (Kebede, 1997). Although there are marine microalgae species, the brackish water species like *Arthrospira maxima* y *Arthrospira platensis* are the ones of greater industrial interest.

Experiments aimed at growing *Spirulina* in saline water (Mary Leema et al. 2010) have shown a strong physiological stress, as evidenced by a decrease in protein synthesis, increased carbohydrates content of biomass, a strong reduction of the content of phycobili proteins and a massive fragmentation of trichomes (Tredici et al. 1986). It has also been observed that the exposure of *A. platensis* to a high salinity medium produces a high energy demand (Vonshak et al. 1988) as well as a drastic inhibition of the photosynthesis, breathing and growth. After a latency period allow growth rate has been established, which correlated inversely with the NaCl culture concentration in the culture medium. Besides from the specific toxic effects of salts in the Luta Valley water, it is necessary to understand the mechanisms of the *Spirulina* isosmotic adaptation to these conditions.

Another key factor is the temperature, for microalgae growth, and it is the most important limiting factor for *Spirulina* production in open cultures (Vonshak, 1997). The temperature of the Lluta Valley water is below the optimum for *A. platensis*, but it is constant, with an isotherm of 25ºC.

In microalgae, the B is a micronutrient or trace element such as Cu, Mn, Zn, Co, important in enzymatic reactions and for the biosynthesis of many compounds (Vonshak, 1997). It is also incorporated into the cells in a range of 0.001 to 0.25 ug mg^{-1} dry weight (Grobbelaar, 2004). In bacteria B is an essential part of signal molecules required for quorum sensing (Goldbach and Wimmer, 2007). So far the mechanism of B toxicity in plants and cyanobacteria is a research matter.

The objective of this study was to evaluate biomass production and biochemical characteristics, using media prepared with brackish water, excess B at 25ºC in the Lluta Valley hydrographic basin for the cultivation and selection between two strains of *A. platensis*, for industrial use.

MATERIALS AND METHODS

The research area where water was sampled was located in the hydrographic basin of the Lluta Valley, Panamerican Crossing (18º23'55.51"S, 70º17'52.14"W), Arica city, Arica Province, Region XV, Chile. The experiments were conducted in the Vegetal Physiology, Tissue Culture and Plant Biotechnology laboratories.

Biological material and growth conditions

The biological material used were two *Arthrospira platens is* strains, thermophilic and vacuolated, P88 and TX98, used for the industrial cultivation by Solarium Biotechnology Company SA (Chile).

The strains were regenerated and maintained according to the conditions described by Vonshak (1997). The experiment was carried out under controlled conditions of salinity 0.02 M NaCl, pH 8.9-9.5, aeration, photoperiod 12:12 hrs 158.56 mol m^{-2} s^{-1} of irradiance and a temperature at 25ºC. The biomass or algal growth was measured daily by optical density at 560 nm. The algal dry weight (gL^{-1}),

was measured as recommended by Vonshak (1997). Strains were adapted to grow in the three treatments before being used in the experiments for a period of 34 days, and the cultures were inoculated at the same biomass concentration (10%, OD_{560} = 0.5). At the end of the study once the biomass was measured samples were kept to perform a total nitrogen analysis using the Kjeldahl method and to determine the B by using the colorimetric method with Azomethine-H.

Water and growth medium

A surface channelled water sample of the Lluta River was taken with high B concentrations (Km 20), in January 2010. The ionic composition of the water is shown in Table 1. The growth medium corresponds to the modified Zarrouk medium (MZM) (Vonshak, 1997; Sánchez et al. 2003). The ionic composition is described in Table 1.

This medium was used as white prepared with distilled water and inorganic salts p.a. (Merck), which represents the two optimal culture conditions. Three other different mediums were also tested. These were prepared with water from the Lluta River basin. One of them like "negative control", with a concentration of 20 mgL^{-1} B and the other with 30 mgL^{-1} and 10 mgL^{-1} of B, respectively as shown in Table 2. The scale of the cultures was in a volume equivalent to 400 ml. The kinetic growing calculations of *Arthrospira*, according to Vonshak (1997). These activities lasted for 24 days.

KNO_3 is included in the three media due to the high sodium sulphate present in the Lluta River water. Besides, according to Sassano et al. (2004), the nitrate was demonstrated to ensure the highest yields of *Arthrospira* sp. biomass, thus justifying the wide use of the media which employ $NaNO_3$ and/or KNO_3. No additional $FeSO_4.7H_2O$ is required because this is already present in the Lluta River water as Fe^{+2} in a concentration of 1.2 mgL^{-1}.

Morphological appearance

A. platensis strains were analyzed using an Olympus BX51 microscope, observing multi cellular filaments of cylindrical blue-green trichomes characteristic of the *Arthrospira* genus, due to the fact that the shape of the filament, can be altered by physical and chemical conditions of the growing medium (Jeeji-Bai, 1985). The observation of the samples using the microscope started the day of the inoculum of the 34 day acclimatization period, and digital photographs were taken every 120 min during the first 24 hrs, and then every 6 days in the three treatments and in the white one as well.

Statistical analysis

Each treatment, including the white with three replicates was compared. To make comparisons between the biomass of the treatments, a two-way ANOVA was used of two repeated ways of measurement or tested, followed by Bonferroni post-tests analysis. The statistical GraphPad Prism, was also used. Significant levels of all the analyzes were set at $P < 0.05$. For the statistical protein analysis and B content a one-way ANOVA was utilized followed by post-hoc analysis with Tukey multiple comparison tests, using the same statistical program. Significant levels of all analyzes were set at $P < 0.05$.

RESULTS AND DISCUSSION

Effect of different sea water media in the growing parameters

Kinetics growth (Figure 1 and Figure 2) of the two *A. platensis* strains grown in three different media of brackish water with B and the MZM White, were evaluated for a 24 day culture period. At a temperature of 25 ± 1ºC the cultures in the three treatments, the TX98 and P88 strain was triplicate. *Arthrospira platensis* showed tolerance at B. It was determined statistically that there are significant differences ($P < 0.05$) in the growth of the two strains; the three treatments and the interrelationship of both.

In Figure 3 the TX98 strain in the brackish water with B20 medium reached a concentration of 1.139 gL^{-1}, the highest and it was significantly higher than ($P < 0.05$) of MZM (control). However, the biomass concentration of *A. platensis* TX98 strain grown in brackish water with B in the B10 and B30 media were not significantly different from the white ($P > 0.05$). The P88 strain did not show significantly differences in the treatments in relation to the white ($P > 0.05$). Similar results were gotten by Faucher et al. (1979) and Mary Leema et al. (2010) who have reported growing rates comparable or superior to the white medium or control (standard mineral medium) for *A. platensis* maximum growth in sea water supplemented with phosphorus and nitrate and addition to the *A. platensis* have shown that external concentrations of sea salt of up to 150% in seawater had little effect on the *A. platensis* strain growth (Warr et al. 1985). According to these researchers the capacity of *A. platensis* to tolerate high salinity levels seems to be an important factor that allows it to survive and to grow in lakes and other similar alkaline brackish water.

According to the results presented in Table 3 the two *A. platensis* strains exhibit an exponential growth, showing a good adaptation to the brackish water growing media in brackish water with B in the culture conditions. The highest specific growing rates (μ_{max}) were obtained with the TX98 strain grown in the B30 and B20 brackish water. And the lowest with the B20 strain in P88 medium.

The μ_{max} obtained for both *A. platensis* strains grown in brackish water with B and in the sea water medium are higher than the values reported by Costa et al. (2000) and Mary Leema et al. (2010), and similar to the values obtained by Vonshak (1997), with M2 and 6MX strains of the same species, with 0.5 M NaCl treatments.

The TX98 strain in brackish B30 medium showed the shortest duplication time (t_d = 0.597 days) lower than the white (t_d = 0.650 days). Shorter duplication time is preferred for the mass cultivation. As the duplication time increases the speed of the cell duplication decreases and this causes the commercial cultivation to become non profitable (Reinehr and Costa, 2006). The duplication time reported by Vonshak (1997) for the *A. platensis* M2 strains grown in 0.5 M NaCl treatment (t_d = 0.663 days). The cost of the nutrients constitutes approximately a 15-20% of the total cost for large scale culture (Vonshak, 1997) therefore the use of media with B brackish water for the culture of *A. platensis* would reduce the production costs considerably.

Effect of the brackish water with B media in the biomass composition

A decrease in protein content and B accumulation was observed in both strains (Table 4). The protein content in *A. platensis* strains cultivated in white medium was not significantly greater than the one of the other treatments ($P > 0.05$). The protein content observed in both strains in the white was about 57% lower than reported by Oliveira et al. (1999) with Zarrouk medium (68.01%) for the same species grown in mineral medium described by Paoletti et al. (1985) at 25ºC and Mary Leemaet al. (2010) (71.16%), but similar to the control medium with the M2 strain Vonshak (1997) (56.1%). The highest protein contents were observed in the P88 strain (57.31 ± 4.16%) in B10 treatment followed by in the ones in the TX98 strain (50.22 ± 1.49) also in B10, which are below the range reported for enriched *Spirulina* in seawater (65.61%) (Olguín et al. 1997) and the enriched salt with the growing media (51-65%) (Tredici et al. 1986). In contrast with the protein content, the content of carbohydrate increased along with the increase in salinity. This could be due to the low molecular weight; the carbohydrates accumulated by *A. platensis* like osmo protectors during its acclimatization process to high concentrations of salt, when cultivated in brackish and salty water Vonshak et al. (1988); Mary Leema et al. (2010).

The boron and the protein concentrations of the TX98 strain in relation to the treatment. There are significant differences ($P > 0.05$), specifically between the white and the B30 brackish water treatment (Figure 4).

The B and the protein concentration of the P88 strain in relation to the treatment. There are significant differences ($P > 0.05$), specifically between white and brackish water treatment with B30 (Figure 5).

Effect of different of sea water media in the morphology of trichomes

The *A. platensis* TX98 and P88 strains maintained the helical shape in the cultures developed using the brackish B water in the three treatments. There was some difference in the trichome length and in the helicity degree. Only during the first acclimatization hours there was a bigger amount of short of trichomes. Nevertheless, after 34 days of acclimatization the trichomes unusually longer dominated the cultures in the brackish B water. Dhiab et al. (2007) have shown that the change in the morphology of the trichome (from helical to straight shape) as well as some modifications in the physiological behaviour of *Arthrospira (Spirulina) platensis* is a response to the increase in the NaCl concentrations in the growing media.

CONCLUDING REMARKS

Arthrospira platensis is a fresh water organism, nevertheless the TX98 and P88 strains used in this study adapted to cultures in brackish B water (B10, B20 and B30).

TX98 strain in the B20 treatment (negative control 20 mgL^{-1} of B) and B30, show a great protein concentration, however, a greater protein concentration (greater than 50%) occurs in the strains in B10 treatment in brackish water, with 10 mg of B. Furthermore they do not show significant differences in the B content with the white. From laboratory to pilot scale, the *A. platensis* TX98 strain is recommended for cultures in brackish water from the Lluta Valley, with 10 mg of B, for commercial production.

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Tables

Table 1. Ionic composition of brackish water in the Lluta Valley and MZM.

	B^{+}	Ca^{+2}	Mg^{+2}	Na^{+2}	K^{+}	Cl^{-}	SO_4^{-2}	HCO_3^{-}	NO_3^{-}
Lluta Water (mgL^{-1})	20.00	229.60	72.23	5,551.28	671.58	658.88	1,091.04	12,444.00	1,837.06
Mineral Water MZM (mgL^{-1})	0.47	14.00	20.00	8,260.00	670.00	585.00	975.00	11,000.00	5,133.00

Table 2. Composition of elements of the media used.

Components	MZM White ($g\ L^{-1}$)	B 10 Medium ($g\ L^{-1}$) 1:1 H_20 destilled	B 20 Medium ($g\ L^{-1}$) Negative Control	B30 Medium ($g\ L^{-1}$)
Macronutrients				
NaCl	1.00	0.50	0.00	0.00
$CaCl_2$	0.04	0.00	0.00	0.00
$NaNO_3$	2.50	1.67	2.01	2.01
KNO_3	0.00	0.98	0.58	0.58
$FeSO_4.7H_2O$	0.01	0.00	0.00	0.00
EDTA-Na	0.08	0.00	0.00	0.00
K_2SO_4	1.00	0.59	0.38	0.38
$MgSO_4.7H_2O$	0.20	0.00	0.00	0.00
$NaHCO_3$	16.80	16.80	16.80	16.80
K_2HPO_4	0.50	0.50	0.50	0.00
Oligoelementos	-			
H_3BO_3	2.86E - 03	0.00E + 00	0.00E + 00	6.10E - 02
$MnCl_2$	1.81E - 03	1.81E - 03	1.81E - 03	1.81E - 03
$ZnSO_4.7H_2O$	2.22E - 04	2.22E - 04	2.22E - 04	2.22E - 04
$CuSO_4.5H_2O$	7.90E - 05	7.90E - 05	7.90E - 05	7.90E - 05
MoO_3	1.00E - 05	1.00E - 05	1.00E - 05	1.00E - 05
$Co(NO_3)_2.6H_2O$	4.40E - 06	4.40E - 06	4.40E - 06	4.40E - 06

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Table 3. Main kinetic parameters obtained from *A. platensis* strain growth in trials in the different treatments at 25ºC.

Treatment	TX98			P88		
	Highest growth rate	Duplicationtime (d)	Maximum biomass (gL^{-1})	Highest growth rate μ (d^{-1})	Duplication time (d)	Maximum biomass (gL^{-1})
White	1.065	0.650	1.617	1.294	0.536	1.430
B 10	1.061	0.653	1.476	1.139	0.608	1.394
B 20	1.142	0.607	1.934	0.946	0.732	1.711
B 30	1.160	0.597	1.759	1.148	0.604	1.625

Table 4. Boron and protein content after 24 days of the *A. platensis* strains in the three treatments and the white.

Treatment Medium	Strain	B (mg Kg^{-1} Sample)	% Protein
MZM White	TX98	59.98 ± 21.15	57.64 ± 2.80
MZM White	P88	89.10 ± 5.71	57.63 ± 2.11
B10	TX98	110.70 ± 67.73	50.22 ± 1.49
B10	P88	95.32 ± 31.16	57.31 ± 4.16
B20	TX98	123.23 ± 18.37	47.41 ± 2.43
B20	P88	102.50 ± 14.66	46.75 ± 7.80
B30	TX98	243.48 ± 17.71	39.84 ± 10.63
B30	P88	246.56 ± 2.18	50.76 ± 2.52

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Figures

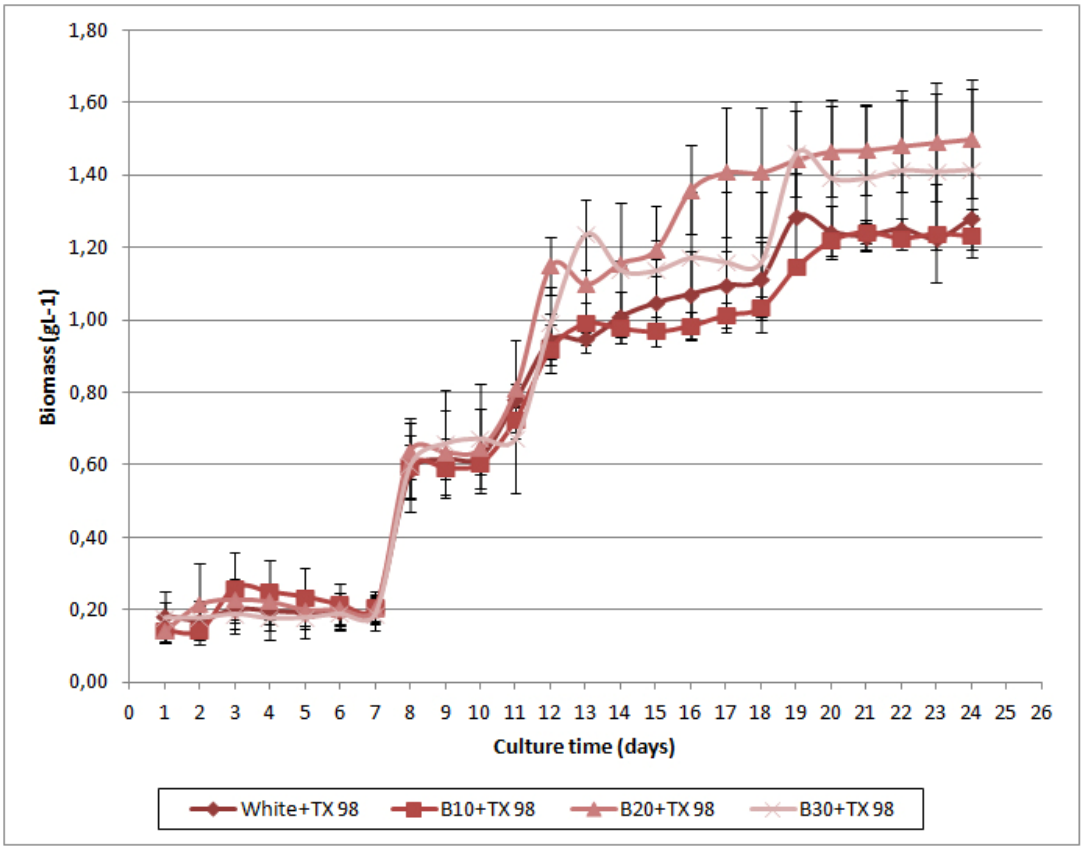

Fig. 1 Autotrophic growth of the culture of the *A. platensis* TX98 strain in the 3 treatments and in the white.

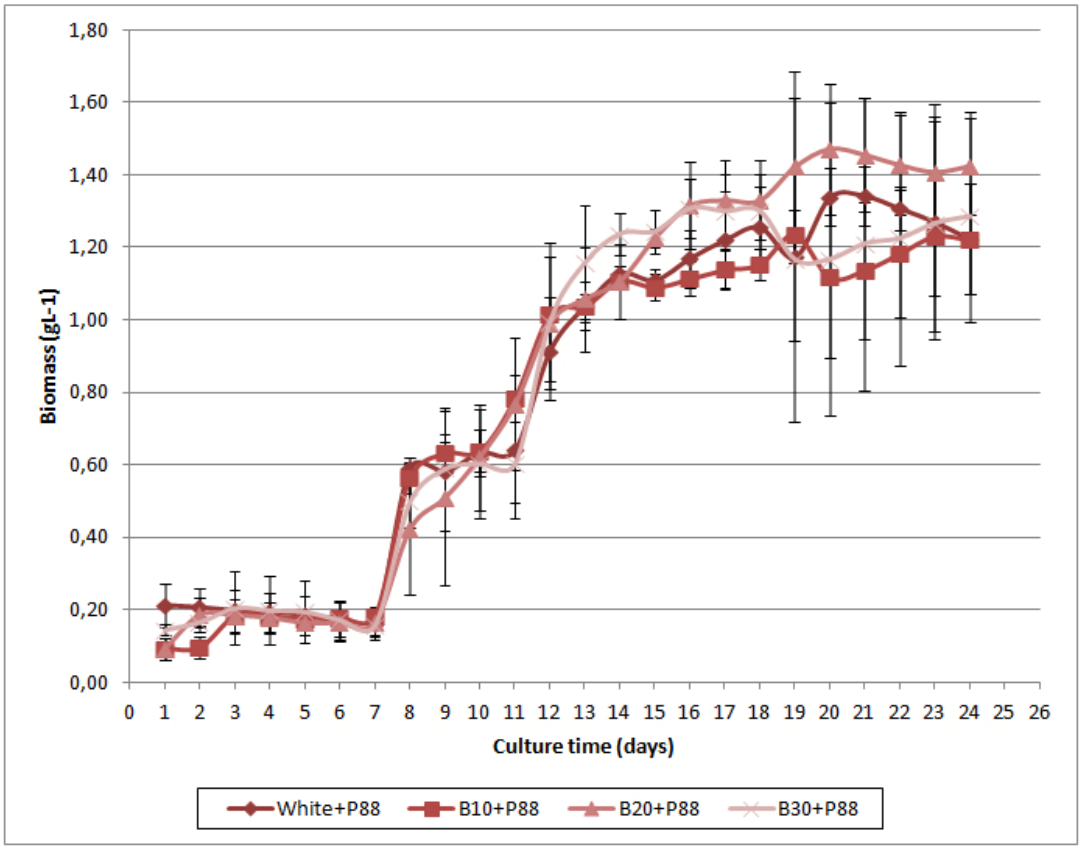

Fig. 2 Autotrophic growth of the culture of the *A. platensis* strain P8 in the 3 treatments and in the white.

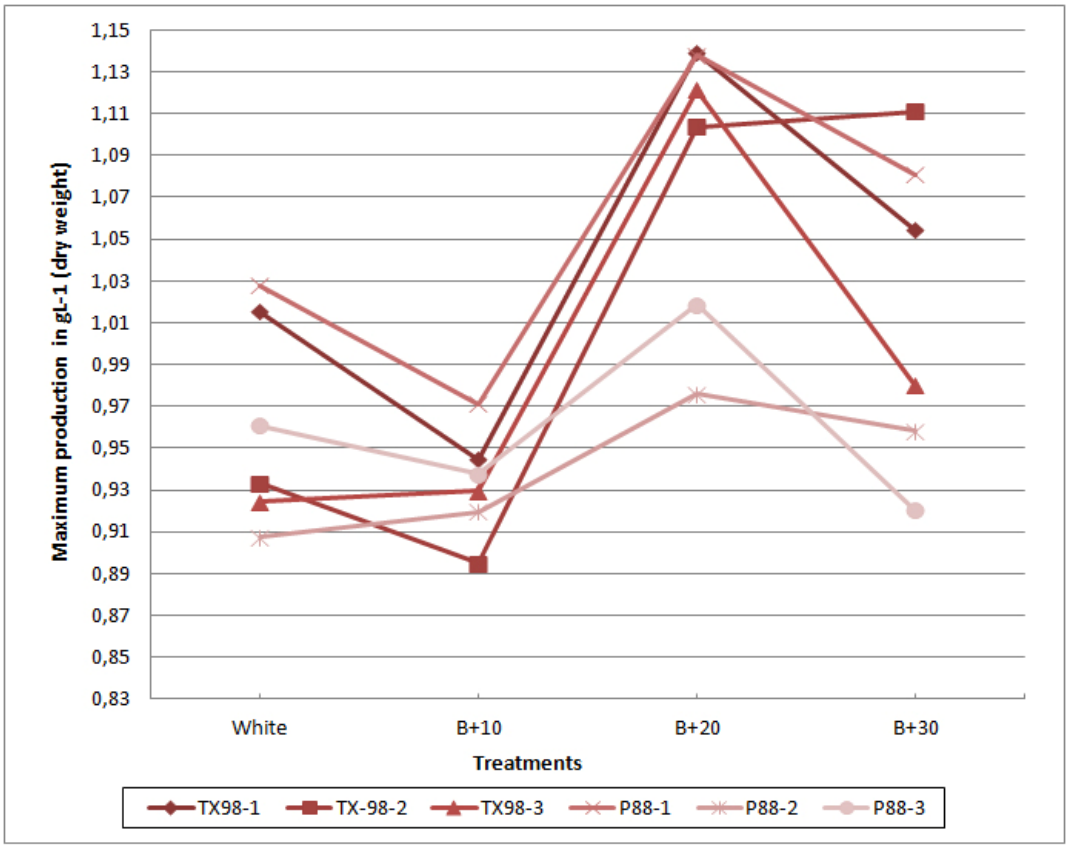

Fig. 3 Maximum production in gL^{-1} (dry weight) of the 6 *A. platensis* strains (2 with triplicates), in the 3 treatments and the white one.

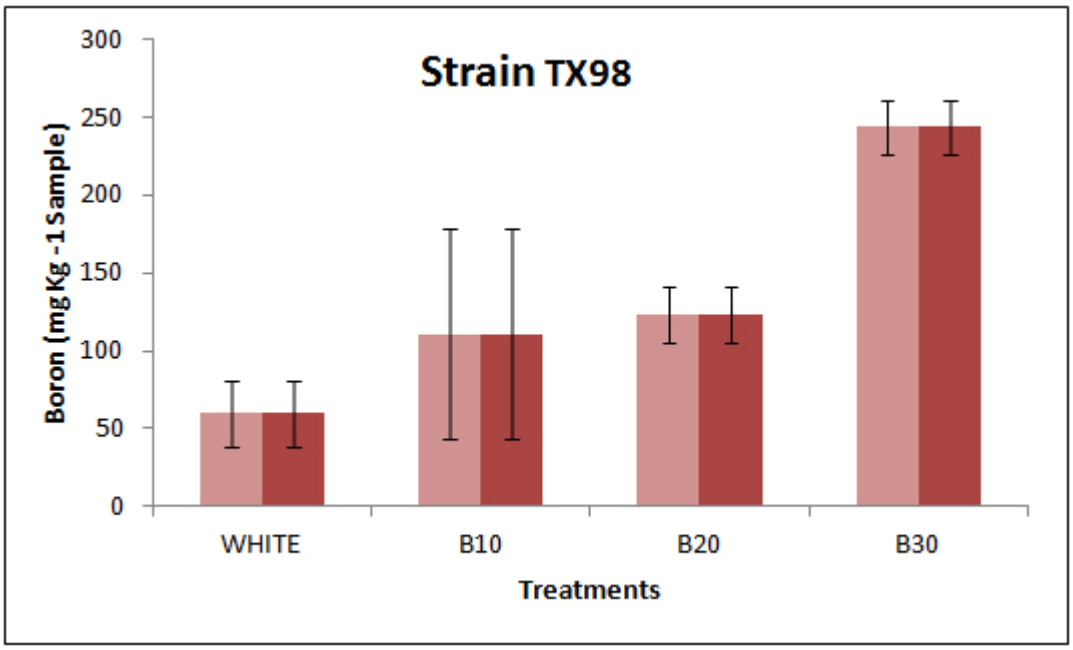

Fig. 4 Boron content (mg Kg^{-1} sample) in the three treatments and in the white in the TX98 strain.

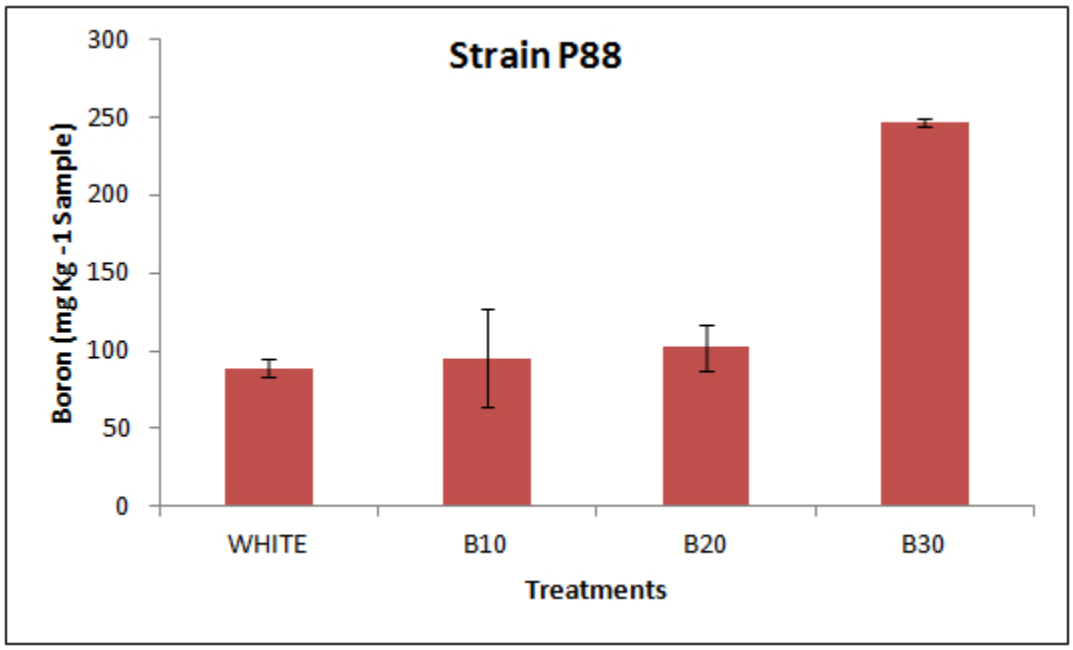

Fig. 5 Boron content (mg Kg^{-1} sample) in the three treatments and in the white in the P88 strain.