

Trehalose and Sucrose Osmolytes Accumulated by Algae as Potential Raw Material for Bioethanol

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ABSTRACT

Currently, obtaining sustainable fuels, such as biodiesel and bioethanol, from cheap and renewable materials is a challenge. In recent years, a new approach being developed consists of producing sugars from algae by photosynthesis. Sugar accumulation can be increased under osmotic stress (osmoregulation). The aim of this study is to show the production of sugars from algae, isolated from natural sources, and the effect of osmotic stress on fermentable sugars accumulation. Strain isolation, production of sugars from each alga and the effect of osmotic stress on growth and sugar production are described. Twelve algal strains were isolated, showing growths between 0.6 and 1.8 g of biomass dry weight /L, all with production of intracellular and extracellular sugars. The strain identified as *Chlorella* sp. showed an increase in sugar production from 23.64 to 421 mg of sugars/g of biomass dry weight after 24 h of osmotic stress with 0.4 M NaCl. Sucrose and trehalose, both fermentable sugars, were the compatible osmolytes accumulated in response to the osmotic stress. The isolated strains are potential producers of fermentable sugars, using the photosynthetic pathway and osmotic stress.

Keywords: Algae, Biofuels, Osmoregulators, Carbohydrates, Bioethanol

1. Introduction

Carbon neutral renewable fuels, as bioethanol, are decisive in contributing to the replacement of petroleum-derived fuels that contribute to global warming. Production of biodiesel from oil crops and bioethanol from sugarcane and crops in large amounts are not sustainable. An alternative offered by microalgae results from photosynthesis-produced sugars. This production can be increased via osmotic stress as osmolytes accumulation in algae. One mechanism developed by microorganisms as a response to osmotic stress, is the ability to accumulate compatible low-molecular-weight organic solutes such as carbohydrates [1-5].

Three categories of microalgae have been proposed according to their response to salt, a) low stress tolerance to salinity (0.7 M NaCl), with production of sucrose and trehalose, b) medium tolerance to salinity (0.7 - 1.8 M NaCl) with production of glucosylglycerol, and c) high tolerance to salinity (more than 2.7 M NaCl) with produc-

tion of amino compounds such as glycine, betaine, or glutamate [6-9]. The mechanism by which microalgae produce sucrose is through the production of a complex enzyme sucrose-phosphate synthetase/phosphatase (SPS/SPP) [10].

The aim of this study is to show the effect of osmotic stress in algae fermentable sugars accumulation, as a potential supply source of raw material for alcoholic fermentation.

2. Material and Methods

2.1. Algae Isolation and Culture

Samples were taken from four different places: the Lake of Texcoco, located in the Metropolitan area of Mexico City (salinity 2000 ppm, pH 8 - 10) [11]; the Lake of Guadalupe, in the State of Mexico (fresh water, pH 7.2, salinity 0.3 ppm); a lake in Tabasco, southeastern Mexico (pH 7.2 - 7.8, salinity 2000 ppm); and the river Thames, in Ontario, Canada (pH 6.7, salinity 0.5 ppm).

Samples were taken in bottles and, upon arriving to the laboratory, pH and salinity were determined through conductivity.

Samples were enriched through cultivation in different media according to their salinity, either BG11, BG11 with 25% NaCl, or Guillard [12]. The culture conditions were, in all experiments: fluorescent light in periods of 12:12 h (100 mol/m² s); 25°C and aeration 4 vvm (in liquid medium). After two replanting procedures, they were plated onto solid medium (the same culture medium with 10% agar-agar). After 15 days of incubation, samples of each separate colony were taken from the plate and seeded again in a liquid medium culture at the same above mentioned conditions. Isolated strains were conserved in liquid medium with natural light at room temperature [12]. The strains were identified by optical microscopy using a LEICA-DMLB microscope. Each strain was observed and compared with a taxonomy library [13].

2.2. Growth Kinetics

A 250-mL flask with 150 mL of selected medium was inoculated (10% inoculum) and cultivated under the previously fixed conditions. Growth was monitored by absorbance (O.D. 600 nm) every 24 h in a colorimeter Genesis 10 UV and by dry weight (dw): culture was filtered through a 0.42 m pre-weighted filter, dried at 80°C for 24 h, and weighted. Culture purity was monitored by optical microscopy.

2.3. Intra- and Extra-Cellular Sugars Analysis

The 15-day cell culture was centrifuged at 5000 rpm, for 20 min at 4°C, in a Beckman J2-MC centrifuge. Liquid phase was separated for total carbohydrate determination. For intracellular sugars, biomass was lyophilized, the dry cells (0.01 - 0.05 g) were extracted with 10 mL of 70% ethanol and incubated at 65°C during 4 h, then they were centrifuged at 10,000 rpm for 15 min in a Sorvall Bio-fugeprimo Centrifuge. The supernatants were collected

and dried at 40°C in an oven. The obtained material was redissolved in 10 mL of distilled water [8,14]. The total carbohydrate content, in both intra- and extra-cellular fractions, was established by the phenol-sulphuric acid method (Dubois Method); and identified by HPLC as established by Müller [8,14]. The supernatants were collected and deionised by shaking with mixed-bed resin (Bio-Rad 501 X8), the solute was filtered through a microfilter (22 µm) prior to injection. A Varian 9002 HPLC with refractive index detector, using a column Phenomenex REYEX organic acid 300X7.8 75985 00H-0138-KO, mobile phase H₂O/H₂SO₄ at a flow rate 0.3 mL/min, was employed.

2.4. Effect of Salt Stress on Growth and Osmotic Shock on Sugar Production

To assess the NaCl concentration effect on growth, isolated strains were grown in BG11 medium with 0, 0.2, 0.4, and 0.6 M NaCl under the same conditions mentioned above. Growth, intra- and extra-cellular, and total carbohydrates were determined during 15 days as indicated before.

The effect of osmotic shock on sugar production was completed by stressing the growth cultures (biomass at 1.5 OD_{600nm}) with the addition of NaCl, as concentrated solution, to achieve 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 M; subsequently shocked cultures were incubated during three additional days, growth and intra- and extra-cellular sugars were determined every 24 h, as indicated before.

3. Results and Discussion

3.1. Photosynthetic Microorganisms Isolation and Its Growth

In the initial stage of this work, twelve different types of photosynthetic microorganisms were isolated and identified, they showed different growths and biomass productions (**Table 1**).

Table 1. Identification of isolated algae, place of origin, and biomass produced (g dw/L).

Orden y Género	Lugar de origen	Máxima biomasa (g/L)	Factor de eficiencia
<i>Chlorococcales Chlorella</i>	Lago de Guadalupe	1.36	29.11
<i>Chyosphaeaceae Chrysochaera</i>	Lago en Tabasco	0.32	5.44
<i>Chlorococcales Trebouxia</i>	Lago de Guadalupe	0.6	6.22
<i>Chlorococcales Chlorella (T)</i>	Lago en Tabasco	0.64	2.70
<i>Chlorococcales Scenedesmus</i>	Río Tamesi, Canadá	1.58	5.37
<i>Chroococcales Synechocystis</i>	Lago de Texcoco	1.74	1.81
<i>Chroococcales Synechocystis (T)</i>	Lago en Tabasco	1.18	0.83
<i>Nostocales Nodularia</i>	Lago de Texcoco	2.16	1.30
<i>Oscillatoriales Lyngbya</i>	Lago de Texcoco	2.56	1.02
<i>Chroococcales Gloeocapsa</i>	Lago en Tabasco	0.9	0.27
<i>Chroococcales Cistococcus</i>	Lago de Texcoco	1.76	0.70
<i>Oscillatoriales Oscillatoria</i>	Río Tamesi Canadá	1.24	2.61

*mg of total sugars/L culture medium

Among the isolated strains, maximum growth (biomass) occurred in increasing order: *Lyngbya*, *Synechocystis*, *Cystococcus*, *Scenedesmus*, *Chlorella*, and *Oscillatoria*; they showed a final biomass (dw) higher than 1 g/L (Figures 1(a) and 1(b)). A second group of strains: *Gloecapsa*, *Chlorella* (T), *Trebouxia*, *Nodularia*, and *Chrysozhaera* showed the lowest biomass dw (<1), i.e., 50% less than the *Lyngbya* strain (Figure 1(c)).

In order to establish the sugars-production efficiency for each strain, the yield of total sugars production per gram of dried biomass per liter of algae culture was calculated for each strain. *Chlorella* sp., *Chrysozhaera*, *Chrysozhaera*, *Trebouxia*, and *Scenedesmus* were the most efficient producer strains (Table 1).

No relationship was found between salinity of the strains place of origin and the natural production (without osmotic stress) of total sugars; for example, strains *Chlorella* sp. and *Trebouxia*, which were isolated from the Guadalupe Lake (fresh water), showed different sugar production rates; from these algae, *Chlorella* produced 4-fold more sugars than *Trebouxia* (Figure 2).

Given its high efficiency in sugar production, *Chlorella* sp. was selected for the latter osmotic stress assays.

3.2. Effect of NaCl Concentration on the Growth and Sugar Production of *Chlorella*

Biomass growth was inhibited at 0.4 and 0.6 M of NaCl, and decreased 28% at 0.2 M (Figure 3). Brown [15] reported a similar phenomenon with *Nannochloris bacillaris* (*Chlorophyceae*), whose growth was inhibited 300% in seawater (0.8 M). Other studies in cyanobacteria mention that this type of photosynthetic microorganism presents growth inhibition at 0.4 M NaCl [16]. *Chlorella vulgaris* decreases growth 50% at 0.6 M, 30% at 0.4, and 10% at 0.2 M [17]. All these species, including *Chlorella* isolated in this study, undergo a strong growth inhibition even under moderate osmotic stress, which means that they are stenohaline.

3.3. Osmotic Shock Effect on Sugar Production

When *Chlorella* was treated under osmotic shock, increments in the production of sugars were observed (Figure 4). Total sugar production was 18-fold higher after 24 h of osmotic shock using 0.4 M NaCl (421 mg/g of biomass dw).

Page *et al.* [8] reported a 15-fold increment of total sugar production under osmotic stress for a strain of *Syntonema*, after 24 h in 0.15 M NaCl. When *Synechocystis* PCC 6714 was grown in BG11, total carbohydrate production increased above 13-fold when the medium was changed to a BG11/100% seawater [18].

After salt stress, sucrose and trehalose concentrations increased from 1.13 to 1.95 and from 0.012 to 0.019

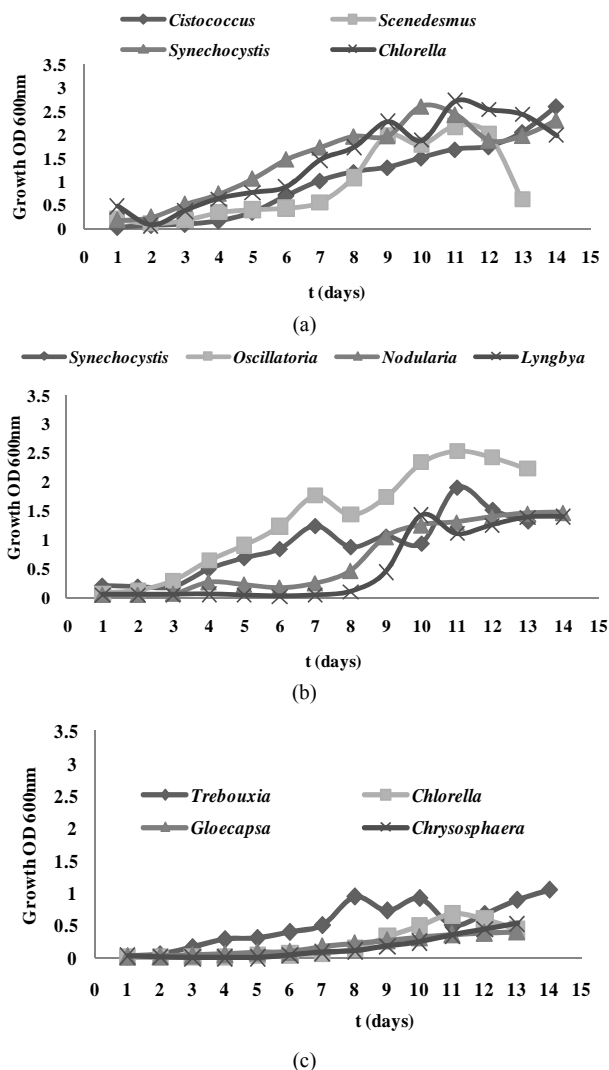


Figure 1. Kinetic growth of isolated strain cultures in BG11 medium.

mg/g biomasse dw, respectively, in *Chlorella*. Under salt stress, *Scytonema* produced sucrose and trehalose, and trehalose concentration was higher than sucrose [8]; perhaps because trehalose has shown superior protein stabilisation capacity [21]. *Chlorella*, under osmotic stress, produced proline and sucrose as osmolytes [19-21], aeroterrestrial "*Chlorella*" *trebouxioides* SAG2142 and "*Chlorella*" *luteoviridis* SAG2196 accumulate the polyols, ribitol and sorbitol [20]. The present study is the first report of trehalose synthesis by *Chlorella* as osmotic response; nevertheless, in this study sucrose concentration was higher than that of trehalose, being the main osmolyte in *Chlorella*.

Algae have been used to obtain different products of interest for humans. Microalgae can provide several different types of renewable biofuels. These include meth-

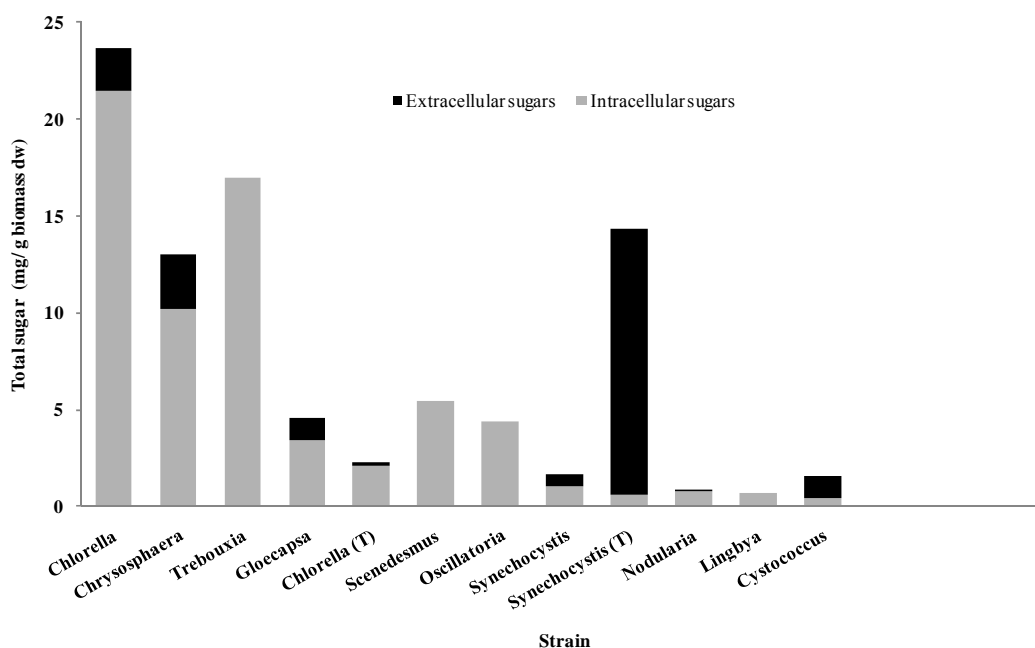


Figure 2. Intra- and extra-cellular sugar production for each isolated strain, grown in BG11 medium without NaCl.

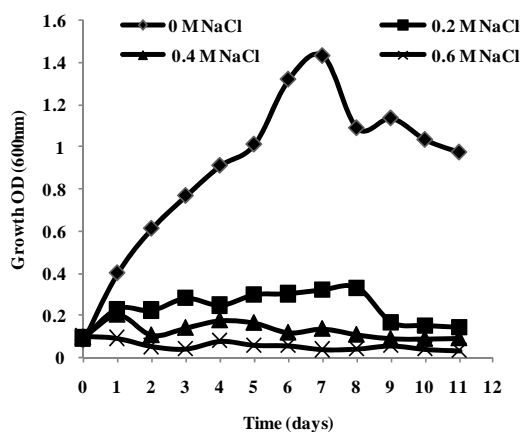


Figure 3. Effect of NaCl concentration on growth of *Chlorella* sp.

ane produced by anaerobic digestion of the algal biomass and biohydrogen produced photobiologically, as well as biodiesel derived from microalgal oil [22], bioethanol from sugars and cellulose [23,24], biomass for combustion or hydrogen and methane production.

Aside from using the sugars produced by algae to obtain bioethanol, other cellular components as lipids and protein can be used for the synthesis of biodiesel and fodder, respectively; making it more feasible economically to obtain biofuels from algae. When *Botryococcus braunii* is grown at different NaCl concentrations, increasing salinity induces a decrease in protein content, but the carbohydrate and lipid contents remain unchanged [25]. Considering the results obtained in the present work re-

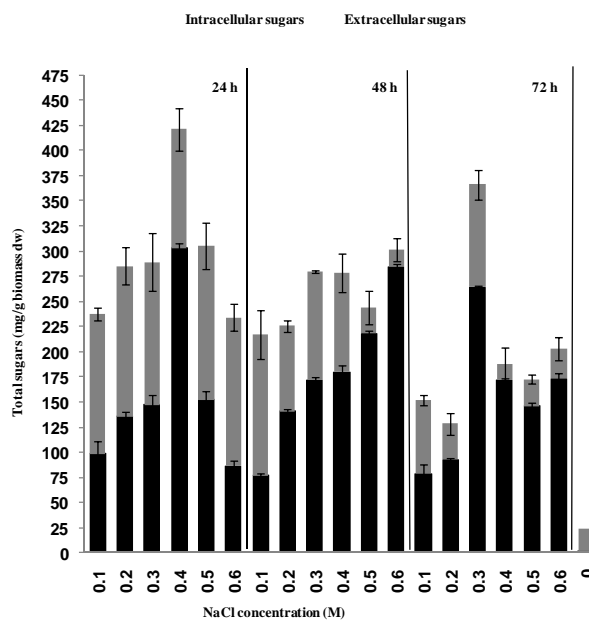


Figure 4. Osmotic stress effect in total sugar production in *Chlorella* sp grown in BG medium .

garding sugars production by *Chlorella*, we estimated the annual production of fermentable sugars, considering a hectare of raceways surface of 0.3 m depth (6000 m³), and a harvested biomass of 20% of the volume per day, under four different scenarios: 50% of the maximum biomass production achieved (equivalent to 0.7 g/L) and the maximum biomass (equivalent to 1.36 g/L), both with a sugars yield without osmotic shock (equivalent to

0.02 kg sugar/kg biomass dw), corresponding to scenarios 1 y 2, respectively. A 50% and 100% of the maximum biomass production achieved with a sugars yield after osmotic shock (equivalent to 0.25 kg sugar/kg dw), corresponds to scenarios 3 and 4, respectively (**Table 2**). Algal sugar production was compared to the sugar cane productivity in Colombia, where the best yield is 80 and 9.6 ton/ha of sugar cane and sugar, respectively [26-29].

As for production of sugars, in the case of *Chlorella* in the most conservative condition (scenario 1), the production of sugars is lower (31%) than sugar cane. Nevertheless, for scenario 2, it is 13% higher than that of sugar cane. The two scenarios with osmotic shock, scenarios 3 and 4 are 7 and 14-fold higher than sugar cane, respectively. However, the dilution of sugars in the culture broth is a limiting factor that needs to be considered contrary to sugar cane juice.

As for *Chlorella*'s by-products, lipids, proteins and residual biomass after extraction (cake) were estimated in a very conservative way (based on our results), the percentage of yields were 10%, 20%, and 50%, respectively. The values of the by-products estimated with *Chlorella* are more than one fold higher than those obtained with African oil palm (*Elaeis guineensis*) (**Table 2**). Nevertheless, many technical challenges remain in regarding to scaling up and adequate process to make the use of algae feasible for the production of biofuels.

4. Conclusions

Twelve strains were isolated from different ecosystems, which produced intra- and extra-cellular carbohydrates. *Chlorella sp.* showed the highest total sugars production, 29.1 mg of sugars/g dw biomass. When salinity level in culture media was increased, culture growth decreased

Table 2. Comparative scenarios production of sugars, lipids and protein, considering a hectare of raceways surface and 0.3 m depth, and a harvested biomass of 20% of the volume per day. Scenario (1) 50% of the maximal biomass production achieved (0.7 g/L); (2) the maximal biomass (1.36 g/L) both with sugars yield without osmotic shock (0.02 kg sugar/kg dw), (3) and (4) 50% and 100% of the maximum biomass production with sugars yield after osmotic shock (0.25 kg sugar/kg dw), scenarios 3 and 4, respectively. Sugar cane from Colombia and Oil Palm from Africa.

Feedstock(scenario)	Biomass	Sugars	(Ton/Ha/year)		Biomass after extraction
			Lipids	Protein	
<i>Chlorella</i> (1)	294	5.9	29.4	59	147
<i>Chlorella</i> (2)	571	11	57.1	114	286
<i>Chlorella</i> (3)	294	73.5*	29.4	59	147
<i>Chlorella</i> (4)	571	142.8*	57.1	114	286
Sugar cane	80	9.6	-	5.2 ⁽³²⁾	26
Oil palm	22 ⁽²⁹⁾	2.6 ⁽²⁹⁾	5.7 ⁽²⁹⁾	3.5 ⁽³⁰⁾	5 ⁽³¹⁾

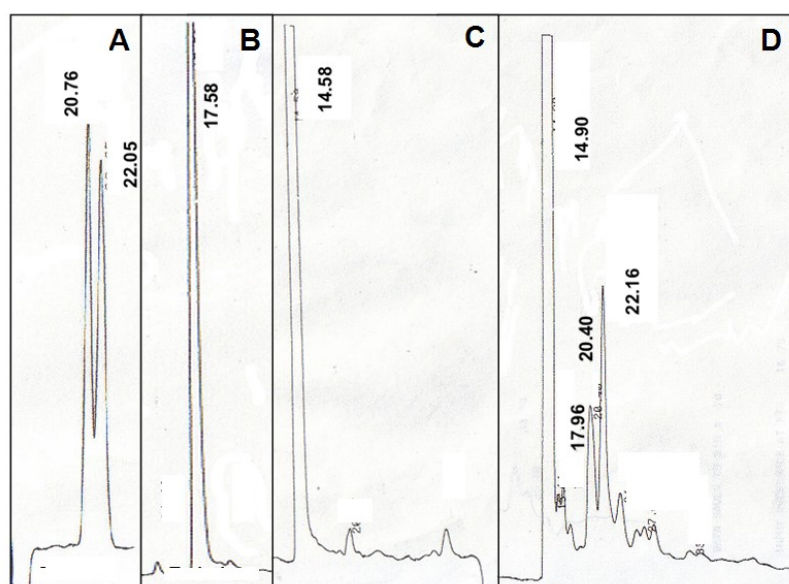


Figure 5. HPLC chromatograms from saccharose standard, A; trehalose standard, B; *Chlorella*'s extract without osmotic stress, C; and *Chlorella*'s extract after 24 h of osmotic stress with 0.4 M NaCl, D.

with increasing NaCl concentration, and was inhibited at concentrations higher than 0.4 M for all strains.

After the osmotic stress of *Chlorella* culture, sugar production increased 14-fold in 0.4 M NaCl after 24 h. The main osmolyte identified was sucrose; however, trehalose was also accumulated (1.93 and 0.019 mg/ biomasse dw, respectively). These results show the potential use of algae as sugar resource of fermentable sugars and the use of osmotic stress to increase the total sugar production. This work shows also trehalose accumulation as osmolyte by *Chlorella*.

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