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Different factors influencing to brown algae hydrolysis to produce mannitol

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Abstract
Seaweeds contain a variety of biologically active substances, which are useful for therapy and medicinal purposes. Brown algae *Endarachne binghamiae* (Phaeophyceae) as well-balanced, harmless, natural sources with a high degree of bioavailability of trace elements are strongly advised for fast grown children and pregnant women. We survey different factors affecting to hydrolysis of brown algae to produce mannitol. Our results are as follows: H_2SO_4 concentration 2%; steaming time 30 minutes; substrate 1.5%; enzyme Cellic 20% – Cellulase 12% – AMG 2%; pH 5; incubation temperature 38 ^oC.

Keywords: Brown algae, hydrolysis, incubation, mannitol.

1. Introduction
The marine brown alga *Endarachne binghamiae* (Phaeophyceae) is widely distributed in temperate and tropical waters. It occurs abundantly on the southern Vietnam. It looks like a tiny kelp with about 10 cm height. *E. binghamiae* is a common sea vegetable and also a good prey for fishing lembus rudder fish by local fishermen. Marine algae have been traditionally used as food and medicine. It is because these marine Algae contain the essential amino acids and polyunsaturated fatty acids, necessary vitamins and minerals, and larger amounts of dietary fibers. Additionally, these contain a variety of biologically active substances, which possess antibacterial (Liao *et al.*, 2003), antiviral (Hudson *et al.*, 1999), agglutinating, and antitumor (Yamamoto et al., 1982; Okai et al., 1997; Nika *et al.*, 2003) activities. Other reports indicated that marine algal polysaccharides and proteinaceous substances have valuable functions in immune modulation and stimulation (Hori *et al.*, 1988; Otterlei *et al.*, 1991; Yoshizawa *et al.*, 1993; Shan *et al.*, 1999; Son *et al.*, 2001), as well as in lowering blood pressure, cholesterol, and glucose level (Hoppe, 1979). Thus, marine algae can be a suitable source of materials for development and utilization of health foods and drugs (Ross *et al.* 2008; Anastasakis *et al.* 2011). Main components of marine seaweeds differ from that of terrestrial biomass (cellulose, hemicellulose, and lignin) and include phytochemically active molecules such as polysaccharides, fatty acids, proteins, vitamins, and mineral elements, which are compounds with potential applications in food, cosmetic, pharmaceutical, and medical fields (O'Sullivan *et al.* 2010; Anastasakis *et al.* 2011; Lordan *et al.* 2011).
There are several studies mentioned to brown algae hydrolysis into different functional products as follows: Rang Huang and Hui-Ting Lee (2005) described immunological properties of the marine brown alga *Endarachne binghamiae* (Phaeophyceae). It was observed that various extracts from the alga effectively stimulated cell proliferation and that the stimulation activity of active substances varied with growth habitat of the alga assayed. The reported alga was found rich in polysaccharides. Of which sodium alginate exhibited strong stimulation activity for macrophage and T cell proliferation, and also alginic acid but to a lesser extent. A glycoprotein isolated from the reported alga was also a strong proliferation stimulant. Additionally, it significantly induced the production of TNF-aand nitric oxide by macrophages and IFN-γby Tcells in a concentration-dependent manner. These assay results suggested that alginate and protein of the reported alga could be promising immune stimulants and modulants.
Sato Minoru, *et al.*, (2005) produced the blood pressure lowing peptides from brown alga (*Undaria pinnatifida*). Brown alga (*Undaria pinnatifida*) was treated with alginate lyase and hydrolyzed using 17 kinds of proteases and the inhibitory activity of the hydrolysates for the angiotensin-I-converting enzyme (ACE) was measured. Four hydrolysates with potent ACE-inhibitory activity were administered singly and orally to spontaneously hypertensive rats (SHRs).

The systolic blood pressure of SHRs decreases significantly after single oral administration of the brown alga hydrolysates by protease S 'Amano' (from *Bacillus stearothermophilus)* at the concentration of 10 (mg protein) (kg body weight)$^{-1}$. In the 17 weeks of feeding experiment, 7-week-old SHRs were fed standard diet supplemented with the brown alga hydrolysates for 10 weeks. In SHRs fed 1.0 and 0.1% brown alga hydrolysates, elevating of systolic bloodpressure was significantly suppressed for 7 weeks. To elucidate the active components, the brown alga hydrolysates were fractionated by 1-butanol extraction and HPLC on a reverse-phase column. Seven kinds of ACE-inhibitory peptides were isolated and identified by amino acid composition analysis, sequence analysis, and LC-MS with the results Val-Tyr, Ile-Tyr, Ala-Trp, Phe-Tyr, Val-Trp, Ile-Trp, and Leu-Trp. Each peptide was determined to have an antihypertensive effect after a single oral administration in SHRs. The brown alga hydrolysates were also confirmed to decrease the blood pressure in humans.

Karim Senni *et al.*, (2006) investigated fucoidan a sulfated polysaccharide from brown algae is a potent modulator of connective tissue proteolysis. They investigated the action of a 16 kDa fucoidan fraction on parameters involved in connective tissue breakdown. This fucoidan is able to inhibit gelatinase A secretion and stromelysin 1 induction by interleukin-1ß on dermal fibroblasts in culture. Furthermore, we observed that fucoidan increases the rate of association of MMPs with their specific inhibitors namely TIMPs. Using tissue sections of human skin in ex vivo experiments, we evidenced that this polysaccharide was able to minimize human leukocyte elastase activity resulting in the protection of human skin elastic fiber network against the enzymatic proteolysis due to this serine proteinase. These results suggested that fucoidan could be used for treating some inflammatory pathologies in which uncontrolled extracellular matrix degradation takes place.

Yasantha Athukorala *et al.*, (2006) verified antiproliferative and antioxidant properties of an enzymatic hydrolysate from brown alga.The potential antiproliferative and antiradical activities of an enzymatic extract of Ecklonia cava together with its crude polysaccharide (CpoF) and crude polyphenolic fractions (CphF) were evaluated in vitro. Tested extracts showed strong selective cell proliferation inhibition on all cancer cell lines tested, especially CphF extract, containing high polyphenol amount, showed 5.1 lg/ml of IC value on murine colon cancer (CT-26) cell line. According to the nuclear staining experiment, antiproliferative effect of CphF was associated with apoptotic cell demise in CT-26. In addition, The CphF at 5 lg/ml scavenged 70% of DPPH radical, which is much higher than those of BHA and BHT at same concentration. Furthermore CphF exhibited interesting antiradical properties, expressed by its capacity to scavenge superoxide anion (O2), hydrogen peroxide (H2O2) and hydroxyl radical (OH). In reducing power assay, CphF extract at 5 lg/ml was found to be as high as that of BHT at same concentration. Also, in total antioxidant assay the effect of CphF at 50 lg/ml was equivalent or slightly higher than those of commercial counterparts at 5 lg/ml concentration. Taken together, the CphF may be a promising alternative to synthetic substances as natural compound with high antiproliferative and antiradical activity.

Ji-Hee Song et al., (2011) investigated butyric acid production from brown algae using *Clostridium tyrobutyricum* ATCC 25755. Butyric acid fermentation by *Clostridium tyrobutyricum* ATCC 25755 using glucose or brown algae as a carbon source was carried out. Initially, different fermentation modes (batch, fed-batch, and semi-continuous) at pH 6 and 37 °C were compared using a model medium containing glucose as a carbon source. By feeding the whole medium containing 40 ~ 50 and 30 g/L of glucose into the fed-batch and semi-continuous fermentations, very similar butyrate yields (0.274 and 0.252 g butyrate/g glucose, respectively) and productivities (0.362 and 0.355 g/L/h, respectively) were achieved. The highest butyrate concentration was about 50 g/L, which was observed in the fed-batch fermentation with whole medium feeding. However, semi-continuous fermentation sustained a longer fermentation cycle than the fed-batch fermentation due to end-product and metabolic waste inhibition. The established conditions were then applied to the fermentation using brown algae, *Laminaria japonica*and *Undaria pinnatifida*, as substrates for butyric acid fermentation. To hydrolyze brown algae, 7.5 ~ 10% (w/v) dried brown algae powder was suspended in 1% (w/v) NaOH or 0.5 ~ 2.5% (w/v) $H_2SO_4$and then autoclaved at 121°C for 30 ~ 90 min. The resulting butyrate concentration was about 11 g/L, which was produced from 100 g/L of *L. japonica* autoclaved for 60 min in 1.5% H_2SO_4 acid solution.

Rui Chen et al., (2012) used an algal hydrolysate to improve enzymatic hydrolysis of lignocellulose. This study investigated the use of acid hydrolyzed algae to enhance the enzymatic hydrolysis of lignocellulosic biomass. The farm-waste grown algal samples were first characterized, and the optimal conditions for algal hydrolysis using dilute sulfuric acid were determined. Neutralized algal hydrolysate was then tested as a reaction medium (replacing the pH buffer solution) for the enzymatic hydrolysis of a lignocellulose, alkali treated anaerobically-digested fiber. Our results showed that net glucose yields from enzymatic hydrolyses containing undiluted algal hydrolysate were at least 65% higher than net glucose yields from control media (bovine serum albumin solution, citrate buffer or distilled water). It is likely that the increase in net glucose yield is due, in part, to the binding of hydrolyzed algal proteins to lignin, which protects cellulase from binding to lignin. This study demonstrates a potential approach of using wastewater-grown algae as a co-substrate to significantly enhance the enzymatic hydrolysis of lignocellulosic materials.

Maria Enquist-Newman *et al.*, (2014) performed efficient ethanol production from brown macroalgae sugars by a synthetic yeast platform. The increasing demands placed on natural resources for fuel and food production require that we explore the use of efficient, sustainable feedstocks such as brown macroalgae. The full potential of brown macroalgae as feedstocks for commercial-scale fuel ethanol production, however, requires extensive re-engineering of the alginate and mannitol catabolic pathways in the standard industrial microbe *Saccharomyces cerevisiae*. Here we present the discovery of an alginate monomer (4-deoxy-L-erythro-5-hexoseulose uronate, or DEHU) transporter from the alginolytic eukaryote *Asteromyces cruciatus*[4]. The genomic integration and overexpression of the gene encoding this transporter, together with the necessary bacterial alginate and deregulated native mannitol catabolism genes, conferred the ability of an *S. cerevisiae* strain to efficiently metabolize DEHU and mannitol. When this platform was further adapted to grow on mannitol and DEHU under anaerobic conditions, it was capable of ethanol fermentation from mannitol and DEHU, achieving titres of 4.6% (v/v) (36.2 g l^{-1}) and yields up to 83% of the maximum theoretical yield from consumed sugars. These results show that all major sugars in brown macroalgae can be used as feedstocks for biofuels and value-added renewable chemicals in a manner that is comparable to traditional arable-land-based feedstocks.

Purpose of our research is to investigate different factors such

as H_2SO_4 concentration; steaming time; substrate; enzyme Cellic – Cellulase – AMG 2%; pH; incubation temperature influencing to the brown algae hydrolysis to produce mannitol.

Fig 1. Brown algae (Phaeophyceae)

2. Material & Method

2.1 Material

Brown algaes are collected in Tra Vinh province. Enzymes Cellic, Cellulase and AMG are purchased from Novozyme Co. Ltd.

2.2 Research method

2.2.1 Effect of different H_2SO_4 concentrations to mannitol hydrolysis

Experiment is conducted by treatment of brown algae hydrolysis at 120ºC in 20 minutes by different H2SO4 concentrations 1%, 1.25%, 1.5%, 1.75%, 2%, 2.5%.

2.2.2 Effect of steaming time to mannitol hydrolysis

Experiment is conducted by treatment of brown algae hydrolysis at 120 ºC with H_2SO_4 2% by different steaming times 20 minutes, 30 minutes, 40 minutes.

2.2.3 Effect of substrate concentration to mannitol hydrolysis

Experiment is conducted by treatment of brown algae hydrolysis at 120 ºC in 30 minutes with H_2SO_4 2% by different substrate concentrations 1.0%, 1.5%, 2.0%, 2.5%

2.2.4 Effect of enzyme concentration to mannitol hydrolysis

Experiment is conducted by treatment of brown algae hydrolysis at 120 ºC in 30 minutes with H_2SO_4 2%, substrate concentration 1.5% by different enzyme formulas as follows.

Table 1: Different enzyme formulas for hydrolysis experiment

	Cellic (%)	Cellulase (%)	AMG (%)
Formula #1	19.0	11.0	1.8
Formula #2	19.5	11.5	1.9
Formula #3	20.0	12.0	2.0
Formula #4	20.5	12.5	2.1
Formula #5	21.0	13.0	2.2
Formula #6	21.5	13.5	2.3

2.2.5 Effect of pH to mannitol hydrolysis

Experiment is conducted by treatment of brown algae hydrolysis at 120 ºC in 30 minutes with H_2SO_4 2%, substrate concentration 1.5%, enzyme (20% cellic - 12% cellulase - 2% AMG) by different pH values: 4.0, 4.5, 5.0, 5.5, 6.0.

2.2.6 Effect of incubation temperature to mannitol hydrolysis

Experiment is conducted by treatment of brown algae hydrolysis at 120 ºC in 30 minutes with H_2SO_4 2%, substrate concentration 1.5%, enzyme (20% cellic - 12% cellulase - 2% AMG), pH 5.0 by different incubation temperatures: 32 ºC, 35 ºC, 38 ºC, 41 ºC, 45 ºC.

2.3 Statistical analysis

All data are processed by Excell 2003

3. RESULT & DISCUSSION

3.1 Effect of different H_2SO_4 concentrations to mannitol hydrolysis

Table 2: Effect of different H_2SO_4 concentrations to mannitol hydrolysis (HMF value)

H_2SO_4 (%)	**Treatment time 0h**	**Treatment time 24h**	**Treatment time 48h**
1.00	0.838	1.056	0.984
1.25	0.971	0.977	0.902
1.50	0.899	1.013	1.089
1.75	0.842	0.840	0.905
2.00	0.491	0.584	0.597
2.50	0.383	0.341	0.438

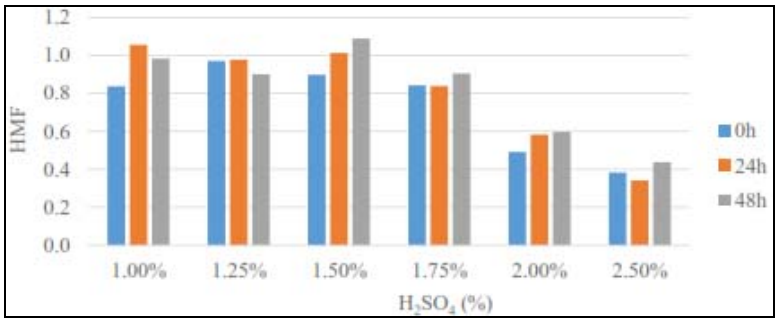

Fig 2: Effect of different H_2SO_4 concentrations to mannitol hydrolysis

Table 3: Effect of different H_2SO_4 concentrations to mannitol hydrolysis (°Brix value)

	oBrix		
H_2SO_4 (%)	**Treatment time 0h**	**Treatment time 24h**	**Treatment time 48h**
1.00	4.51	4.54	3.66
1.25	4.47	5.01	3.84
1.50	4.36	5.24	4.05
1.75	4.44	5.29	4.12
2.00	6.31	6.66	5.31
2.50	5.49	5.80	4.29

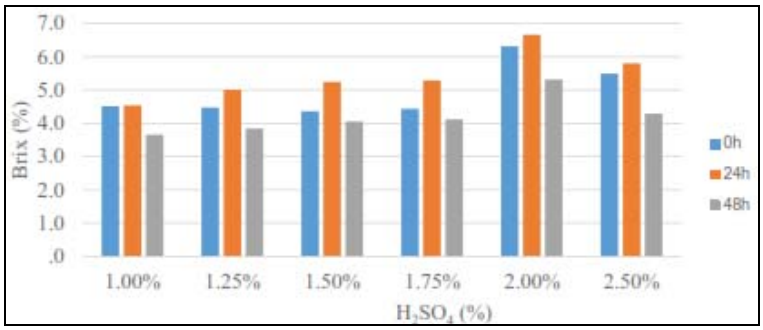

Fig 3: Effect of different H_2SO_4 concentrations to mannitol hydrolysis (°Brix value)

Table 4: Effect of different H_2SO_4 concentrations to mannitol hydrolysis (mannitol %)

	Mannitol (%)		
H_2SO_4 (%)	**Treatment time 0h**	**Treatment time 24h**	**Treatment time 48h**
1.00	22.2	26.2	23.6
1.25	26.0	27.8	22.1
1.50	28.3	33.9	33.2
1.75	35.4	36.3	36.4
2.00	39.8	52.4	49.7
2.50	38.3	48.0	46.3

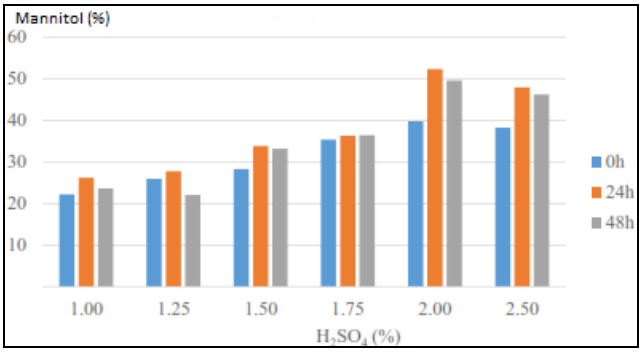

Fig 4: Effect of different H_2SO_4 concentrations to mannitol hydrolysis (mannitol %)

H_2SO_4 2% hydrolizes the brown algae in maxium amount so this concentration is selected for further experiments.

3.2 Effect of steaming time to mannitol hydrolysis

Table 5: Effect of steaming time to mannitol hydrolysis (HMF value)

	HMF (5 – hydroxymethyl furfual) value		
Steaming time	**Treatment time 0h**	**Treatment time 24h**	**Treatment time 48h**
20 minutes	0.35191	0.40925	0.45958
30 minutes	0.38696	0.39573	0.47602
40 minutes	0.40692	0.49293	0.51594

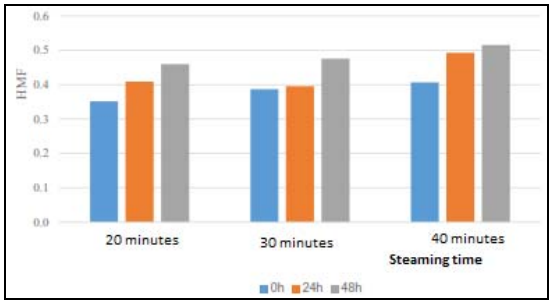

Fig 5: Effect of steaming time to mannitol hydrolysis (HMF value)

Table 6: Effect of steaming time to mannitol hydrolysis (°Brix)

	oBrix		
Steaming time	**Treatment time 0h**	**Treatment time 24h**	**Treatment time 48h**
20 minutes	5,42	6.10	5.89
30 minutes	5.35	6.53	6.31
40 minutes	5.40	6.08	5.88

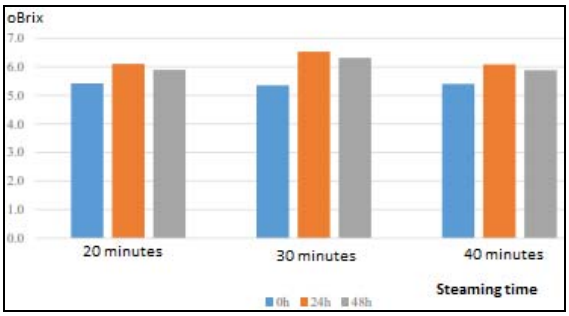

Fig 6: Effect of steaming time to mannitol hydrolysis (°Brix)

Table 7: Effect of steaming time to mannitol hydrolysis (mannitol %)

	Mannitol %		
Steaming time	**Treatment time 0h**	**Treatment time 24h**	**Treatment time 48h**
20 minutes	49.847	70.538	65.172
30 minutes	54.451	82.244	80.379
40 minutes	56.432	76.068	71.231

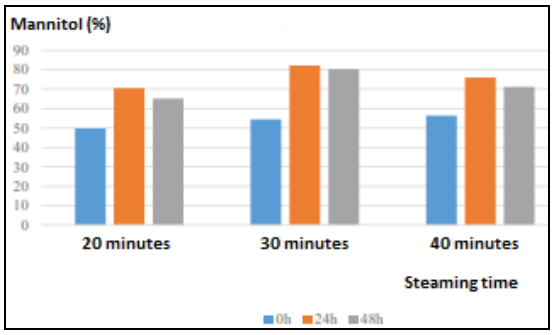

Fig 7: Effect of steaming time to mannitol hydrolysis (mannitol %)

Mannitol hydrolysis is maximum 82.244% at steaming time 30 minutes. So we choose this value for further experiments.

3.3 Effect of substrate to mannitol hydrolysis

Table 8: Effect of substrate to mannitol hydrolysis (HMF value)

	HMF (5 – hydroxymethyl furfual) value		
Substrate	**Treatment time 0h**	**Treatment time 24h**	**Treatment time 48h**
1.0%	0.31494	0.34643	0.33993
1.5%	0.35277	0.39483	0.39243
2.0%	0.38182	0.45121	0.42959
2.5%	0.42677	0.46173	0.40859

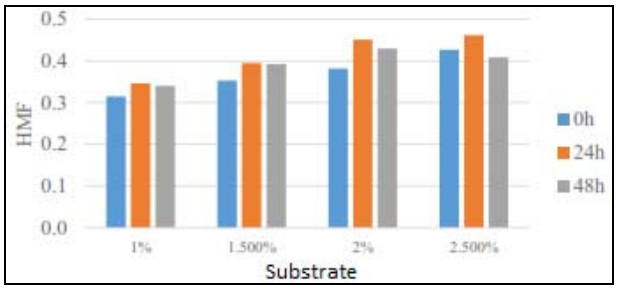

Fig 8: Effect of substrate to mannitol hydrolysis (HMF value)

Table 9: Effect of substrate to mannitol hydrolysis (oBrix)

	oBrix		
Substrate	**Treatment time 0h**	**Treatment time 24h**	**Treatment time 48h**
1.0%	5.53	6.57	6.31
1.5%	5.31	6.79	6.56
2.0%	5.28	5.92	6.14
2.5%	5.26	6.35	6.60

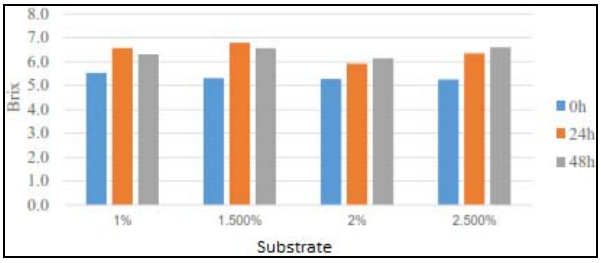

Fig 9: Effect of substrate to mannitol hydrolysis (oBrix)

Table 10: Effect of substrate to mannitol hydrolysis (Mannitol %)

	Mannitol %		
Substrate	**Treatment time 0h**	**Treatment time 24h**	**Treatment time 48h**
1.0%	59.724	81.865	77.139
1.5%	59.271	84.131	81.185
2.0%	52.473	78.369	79.405
2.5%	56.292	75.974	71.416

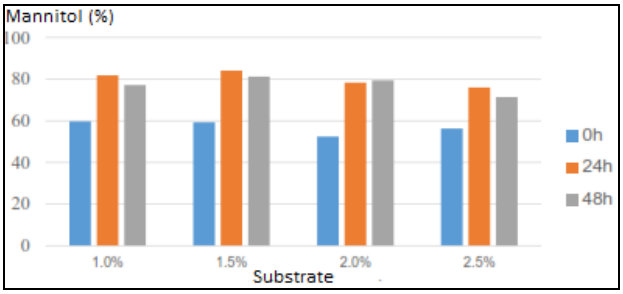

Fig 10: Effect of substrate to mannitol hydrolysis (Mannitol %)

At 1.5% substrate, the hydrolysis efficiency is maximum at 84.131% after 24 hours.

3.4 Effect of enzyme ratio to mannitol hydrolysis

Table 11: Effect of enzyme ratio to mannitol hydrolysis (HMF value)

	HMF (5 – hydroxymethyl furfual) value		
Enzyme	**Treatment time 0h**	**Treatment time 24h**	**Treatment time 48h**
Formula #1	0.38215	0.41306	0.48214
Formula #2	0.37167	0.40143	0.54114
Formula #3	0.37538	0.41257	0.42091
Formula #4	0.35071	0.48532	0.55809
Formula #5	0.40231	0.53768	0.48313
Formula #6	0.39636	0.50628	0.57991

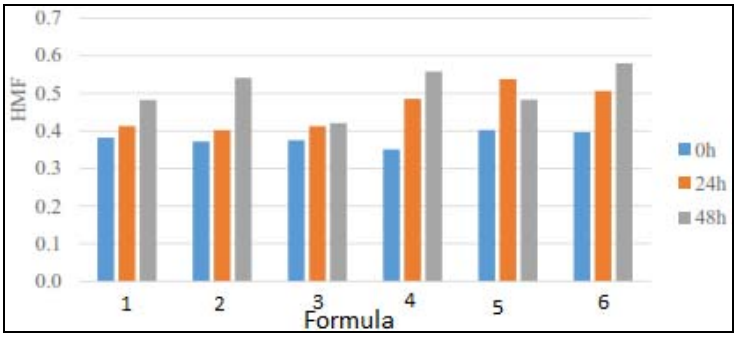

Fig 11: Effect of enzyme ratio to mannitol hydrolysis (HMF value)

Table 12: Effect of enzyme ratio to mannitol hydrolysis (°Brix)

	°Brix		
Enzyme	**Treatment time 0h**	**Treatment time 24h**	**Treatment time 48h**
Formula #1	5.20	5.98	5.79
Formula #2	5.23	6.05	6.62
Formula #3	5.21	6.90	6.47
Formula #4	5.36	5.92	5.56
Formula #5	5.51	6.45	6.60
Formula #6	5.30	6.27	5.78

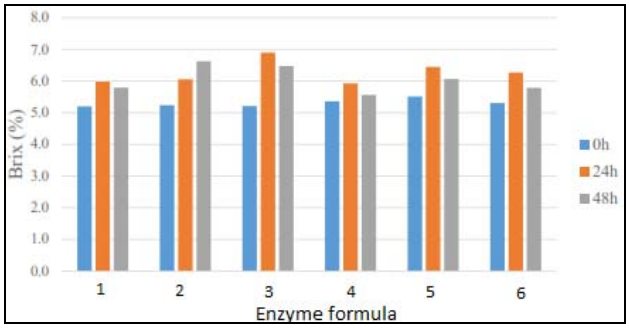

Fig 12: Effect of enzyme ratio to mannitol hydrolysis (°Brix)

Table 13: Effect of enzyme ratio to mannitol hydrolysis (Mannitol %)

	Mannitol (%)		
Enzyme	**Treatment time 0h**	**Treatment time 24h**	**Treatment time 48h**
Formula #1	58.879	80.146	75.135
Formula #2	55.849	75.601	80.006
Formula #3	56.840	75.601	83.555
Formula #4	59.054	82.710	78.235
Formula #5	65.929	77.466	72.030
Formula #6	67.619	81.720	76.5417

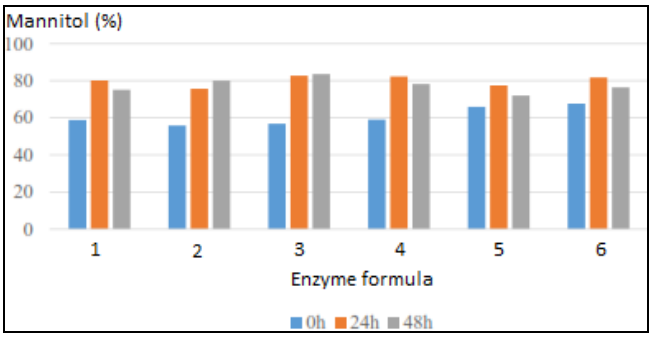

Fig 13: Effect of enzyme ratio to mannitol hydrolysis (Mannitol %)
At enzyme formual #3 (20% cellic - 12% cellulase - 2% AMG), mannitol hydrolized highest at 83.555% after 48 hours.

3.5 Effect of pH mannitol hydrolysis

Table 14: Effect of pH mannitol hydrolysis (HMF value)

	HMF (5 – hydroxymethyl furfual) value		
pH	**Treatment time 0h**	**Treatment time 24h**	**Treatment time 48h**
4.0	0.34690	0.48305	0.48179
4.5	0.35586	0.46619	0.45774
5.0	0.39538	0.43257	0.42091
5.5	0.39614	0.47067	0.46824
6.0	0.37155	0.40720	0.41609
6.5	0.35291	0.46251	0.44577

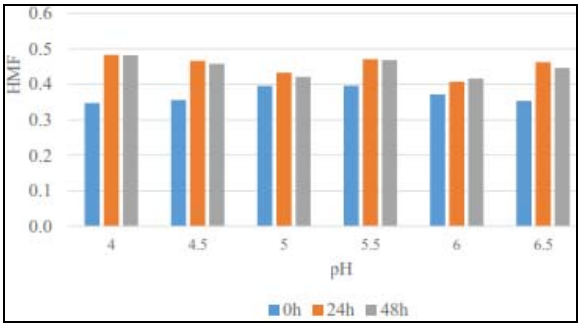

Fig 14: Effect of pH mannitol hydrolysis (HMF value)

Table 15: Effect of pH mannitol hydrolysis (°Brix)

	°Brix		
pH	**Treatment time 0h**	**Treatment time 24h**	**Treatment time 48h**
4.0	5.56	6.22	6.01
4.5	5.65	6.59	6.65
5.0	5.62	6.71	6.48
5.5	5.52	6.46	6.35
6.0	5.86	6.35	6.11
6.5	5.59	6.16	6.31

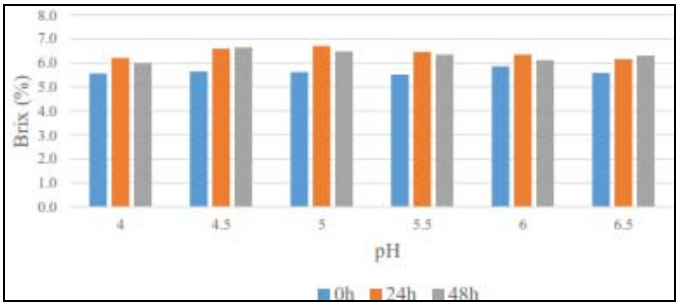

Fig 15: Effect of pH mannitol hydrolysis (°Brix)

Table 16: Effect of pH mannitol hydrolysis (Mannitol %)

	Mannitol %		
pH	**Treatment time 0h**	**Treatment time 24h**	**Treatment time 48h**
4.0	58.24	78.95	76.59
4.5	65.11	83.87	75.78
5.0	60.34	89.70	83.55
5.5	60.98	76.35	73.56
6.0	61.73	70.01	75.43
6.5	62.61	74.67	78.55

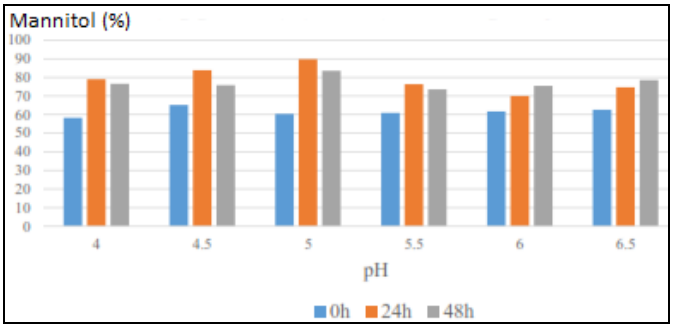

Fig 16: Effect of pH mannitol hydrolysis (Mannitol %)
Optimal pH for hydrolysis is 5; mannitol is 89.702%; Brix is 6.71%.

3.6 Effect of incubation temperature to mannitol hydrolysis

Table 17: Effect of incubation temperature to mannitol hydrolysis (HMF value)

	HMF (5 – hydroxymethyl furfual) value		
Incubation temperature	**Treatment time 0h**	**Treatment time 24h**	**Treatment time 48h**
32°C	0.35114	0.44075	0.44853
35°C	0.34454	0.45091	0.47369
38°C	0.39765	0.49765	0.48093
41°C	0.34976	0.54765	0.59304
44°C	0.35546	0.59612	0.65016

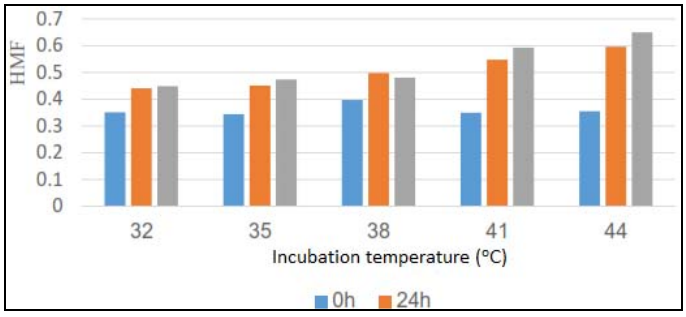

Fig 17: Effect of incubation temperature to mannitol hydrolysis (HMF value)

Table 18: Effect of incubation temperature to mannitol hydrolysis (°Brix)

	°Brix		
Incubation temperature	**Treatment time 0h**	**Treatment time 24h**	**Treatment time 48h**
32°C	5.5	6.6	6.7
35°C	5.7	6.9	6.9
38°C	5.7	7.3	7.1
41°C	5.6	6.9	6.6
44°C	5.6	6.6	6.4

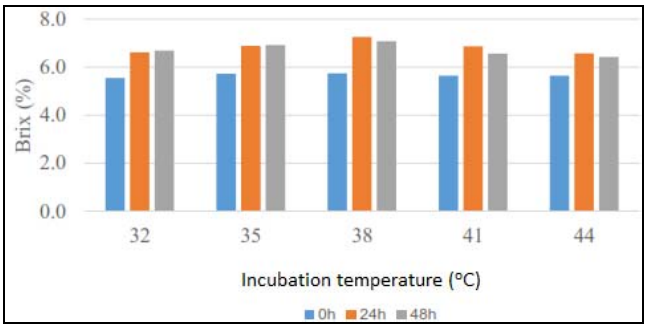

Fig 18: Effect of incubation temperature to mannitol hydrolysis (°Brix)

Table 19: Effect of incubation temperature to mannitol hydrolysis (Mannitol %)

	Mannitol (%)		
Incubation temperature	**Treatment time 0h**	**Treatment time 24h**	**Treatment time 48h**
32°C	55.185	78.181	72.717
35°C	60.416	82.480	82.905
38°C	55.595	89.265	87.452
41°C	59.997	79.580	77.767
44°C	57.449	76.938	70.273

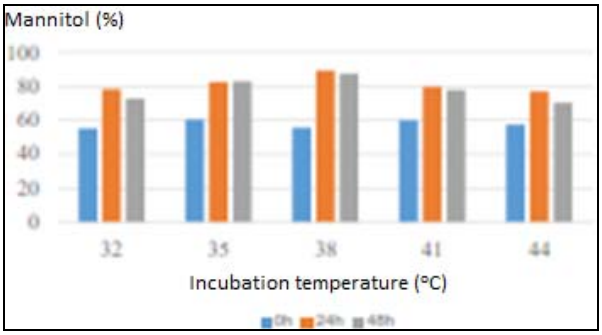

Fig 19: Effect of incubation temperature to mannitol hydrolysis (Mannitol %)
Samples incubated in 24h at temperature 38 °C have the maximum mannitol 89.3%.

4. Conclusion

In recent years, great attention has been given to the use of marine seaweed biomass. Such interest has been supported by important advantages that the use of this kind of biomass represents: (a) low future fluctuations in biomass demand are expected due to overpopulation; (b) feasibility of growing fast in the open ocean; (c) higher photosynthetic efficiency than terrestrial biomass; (d) no limitation by water and to a lesser extent by temperature; and (e) low costs of collection In order to use algal proteins and carbohydrates for value-added fuel/chemical production, a hydrolysis step is needed to break down algal cell walls and convert large molecules of carbohydrates and proteins into smaller molecules of mono-sugars, peptides, and amino acids that are able to be utilized by various biological conversion processes.

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