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The Biorefinery Concept: Production of Bioactive Compounds and Biofuels

Tesis que presenta

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Para obtener el grado de

Doctor en Ciencias en Biología Molecular

Director de la Tesis:

Dr. Antonio De León Rodríguez

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Constancia de aprobación de la tesis

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Angel Mario López Hidalgo

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The Biorefinery Concept: Production of Bioactive Compounds and Biofuels

Ha sido descrito con anterioridad que la biomasa tiene el potencial de reemplazar una gran fracción de los recursos fósiles como materia prima para producciones industriales. Sin embargo, para que la conversión de biomasa sea rentable es necesario integrar la producción de combustibles, energía y productos químicos de valor agregado, esta integración es conocida como biorrefinería. Por lo anterior, durante el desarrollo de este trabajo se propone la conversión de biomasa a biocombustibles y compuestos bioactivos, en procesos independientes que pueden integrarse en un futuro bajo el concepto de biorrefinería. En esta tesis se demostró la capacidad natural de bacterias psicrófilas para producir hidrógeno y etanol a partir de glucosa. También, se determinó que la producción de hidrógeno por lodos granulares anaerobios utilizando mezclas de residuos agroindustriales no es afectada por el cambio de volumen de trabajo. Aunado a lo anterior, se optimizó y escaló exitosamente la producción simultánea de etanol e hidrógeno a partir de hidrolizado de paja de trigo por cepas recombinantes de *Escherichia coli*. Finalmente, se realizó la optimización de la extracción asistida por ultrasonido de pigmentos y compuestos fenólicos provenientes de algas marinas a fin de obtener extractos crudos con actividad antioxidante, demostrándose que pueden ser usados como antioxidantes o antimicrobianos, o bien, en la terapia contra cáncer de colón o la virulencia de *Staphylococcus aureus*. Todos los procesos anteriormente mencionados se evalúan bajo un concepto de una biorrefinería multinivel.

Palabras clave: biocombustibles, compuestos bioactivos, hidrolizado de paja de trigo, suero de leche, macroalgas

Abstract

The Biorefinery Concept: Production of Bioactive Compounds and Biofuels

Have been described that biomass has the potential to replace a large fraction of fossil resources as feedstocks for industrial productions. However, to conversion of biomass been profitable it is necessary integrate the production of fuels, power and added value chemical products. The integration of these processes is known as biorefinery. Therefore, during the development of this work was proposed the biomass conversion to biofuels and bioactive compounds, in independent processes that could be integrated in the future under the biorefinery concept. In this thesis was demonstrated the natural ability of psychrophilic bacteria to produce hydrogen and ethanol from glucose. Also, it was determined that the working volume doesn't affect the hydrogen production by anaerobic granular sludge from agro-industrial waste mixtures. In addition, the simultaneous production of ethanol and hydrogen by recombinant *Escherichia coli* strains from wheat straw hydrolysate was optimized and scaled up successfully. Finally, the optimization of ultrasonic assisted extraction of pigments and phenolic compounds from marine algae was achieved, demonstrating that it could be used as antioxidants and antimicrobials, or in anti-virulence therapies of Staphylococcus aureus or against colon cancer. All the aforementioned processes are evaluated under a concept of a multilevel biorefinery.

Keywords: biofuels, bioactive compounds, wheat straw hydrolysate, cheese whey, macroalgae

Background

The need for energy is continuously increasing, due to increases in industrialization and population. The growth of the world's energy demand raises urgent problems. The use of biomass for energy purposes is central to the secure and sustainable supply of energy. However, to achieve a successful implementation of bioenergy must be new and sustainable biomass resources and corresponding conversion technologies are identified and developed. Terrestrial and aquatic plants use photosynthesis to convert solar energy into chemical energy. It is stored in the form of oils, carbohydrates, proteins, etc. This plant energy is converted to biofuels. Hence, biofuels are primarily a form of solar energy. For biofuels to succeed at replacing large quantities of petroleum fuel, the feedstock availability needs to be as high as possible. There is an urgent need to design integrated biorefineries that are capable of producing transportation fuels and chemicals.

Objectives and thesis structure

Based on the challenges described, the main objective of this PhD project were evaluate process environment friendly to produce high added value chemicals with bioactive potential and liquid and gaseous biofuels using lignocellulosic raw material and seaweed biomass. In order to fulfil the main objective, the following specific objectives were:

- Determine the ability of psychrophilic microorganisms to produce hydrogen and ethanol
- Optimize the hydrogen production by anaerobic granular sludge using mixtures of agro-industrial residues as substrates

- Optimize and scale up the simultaneous production of ethanol and hydrogen by genetically modified bacteria using an agro-industrial residue
- Optimize the ultrasonic assisted extraction of bioactive compounds from seaweed species
- Determine the antioxidant, antimicrobial, anti-virulence and anticancer activities of seaweed extracts

In Chapter 1 results of the ability of psychrophilic bacteria to produce hydrogen and ethanol are given. In Chapter 2 the optimization of the co-culture of two agro industrials residue to produce hydrogen by anaerobic granular sludge are presented. Chapters 3 and 4 contains the optimization and scaling up of simultaneous production of ethanol and hydrogen by recombinant *E. coli* strains using wheat straw hydrolysate as substrate. Chapters 5 and 6 are dedicated to the optimization of ultrasonic assisted extraction of bioactive compounds from seaweed and to the determination their potential antioxidant, antimicrobial, anti-virulence and anticancer activities.

1 Biohydrogen production using psychrophilic bacteria isolated from Antarctica

The climate-driven changes have intensified the search for alternative energy sources, among which hydrogen stands out. It is an attractive option because is a renewable energy source, its combustion generates only water and heat, and has a high-energy yield of 122 kJ/g [1]. One of the important options to produce hydrogen is through microbial fermentation (biohydrogen), which can be classified as biophotolysis, dark fermentation, photofermentation, and microbial electrolysis cell [2]. Among these, dark fermentation and photofermentation technologies are processes that are being studied widely. Compared with photofermentation, anaerobic dark fermentation has the advantages of not requiring solar input and accepting a variety of substrates, such as organic waste, agricultural crops, or their byproducts, and using a very simple reactor technology [2].

Although the current biohydrogen yields are low, it is expected that with improvements in technology and genetic engineering, the amount of generated biohydrogen could be enhanced tremendously [3]. Highly efficient biohydrogen producing strains still need to be screened. Anaerobic fermentative microbial species may be obtained from the natural environment and screening in the laboratory [4]. Most full-scale anaerobic digesters operate under mesophilic or thermophilic conditions and most rarely in the psychrophilic temperature ranges. However, it is desirable to find psycrophilic microorganisms that improve biofuel production and reduce the energy consumption for heating of the digesters [5–8]. On this particular issue, some authors have also noticed that biohydrogen could be produced at low temperatures [9, 10].

The harsh environmental conditions of continental Antarctica have done that organisms face severe conditions such as low water and nutrient availability, extremely cold temperatures, oxidative stress, frequent freeze thaw cycles, periods of prolonged darkness in winter, and exposure to high levels of ultraviolet radiation in summer. Therefore, Polar Regions such as the Antarctica represent a vast resource of novel psychrophilic microorganisms [11, 12].

Psychrophilic bacteria and their enzymes are of commercial interest because their possibility of use at low temperatures and to scientific interest due to their relationship between protein structure and thermal stability [13, 14]. The main areas of potential applications are food technologies, medical uses, bioremediation and environmental sciences [14]. However, biotechnological applications of psychrophile for biofuel production, such as biohydrogen, have not been assessed. Thus, the capability of cultivable psychrophilic microorganisms for biohydrogen production was studied.

1.1 Material and methods

1.1.1 Strains and culture media

The psychrophilic microorganisms isolated from Antarctica [15] used in this study are showing in Table 1.1. Each strain was grown separately in YPG agar plates and maintained at 4 °C. Biohydrogen production experiments were done in a rich production medium containing 2.75 g/L Bacto-tryptone (Difco), 0.25 g/L yeast extract (Difco) and 20 g/L glucose (Sigma) [16].

Table 1.1 Taxonomic classification and biohydrogen production parameters for the psychrophilic bacteria cultured with 20 g/L of glucose, pH 6.5 and 25 °C.

Strain	Accession number	Closest relative according to the NCBI	Identity (%)	Biohydrogen production (mL)	Production rate (mL/L/h)	Yield (mol H_2/mol$_{glucose}$)
M02	EU636032	*Sejongia marina* (EF554366)	97.9	34.8	3.99	0.32
L2	HQ226068	*Bacillus simplex* (EU236732)	99.9	48.5	3.87	1.06
G088	EU636029	*Polaromonas rhizosphaerae* (EF127651)	98.7	141.5	11.38	0.62
GA0G	EU636051	*Pseudomonas antarctica* (AJ537601)	99.4	47.83	4.35	0.82
G024	EU636026	*Polaromonas jejuensis* strain JS12-13 (NR044379)	99.0	70.17	5.61	1.57
G057	EU636044	*Janthinobacterium agaricidamnosum* (Y08845)	98.6	42.33	3.57	1.07
N92	EU636058	*Rhodobacter ovatus* (AM690348)	96.1	228.83	14.29	0.88
N25	EU636053	*Pseudomonas frederiksbergensis* (AJ249382)	98.6	195.5	11.11	0.89
R19	EU636063	*Pedobacter aurantiacus* (DQ235228)	98.4	178	12.30	1.19
A02	EU636035	*Devosia limi* strain R-21940 (NR042324)	98.0	236.3	15.42	0.80
N04	EU636031	*Flavobacterium limicola* (AB075230)	95.8	219.67	14.96	0.74
GA0L	EU636046	*Actimicrobium antarcticum* (HQ699437)	97.7	43.67	5.08	0.52
GA051	EU636048	*Janthinobacterium agaricidamnosum* (Y08845)	98.0	253.33	16.64	0.86
GA0F	EU636050	*Pseudomonas meridiana* (AJ537602)	99.7	151.5	9.05	0.51

1.1.2 Biohydrogen production screening

Experiments were performed in 120 ml serological bottles (Prisma, DF, Mex) with a working volume of 110 ml of production medium, incubated at 25 °C, and using each psychrophilic bacterium as inoculum at pH 6.5 (Table 1.1). *Escherichia coli* WDHL, a biohydrogen overproducing strain obtained by genetic engineering, was used as a reference [17]. Each pre-inoculum was grown overnight at 25 °C and the volume needed to start the experiments with an optical density (OD600nm) of 1,

was used. Determination of biohydrogen was performed by NaOH 1N displacement and Gas Chromatography as described elsewhere [17]. All the experiments were carried out in triplicate. Samples of 1 ml were taken at different times during fermentation, then they were centrifuged and the supernatants were diluted and filtered through a 0.22 mm membrane (Millipore, Bedford, Massachusetts, USA) for metabolite analysis.

1.1.3 Analysis of metabolites

Remaining substrate and fermentation end products (glucose, succinic acid, lactic acid, and acetic acid) were analyzed by High Performance Liquid Chromatography (HPLC, Infinity LC 1220, Agilent Technologies, Santa Clara, CA USA) using a Refraction Index Detector (Agilent Technologies, Santa Clara, CA USA), and column Phenomenex Rezex ROA (Phenomenex, Torrance, CA, USA) at 60 °C, and using 0.0025 M H2SO4 as mobile phase at 0.55 ml/min. Ethanol, acetoin, propionic acid, and butyric acid were analyzed by injecting a 1 µl sample in a Gas Chromatograph 6890N (Agilent Technologies, Wilmington, DE, USA) equipped with an auto-sampler 7863 (Agilent, Wilmington, DE, USA) and a capillary column HP-Innowax (30 m × 0.25 mm i.d. × 0.25 m film thickness; Agilent, Wilmington, DE, USA). Helium was used as the carrier gas at a flow rate of 25 ml/min. Temperatures of the injector and flame ionization detector (FID) were 220 and 250 °C, respectively. The analyses were performed with a split ratio of 5:1 and a temperature program of 25 °C for 10 min, 175 °C for 1 min increased at 5 °C/min to 280 °C, and was maintained at this temperature for a final time of 10 min.

1.1.4 Statistics

The statistical analysis of the treatments was determined by analysis of variance (ANOVA) and unpaired Student's t-test. Treatments with $p < 0.05$ were statistically significant. The statistical analysis was performed using Microsoft Excel v 14.0.

1.2 Results and discussion

The growing interest in biohydrogen has intensified the search for novel and efficient microorganisms to produce it. Typically mesophilic and thermophilic microorganisms have been used as inocula for this purpose. However, they demand external energy to maintain the optimal fermentation temperature, which is in the range of 37–74 °C [7]. Meanwhile, the use of strict psychrophilic microorganisms, which only grown below 4 °C, has the inconvenience of having a very low substrate consumption rate and therefore an insignificant biohydrogen production [8]. Therefore, it is strongly preferred the use of psychrotolerant microorganisms capable of growing at room temperature. For this reason, we decided to evaluate only psychrophilic strains from our collection which are capable of growing at 25 °C.

Typical batch cultures using WDHL, M02, G088, GA051 strains for biohydrogen production are shown in the Figure 1.1. Psychrophilic bacteria produced 34.8, 141.5, and 253.3 mL, whereas WDHL produced only 20.1 mL. Psychrophilics started biohydrogen production after 36 h of culture, whereas the *E. coli* WDHL started immediately, because this strain is a genetically modified to produce biohydrogen from carbohydrates such as glucose, galactose or lactose [18].

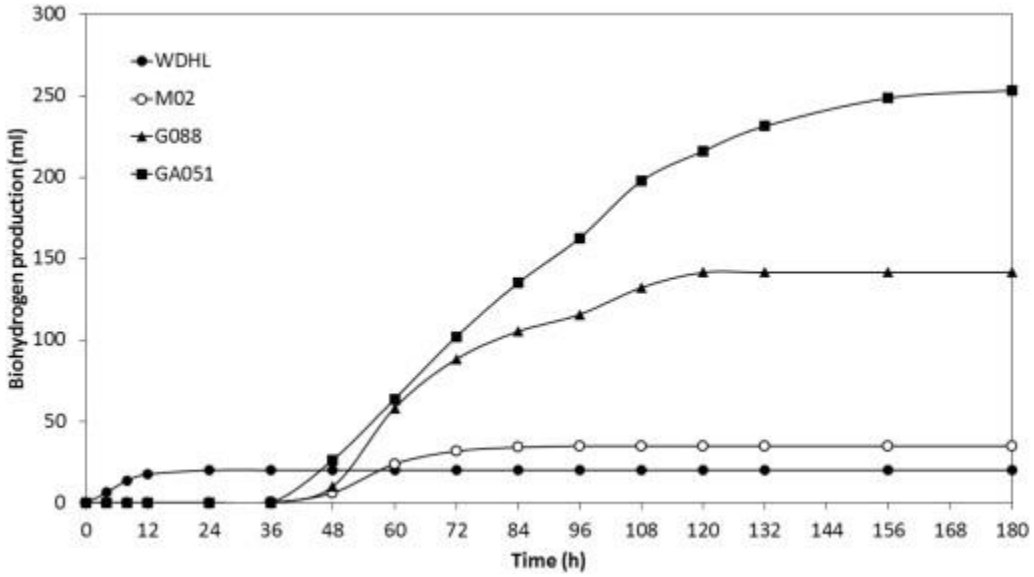

Figure 1.1 Kinetics of biohydrogen production by some psychrophilic bacteria and reference culture (▪ GA051, ▲ G088, ○ M02 and ● WDHL) using 20 g/L glucose as carbon source

From the bacteria analyzed here, 12 strains had a higher production compared to the reference strain (Figure 1.2). For instance, N92 and GA051 produced 1139 ± 260%, and 1261 ± 164%, respectively. Whereas M02 and G024 produced 173 ± 27% and 252 ± 21%, respectively, which were not statistically significant ($p < 0.05$) with respect to the WDHL. A summary of biohydrogen production parameters is shown in Table 1.1. For the M02 strain, which is related to genus *Sejongia*, the low production rate may be due to low glucose consumption and its optimal growth temperature is 15 °C [19]. G057, GA0G, GA0L and L2 had similar biohydrogen production levels below 50 mL. It is known that the species of the genus *Janthinobacterium*, which is related to G057 strain, can reduce nitrate and nitrite consuming hydrogen; however this should be confirmed. Three strains are closely

related to *Pseudomonas* genus (GA0G, N25, GA0F). Reddy et al. [20] mentioned that around 100 species of the genus *Pseudomonas* have been reported from various habitats, including Antarctica. The results shown here indicate that some species of *Pseudomonas* could be candidate for biohydrogen production such as the N25 strain. G088, R19, N25, N04, N92, A02, and GA051 were bacteria with the biohydrogen production between 141.5 and 253.3 mL. These strains are closely related to the genera *Polaromonas*, *Flavobacterium*, *Devosia*, *Rhodobacter* and *Janthinobacterium*. GA051 was the best biohydrogen producer strain and it is closely related to *Janthinobacterium agaricidamnosum* (Y08845) with a 98% of identity. This strain also presented the highest production rate of 16.64 mL/L/h and a yield of 0.86 mol H_2/mol glucose. Whereas the highest yield of 1.57 mol H_2/mol glucose was for the GA024 strain, which is closely related to *Polaromonas jejuensis* strain JS12-13 (NR044379) with an identity of 99%. Interestingly, the G057 strain is also closely related to *J. agaricidamnosum* (Y08845) with 98.6% of identity, however this strain was a bad biohydrogen producer. It is important to remark that G051 and G057 are distinct clones with different genetic background and therefore different metabolic behavior.

Srinivas et al. [21] showed that *Rhodobacter ovatus* (related to N92 strain) grows under anaerobic conditions in the light or under aerobic conditions in the dark using acetate, propionate, butyrate, valerate, caproate, succinate, mannitol, sorbitol, butanol, peptone and casein hydrolysate as a carbon source. Thus, this strain could be a candidate for additional studies on biohydrogen production by photo-fermentative pathway.

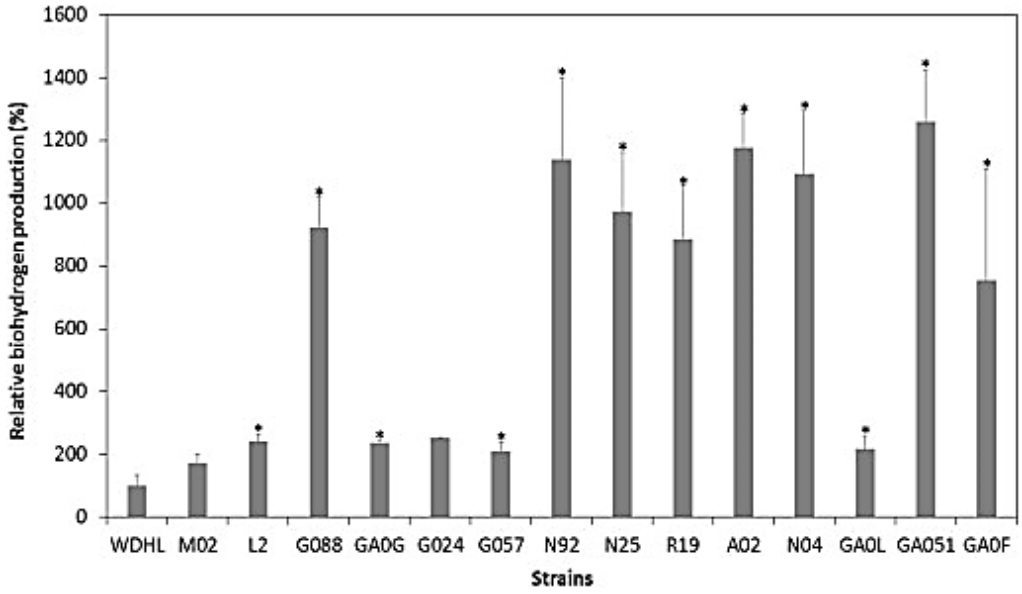

Figure 1.2 Relative biohydrogen production by psychrophilic bacteria using 20 g/L glucose as substrate. Biohydrogen production by *E. coli* WDHL strain [17] was used as reference. Values are expressed as mean ± standard deviation (n = 3). *Statistically different

To our knowledge there is no previous reports on the use of psychrophilic bacteria isolated from Antarctica for biohydrogen production. Debowski et al. [10] analyzed 12 psychrophilic strains isolated from underground water and demersal lake water samples. They used Gram-negative bacteria belonging to the class *Gammaproteobacteria-Rahnella aqualitis* (denominated RA1-RA9) and Gram-positive bacteria belonging to the phylum *Firmicutes*: *Carnobacterium maltaromaticum* (CM), *Trichococcus collinsii* (TC) and *Clostridium algidixylanolytium* (CA). RA7 strain had the highest total hydrogen volume at 66.93 ml, meanwhile in our work; the best strain had a hydrogen production of 253.3 ml, which is 3.8 times higher than RA7.

Table 1.2 compares the biohydrogen production parameters for different type of microorganisms depending on the temperature of growth. The highest production rate and yield of 1162 mL/L/h and 2.53 mol H_2/mol hexose are for the cultures at temperature above 50 °C, since they use a high temperature for the culture, large-scale implementation is a cost-energy challenge. Yield for psychrophilic microorganisms are comparable to those reported for mesophilic, but production rate are below. However, these values could increase under optimal operative conditions such as sugar concentration and pH. For example, G088 strain produced 2.5 times more biohydrogen when it was cultured with 10 g/L of glucose, pH 5.5 and 25 °C (data not shown) in comparison with that attained under the screening conditions (Table 1.1).

Since bacteria used in this study, have not been characterized previously, there is little information about them, i.e. substrate consumption and metabolites produced. Main cumulated metabolites at the end of the fermentation were analyzed and they are shown in Table 3. Ethanol was the main product followed by lactic and succinic acid. A02, N04, GA051 and GAOF, cumulated between 6.28 and 6.76 g/L ethanol, which corresponded to a yield of 0.31–0.35 g ethanol/g glucose. These values are comparable to those reported for the alcohologenic microorganisms used for bioethanol production [22]. This finding is also relevant since it is possible to develop processes using these strains for simultaneous production of hydrogen and ethanol, which today is the more important produced biofuel. Despite G057 and GA51 strains are closely related to *J. agaricidamnosum*, they showed different metabolite profiles supporting the fact that they are different microorganisms.

Table 1.2 Comparison of biohydrogen production parameters of diverse microorganisms cultured to different temperatures

Microorganism	Temperature (°C)	Working volume (ml)	Glucose (g/l)	Biohydrogen production (ml)	Production rate (ml/l/h)	Yield (mol H_2/mol glucose)	Reference
Microbial community[b]	74	450	18	NR	38.46[a] (1.35 mmol/l/h)	0.42	[23]
Thermoanaerobacterium thermosaccharolyticum	60	100	20[c]	NR	331.33 ml H_2/l/h[a] (12.12 mmol/l/h)	2.53	[24]
Clostridium ramosum	55	50	9	60	82[a] (4.1 ml H_2/h)	1	[25]
Microbial community	52	50	9	90	1162[a] (58.1 ml H_2/h)	1.52	[26]
Clostridium sp. 6A-5	43	100	16	281[a] (2727 ml H_2/l)	269.3	2.50	[27]
Microbial community	40	100	10	269.9	300 (30 ml H_2/h[a])	1.93[a] (275.1 ml H_2/g)	[28]
Microbial community	37	80	15	176.8	218 (8.9 mmol H_2/l/h)	1.46	[29]
Microbial community	37	50	9	70	114[a] (5.7 ml H_2/h)	1.23	[30]
E. coli DJT135	35	350	10	1011 (40 mmol H_2[a])	NR	1.51	[31]
Clostridium sp. 6A-5	25	100	20	32.7	29	0.48	[27]
E. coli WDHL	25	110	20	20.1	15.63	0.07	This study
GA051	25	110	20	253.3	16.64	0.86	This study
G024	25	110	20	70.17	5.61	1.57	This study
N92	25	110	20	228.83	14.29	0.88	This study
Rahnella aquatilis strain 7	20	500	10[d]	66.93	NR	NR	[10]

NR: Not reported. [a]Converted units from the original data. [b]Continuous Flow 0.95 g/l/h. [c]Sucrose. [d]g/COD Cheese whey.

1.3 Summary

This study has shown that psychrophilic microorganisms isolated from Antarctic samples are natural biohydrogen producers. Some of them have production parameters comparable to those reported for mesophilic microorganisms, and therefore could be novel microorganisms for biohydrogen production at room temperature. GA051 is the best biohydrogen producer strain and it is closely related to *J. agaricidamnosum* (Y08845) with a 98% of identity. This microorganism also produces ethanol from glucose fermentation, which makes it attractive as a potential candidate for further investigation including the optimization of the fermentation operational conditions, studies of assimilation of other carbon sources such as pentoses, and complex carbohydrates.

2 Optimization of the biohydrogen production by anaerobic granular sludge using mixtures of wheat straw hydrolysate and cheese whey as substrates

Exhaustion of fossil fuel resources and environmental damages owing to petroleum production and consumption highlight the importance of a shift to renewable sources for fuels. Biohydrogen is a sustainable form of energy as it can be produced from organic wastes through fermentation processes such as dark fermentation. This process also produces fermented by-products (fatty acids and solvents), thus there is an opportunity for further combining with other processes that yield more bioenergy [32]. Organic wastes are abundant sources of renewable and low cost substrate that can be efficiently fermented by microorganisms. The main criteria for the selection of waste materials to be used in biohydrogen production are the availability, cost, carbohydrate content and biodegradability. Simple sugars such as glucose, sucrose and lactose are readily biodegradable and preferred substrates for hydrogen production. However, pure carbohydrate sources are expensive raw materials for hydrogen production [1]. The advantages of using organic wastes for biohydrogen are: reduction of CO_2 and other pollutants emissions, added value agricultural waste, partial substitution of fossil fuels with sustainable biomass fuel, and reduction of environmental and economic costs for diverging the disposition of municipal solid wastes [32]. The production of renewable energy, a reduction of waste and prevention of environmental pollution promote the industrial application of anaerobic co-digestion for the treatment of agroindustrial organic waste. Co-digestion is defined as the anaerobic treatment of a mixture of at least two different waste types with the aim of improving the

efficiency of the anaerobic digestion process [33]. Despite the large body of literature on biogas production from waste materials, only a limited number of co-digestion studies are available [34]. In previous work in our laboratory we evaluated the use of a waste residue (cheese whey) as a substrate for biohydrogen production with a good yield of 2.74 mol H_2 mol lactose^{-1} consumed [17], but we are interested in to evaluate the use of mixtures of waste materials such as wheat straw hydrolysate (WSH) and cheese whey (CW). According to SIACON- SIAP in Mexico in 2011 was reported a production of 4,407,436 ton of wheat straw [35] and 11,129,921 thousands of liters (187,075 ton approximately) of milk of which it is estimated that 259,076 are cheese whey [36]. The first agroindustrial residue is rich in cellulose (35-45%), hemicellulose (20-30%) and lignin (18-15%) [37]. Pretreatment of wheat straw is necessary to break down the lignocellulose into the three major polymeric constituents [38]. The thermal pretreatment of biomass results in two main streams: the solid fraction mainly consisting of cellulose (hexose: glucose) and liquid phase (hydrolysate) mainly consisting of hemicellulose (pentose: xylose and arabinose) [39]. Meanwhile CW is one of the polluting residues in the dairy industry that can negatively affect the environment and biological processes in wastewater treatment. This residue is a liquid that separates from the milk coagulation during cheese manufacture. CW is considered a residue of the dairy industry and corresponds to around 85–90% of the total volume of processed milk [40]. In a dry basis, bovine whey contains 70-80% of lactose, 9% of proteins, 8-20% of minerals and other minor components, such as some hydrolyzed peptides of k-casein and lipids [41]. Therefore, the treatment of the degradable fraction of solid wastes or biowaste, allows for the

generation of carbon-neutral bioenergy, nutrients and other resources or valuable bioproducts, such as enzymes [32]. Therefore, we decided to evaluate the use of mixtures of cheese whey (CW) and wheat straw hydrolysate (WSH) as a substrate for biohydrogen production using anaerobic sludge as inoculum.

2.1 Material and Methods

2.1.1 Substrates and inoculum

CW was purchased from Land O'Lakes Inc. (Arden Hills, Minnesota) and WSH was obtained from CUCBA (University of Guadalajara, Jalisco, Mex). The lactose content of CW solution was 6.9 g L^{-1}. WSH contained 21, 1.54, 13.96 and 1.93 g L^{-1} of reducing sugars, glucose, xylose and arabinose, respectively. Anaerobic granular sludge was obtained from a wastewater treatment plant in San Luis Potosi, Mexico. The granular sludge was washed with three volumes of tap water and then boiled for 40 min to inactivate methanogenic microflora and stored at 4°C before use.

2.1.2 Experimental design

A Central Composite experimental design with six central points (Table 2.1) was used to find the optimal conditions for biohydrogen production using mixtures of CW and WSH as substrate. The independent variables were pH, temperature and concentration of CW and WSH. Three levels for each variable were included and 2 star points. The response variable was biohydrogen production (H_2). The experiments were performed in 120 mL anaerobic serological bottles with a

Table 2.1 Central Composite experimental design and corresponding results by anaerobic granular sludge using mixtures of wheat straw hydrolysate and cheese whey as substrate

Experiment	WSH[a] (g L^{-1})	CW[b] (g L^{-1})	Temperature (ºC)	pH	H_2[c] (mL H_2 L^{-1})
1	10	20	28	7.5	4,963.6
2	10	20	46	7.5	4.5
3	15	15	37	6.5	3,709.1
4	20	10	28	5.5	0.0
5	20	20	28	7.5	4,731.8
6	10	10	28	7.5	3,425.5
7	20	10	46	7.5	0.0
8	20	20	46	7.5	0.0
9	15	15	37	4.5	9.1
10	15	15	37	6.5	3,572.7
11	20	10	28	7.5	5,359.1
12	15	15	19	6.5	4.5
13	15	15	37	6.5	3,718.2
14	20	20	46	5.5	4.5
15	5	15	37	6.5	2,972.7
16	15	15	37	6.5	3,618.2
17	15	15	55	6.5	45.5
18	15	15	37	6.5	3,666.4
19	10	20	46	5.5	36.4
20	20	20	28	5.5	63.6
21	15	15	37	6.5	3,269.1
22	10	10	28	5.5	27.3
23	15	5	37	6.5	3,450.0
24	10	20	28	5.5	3,750.0
25	15	25	37	6.5	4,518.2
26	15	15	37	8.5	3,601.8
27	20	10	46	5.5	9.1
28	10	10	46	7.5	0.0
29	25	15	37	6.5	4,559.1
30	10	10	46	5.5	0.0

[a] Wheat straw hydrolysate. [b] Cheese whey. [c] Biohydrogen production.

working volume of 110 mL, all bottles containing medium B [41], 2.75 g L^{-1} yeast extract, and CW and WSH at the determined concentrations of experimental design. The cultures were shaken at 175 rpm during the period of experiment. Consequently, the data was analyzed by the response surface methodology

(RSM). Analysis of variance (ANOVA), RSM and the optimum conditions were performed using Design-Expert® Version 7.0 (Stat-Ease, Inc.). ANOVA *F* test was used to assess the adjusted models. The significance of each coefficient was determined with the *t* test with a *P* value less than 0.05.

2.1.3 Batch cultures on bioreactor

Batch fermentations were performed using a mixture of WSH and CW (25 g L^{-1} and 5 g L^{-1}, respectively) in 1-L and 4-L bioreactors (Applikon, Foster City, CA) equipped with two six-blade Rushton turbines, pH was monitored using an autocleavable electrode (Applikon) and controlled at 6.5 by a Bioconsole ADI 1035/Biocontroller 103 (Applikon). BioXpert 1.3 software (Applikon) was used for data acquisition. The experiments were performed at 26.3ºC and stirred at 175 rpm. Culture samples of 1 mL were taken every 4 h from the bioreactors and centrifuged at 600 rpm. The supernatant was filtered through a 0.22 μm syringe filter (Millipore, Bedford, MA, USA) before analysis of fermentation products.

2.1.4 Analytical methods

The gas production was measured by 1N NaOH displacement in an inverted burette connected to the bioreactor or to serological bottles with rubber tubing and a needle. Hydrogen content in the gas phase was measured by Gas Chromatograph model 6890N (Agilent Technologies, Wilmington, DE) as described elsewhere [29]. Remaining substrates and fermentation end products (glucose, succinic acid, lactic acid, formic acid, acetic acid, methanol, propanol, and butanol)

were analyzed by High Performance Liquid Chromatography (HPLC, Infinity LC 1220, Agilent Technologies, Santa Clara, CA, USA) using a Refraction Index Detector (Agilent Technologies, Santa Clara, CA, USA), and column Phenomenex Rezex ROA (Phenomenex, Torrance,CA, USA) at 60ºC, and using 0.0025 mM H_2SO_4 as mobile phase at 0.55 mL min^{-1} flow rate. Ethanol, acetoin, propionic acid, and butyric acid were analyzed by injecting a 1 µl sample in a Gas Chromatograph 6890N (Agilent Technologies) equipped with capillary column HP-Innowax (30 m x 0.25 mm i.d. x 0.25 m film thickness; Agilent, Wilmington, USA). Helium was used as carrier gas at a flow rate of 25 mL min^{-1}. Temperatures for the injector and flame ionization detector (FID) were 220 and 250ºC, respectively. The analyses were performed with a split ratio of 5:1 and a temperature program of 25ºC for 10 min, 175ºC for 1 min increased at 5ºC min^{-1} to 280ºC, and maintained at this temperature to a final time of 10 min.

2.2 Results and discussion

2.2.1 Optimization of the culture conditions to improve biohydrogen production

The effect of substrates concentrations, temperature and pH in biohydrogen production was evaluated with a Central Composite experimental design (Table 2.1). Central points attained a production average of 3,592.3 ± 167.6 mL H_2 L^{-1}. The highest production was obtained in experiment 11 with 5,359.1 mL H_2 L^{-1}. Experiments with pH ≤ 5.5 and temperatures < 28°C or ≥ 46°C, obtained less than 70 mL H_2 L^{-1}. With the ANOVA it was established that H_2 was affected significantly

by temperature and pH (Table 2.2). These results indicate that the concentration of the substrates does not have effect on response variables that are studied.

Table 2.2 Analysis of variance for biohydrogen production

Source	SS[a]	DF[b]	MS[c]	F-value	*p*-value
Model	9.963E+007	14	7.117E+006	4.77	0.0024
WSH	53657.13	1	53657.13	0.036	0.8521
CW	1.966E+006	1	1.966E+006	1.32	0.2687
T	2.051E+007	1	2.051E+007	13.76	0.0021
pH	2.050E+007	1	2.050E+007	13.75	0.0021
WSH•CW	2.154E+006	1	2.154E+006	1.44	0.2480
WSH•T	2.462E+005	1	2.462E+005	0.17	0.6902
WSH•pH	1.845E+006	1	1.845E+006	1.24	0.2834
CW•T	1.358E+006	1	1.358E+006	0.91	0.3550
CW•pH	5.266E+005	1	5.266E+005	0.35	0.5611
T•pH	1.348E+007	1	1.348E+007	9.04	0.0088
WSH^2	4.419E+005	1	4.419E+005	0.30	0.5941
CW^2	1.434E+005	1	1.434E+005	0.096	0.7607
T^2	3.094E+007	1	3.094E+007	20.76	0.0004
pH^2	9.612E+006	1	9.612E+006	6.45	0.0227
Residual	2.236E+007	15	1.491E+006		
Lack of Fit	2.222E+007	10	2.222E+006	79.07	< 0.0001
Pure Error	1.405E+005	5	28099.41		
Cor Total	1.220E+008	29			

[a]Sum of squares, [b]Degree freedom, [c]Mean square

In Table 2.1 is possible observe that the higher is the concentration of substrates higher is the biohydrogen production, this can be seen by comparing the following pairs of experiments 15 and 23, 1 and 11, 25 and 29, in which substrate concentration was 20, 30 and 40 g L^{-1}, respectively. Nevertheless, if the substrate concentration is doubled the increase in H_2 is not the double, for to notice this consider that the biohydrogen production in experiment 5 was just 38% higher than obtained in experiment 6; the concentration of substrates is 20 and 10 g L^{-1},

respectively. This appears indicate that the effect of the concentration was not linear. Also, it can also be noted that temperature and pH have a more important role on biohydrogen production because this can be inhibited if these variables doesn't have a value adequate. Observe the results of experiments 1, 2 and 24 all these have the same concentration of substrates, but the temperature in the experiment 2 and pH in the experiment 24 reduced the biohydrogen production. The mathematical model representing the variable response as a function of the evaluated variables in the experimental region are expressed by the following equation:

$$H_2\ (mL\ H_2\ L^{-1}) = -71{,}174.90531 - 161.58097 \cdot WSH + 839.50917 \cdot CW + 1{,}586.19349 \cdot T + 11{,}918.30972 \cdot pH - 14.67500 \cdot WSH \cdot CW + 2.75639 \cdot WSH \cdot T + 67.92250 \cdot WSH \cdot pH - 6.47333 \cdot CW \cdot T - 36.28500 \cdot CW \cdot pH - 101.97639 \cdot T \cdot pH - 5.07733 \cdot WSH^2 - 2.89233 \cdot CW^2 - 13.11214 \cdot T^2 - 591.97083 \cdot pH^2$$

With the RSM, contour and response surface plots for biohydrogen production (Figures 2.1 and 2.2) were obtained. From the plots it can be revealed that temperature and pH have great influence on biohydrogen production. Maximum biohydrogen production was found to be approximately in a range of 5,200-5,700 mL H_2 L^{-1} at range of concentration of substrates of 5-10 g TRS L^{-1} WSH and 20-25 g L^{-1} CW, incubation temperature of 25-31°C and pH initial of 6.5-8.5. Decreasing in the WSH concentration and increasing in the CW concentration, as well as, keep incubation temperatures near the room temperature and initial pH up

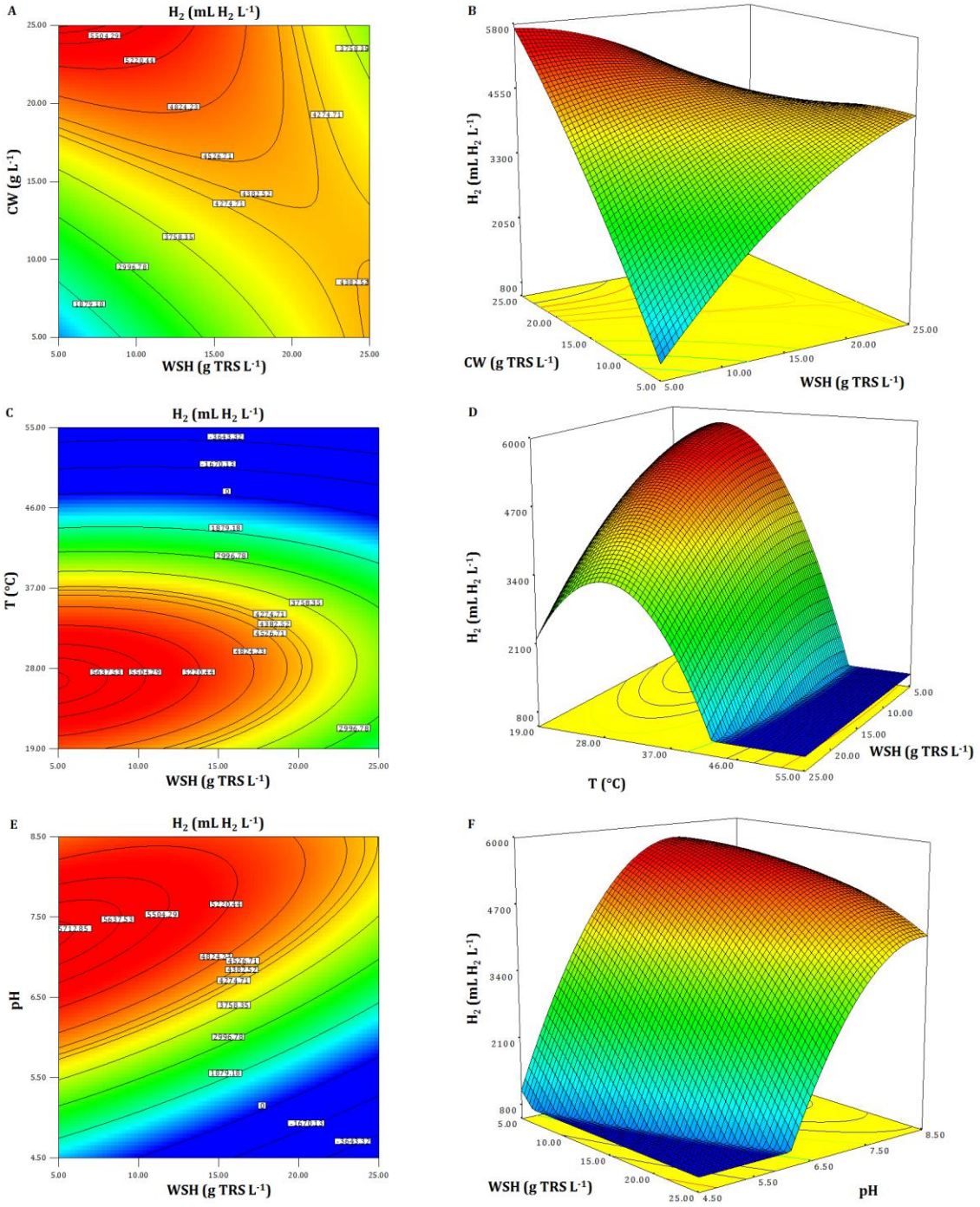

Figure 2.1 Contour and response surface plots of biohydrogen production by anaerobic granular sludge under optimized conditions. Temperature was fixed at 26.6°C and pH adjusted to 7.25 in A and B, concentration of CW was fixed at 25 g L^{-1} and pH adjusted to 7.25 in C and D, concentration of CW was fixed at 25 g L^{-1} and temperature fixed at 26.6°C in E and F.

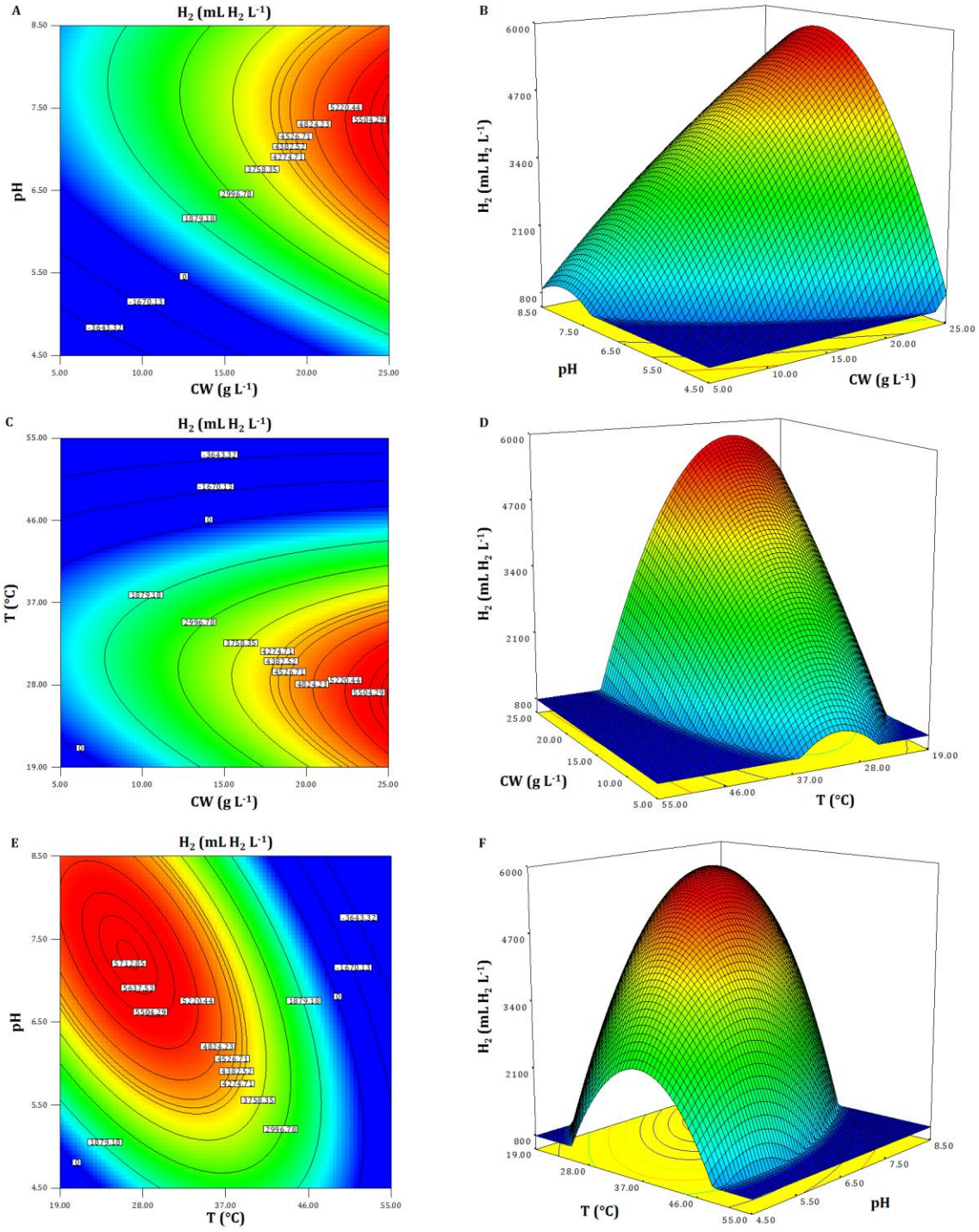

Figure 2.2 Contour and response surface plots of biohydrogen production by anaerobic granular sludge under optimized conditions. Concentration of WSH was fixed at 5 g L^{-1} and temperature was fixed at 26.6°C in A and B, concentration of WSH was fixed at 5 g L^{-1} and pH adjusted to 7.25 in C and D, concentration of WSH was fixed at 5 g L^{-1} and concentration of CW was fixed at 25 g L^{-1} in E and F.

above 6.5 leads to maximize H_2 production, which proves that the evaluated parameters affect the biohydrogen production considerably. Hence, from these results, biohydrogen production was optimized to get the optimum values of WSH concentration, CW concentration, temperature and pH for maximum values of H_2. According to the mathematical model, maximum biohydrogen production of 5,724.5 mL H_2 L^{-1} (95% CI: 3,375.53-6,722.02 mL H_2 L^{-1}) can be attained at WSH 5 g TRS L^{-1}, CW 25 g L^{-1} CW, 26.6°C and initial pH 8.5. To verify the predicted results, additional experiments were performed by triplicate using these optimized conditions and the biohydrogen production attained was 4,554.55 ± 10.9 mL H_2 L^{-1} (Figure 2.3).

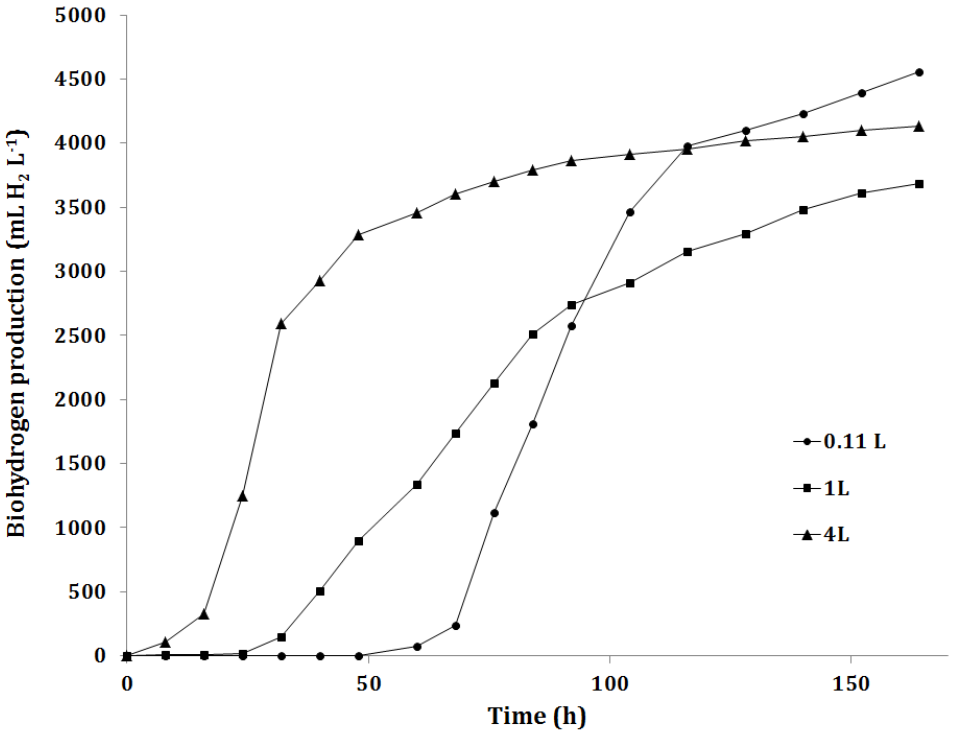

Figure 2.3 Biohydrogen production in batch culture of anaerobic sludge at optimal conditions (5 g L^{-1} WSH, 25 g L^{-1} CW, 26.6ºC and pH 7.25) in 0.11L, 1L and 4L bioreactors.

The optimization of operational conditions using RSM was successful because the result obtained is within the confidence interval. Davila-Vazquez *et al*. reported 2,133.8 [41] and 3,812.5 [29] mL H_2 L^{-1} using anaerobic granular sludge and CW as substrate, the biohydrogen production obtained by us in optimal conditions increased in 113.5% and 19.5% in comparison with these two works. In other studies in which mixed culture was used for biohydrogen production and rice straw hydrolysate, sucrose, kitchen wastes, fruit-vegetable waste and rotten wheat straw were employed as substrates was reported H_2 between 2-1,500 mL H_2 L^{-1} [42–46], values lower than obtained in the present study. Wu *et al.* [47], reported a higher biohydrogen production using bagasse as substrate, 8,105 mL H_2 L^{-1}. The incubation temperatures used in all these studies oscillate between 35-60°C, which are higher compared with the one used by us (26.6°C).

2.2.2 Biohydrogen production from a mixture wheat straw hydrolysate with cheese whey under optimal operating conditions

After to confirm that the optimal conditions were appropriate for maximize biohydrogen production, these were tested in 1-L and 4-L bioreactors. In 1-L and 4-L bioreactors (Figure 2.3) the biohydrogen production started at 8 h reaching 3,685 ± 305 and 4,132.1 ± 37.8 mL H_2 L^{-1}, respectively. In 1-L bioreactor the carbohydrates were totally consumed at the end of the fermentation (164 h) while in 4-L bioreactors the carbohydrates were totally consumed in 96 h. Table 2.3 summarizes a comparison of production, production rate and yield of biohydrogen between results obtained in others experiments and those achieved by us. A fermentation in three stages reported a biohydrogen production of 1,023 mL H_2 L^{-1}

and 63.7 mL H_2 L^{-1} h^{-1} by *Escherichia coli* EGY and *Clostridium acetobutylicum* ATCC using rotting date palm fruits as substrate [48], and another which attained 2,327 mL H_2 L^{-1} and 212.2 mL H_2 L^{-1} h^{-1} using a mixed culture and glucose as substrate [49]. The incubation temperatures used in the above mentioned studies were 30 and 37°C, respectively, whereas with the use of 26.6°C in this work a higher production as well as a production rate of 81 mL H_2 L^{-1} h^{-1} were obtained. Previous reported works using a mixed culture (or co-culture) as well as sweet sorghum or glucose/xylose as substrate attained biohydrogen production rates of 212.5 [50] and 135.2 [51] mL H_2 L^{-1} h^{-1}, respectively, these values are higher than that obtained by us. The yield obtained by us was 199 mL H_2 g TRS^{-1}, this value is 1.7-times higher than the attained in other work using a mixed culture that metabolized sweet sorghum with a yield range of 98.2 - 120.7 mL H_2 g $glucose^{-1}$ [50]. However, others authors reported higher yields than the achieved by us, using single carbohydrates, *i.e.* 213.1 mL H_2 g $glucose^{-1}$ [49], 218 mL H_2 g $sucrose^{-1}$ [48], 278 mL H_2 g $hexose^{-1}$ [52], 443.3 mL H_2 g $sucrose^{-1}$ [48] and 578.3 mL H_2 g $hexose^{-1}$ [51]. In the latter work with highest yield was used a co-culture of *Caldicellulosiruptor saccharolyticus* DSM 8903 and *C. kristjanssonii* DSM 12137 and glucose/xylose mixture as substrate. Using a *t*-student analysis was obtained absence of significant statistical difference ($p < 0.05$) in the results achieved in 0.11-L serological bottles, 1-L and 4-L bioreactors; so is possible conclude that optimal operating conditions can be scaled successfully. Nevertheless, for maximize production rate and yield more experiments are necessary and find the conditions that permit high production as well as high production rate and high yield.

Table 2.3 Comparison of production, production rate and yield of biohydrogen from different microorganisms and substrates

Microorganisms	T (°C)	Substrate	Concentration	H_2 (mL H_2 L^{-1})	r_{H_2} (mL H_2 L^{-1} h^{-1})	Y_{H_2} † (mL H_2 g^{-1})	Reference
Escherichia coli EGY, *Clostridium acetobutylicum* ATCC	30	Rotting date palm fruits	10 g L^{-1} sucrose	1,023* (2 L accumulated, 1.95 L V_w)	63.7* (2.56 mmol H_2 L^{-1} h^{-1})	218* (3 mol H_2 mol sucrose^{-1})	[48]
			2.5 g L^{-1} sucrose	NR	87.2* (1.2 mmol H_2 L^{-1} h^{-1})	443.3* (6.1 mol H_2 mol sucrose^{-1})	
Mixed culture	35	Sweet sorghum	0.45 g L^{-1} glucose	NR	212.5* (2550 mL H_2 d^{-1}, 0.5 L V_w)	98.2* (0.70 mol H_2 mol glucose^{-1})	[50]
			0.47 g L^{-1} glucose	NR	122.5* (1740 mL H_2 d^{-1}, 0.5 L V_w)	120.7* (0.86 mol H_2 mol glucose^{-1})	
Thermoanaerobacterium aotearoense SCUT27/*Δldh*	55	Sugarcane bagasse (SCB)	2 L of nonsterilized SCB hydrolysate	4,017.5* (298.4 mmol accumulated, 2 L V_w)	520	278* (1.86 mol H_2 mol hexose^{-1})	[52]
Caldicellulosiruptor saccharolyticus DSM 8903, *C. kristjanssonii* DSM 12137	70	Glucose/xylose	NR	NR	135.2* (4.8 mmol H_2 L^{-1} h^{-1})	578.3* (3.7 mol H_2 mol hexose^{-1})	[51]
Mixed culture	37	Glucose	20 g L^{-1}	2,327* (24.8 L accumulated gas, 56.3 % H_2 content, 6 L V_w)	212.2	213.1	[49]
Anaerobic granular sludge	26.6	Wheat straw hydrolysate	5 g TRS L^{-1}	4,132.1 ± 37.8 (4L V_w)	81	199	This work
		Cheese whey	25 g L^{-1}				

†Biohydrogen yield. *Converted units from the original data. V_w: "Working" volume. NR: Not reported.

2.2.3 Production of soluble metabolites

Biohydrogen production is typically accompanied by the generation of organic acids and ethanol during dark fermentation processes. Hence, the composition and concentration of the produced soluble metabolites are useful indicators for monitoring the biohydrogen production process [45]. The investigation of the soluble metabolites at the end of the hydrogenogenic process is shown in Table 2.4.

Table 2.4 Soluble metabolite concentrations accumulated during biohydrogen production process

Metabolite	Concentrations (g L^{-1})		
	0.11-L Serological Bottles	**1-L Bioreactor**	**4-L Bioreactor**
Lactic acid	-	0.47 ± 0.06	1.14 ± 0.18
Formic acid	-	1.03 ± 0.07	0.41 ± 0.08
Acetic acid	6.09 ± 1.11	2.84 ± 0.18	3.58 ± 0.33
Propionic acid	0.60 ± 0.02	1.76 ± 0.11	0.57 ± 0.08
Butyric acid	3.73 ± 1.21	1.20 ± 0.11	0.41 ± 0.06
Ethanol	0.42 ± 0.04	0.59 ± 0.04	0.56 ± 0.04
Propanol	-	-	0.70 ± 0.13

We can observe that the acetic acid is the main organic acid produced; other organic acid and ethanol are also produced. Similar results for metabolic products were reported using sweet sorghum and indigenous micro flora; 5.55 g L^{-1} butyric acid, 3.5 g L^{-1} acetic acid and others metabolites with values lower than 1.55 g L^{-1} (propionic acid, ethanol, lactic acid) were produced [50]. In a study in which two thermophilic bacteria were used, the most abundant byproduct was butyric acid with a concentration of 1.06 g L^{-1} at the fermentation end [46]. Another work reported that the dominant byproducts in fermentation were butyric acid (9.5 g L^{-1})

and acetic acid (3.8 g L^{-1}) by anaerobic granular sludge when the substrate was kitchen waste [44]. So too, a work in which “piggery anaerobic digested residues” was used as inoculum the formation of butyric and acetic acids were favored, fruit-vegetable waste was used as substrate; ethanol, propionic and lactic acids were detected at lower values of 0.5 g L^{-1} [45]. With using *Klebsiella oxytoca* ***ΔadhE*** HP1, Wu *et al*. [47] reported that the byproducts on the fermentation of bagasse were acetic acid, lactic acid and ethanol.

2.3 Summary

Biohydrogen production using the co-digestion of two different sources of carbohydrates by anaerobic granular sludge was successful. Through ANOVA analysis we observe that temperature and pH are the most important variables in the biohydrogen production. Also the proposed mathematical model proved to be valuable for optimizing the biohydrogen production with the optimal conditions of 5 g L^{-1} WSH, 25 g L^{-1} CW, 26.6ºC and pH 7.25. The optimization of operational conditions was successful because the biohydrogen production it is doubled in compared with experiments in operational conditions without optimize. These results demonstrate that it is possible to use mixtures of agro-industrial wastes to generate biofuels through a cheap process that it is also industrially scalable.

3 Simultaneous production of bioethanol and biohydrogen by *Escherichia coli* WDHL using wheat straw hydrolysate as substrate

Due to the recent energy crisis and rising concern over climate change, the development of clean alternative energy sources is of significant interest. Today's energy supply depends heavily on fossil fuels, although significant efforts are being made to use fuels produced from renewable feedstock, as these have fewer greenhouse gas emissions during both fuel production and use [53]. Nowadays, biomass is a promising source of renewable energy, since the feedstock comes from non-food crops or agricultural waste (second generation feedstock), to avoid competition with food sources and arable land. Second-generation biofuels use lignocellulosic materials mainly for the production of liquid or gaseous fuels because they can be degraded to monomeric carbohydrates such as glucose, arabinose and xylose. Wheat straw is one of the most abundant agricultural waste components and has potential for the production of biofuels [53–55]. It is a well-known fact that the dark fermentation is a phenomenon occurring under anoxic conditions and substrates may be used as a wide variety of carbon sources to produce valuable metabolites such as butyric, lactic acid, acetic acid and ethanol [56]. Consequenly, bioethanol and biohydrogen production by dark fermentation from cellulose hydrolysates has been tested [57], as well as simultaneous production of bioethanol with biogas from energy crops (*e.g*. rye, canola and beans) was reported [58]. However, there is still a need for studies on multibiofuels production (bioethanol, biohydrogen and biogas) that could assist in the evaluation of new concepts such as biorefineries using lignocelullose feedstocks*, i.e*. production of biofuels from agricultural waste [38]. Since, the optimization of the

fermentation conditions, nutritional and environmental parameters are of primary importance for the development of bioprocesses [59], the goals of this research are to explore the use of wheat straw hydrolysate for the simultaneous production of bioethanol and biohydrogen and to obtain optimal production conditions for both biofuels.

3.1 Materials and methods

3.1.1 Production of Wheat Straw Hydrolysate

Wheat straw from La Barca (Jalisco, Mex) was milled with a hammer mill (Azteca 301012), classified with a vibratory sieve (Alcon, Guadalajara, Mex; 8, 16, and 40 US Mesh) and stored at room temperature. Straw composition was determined at CUCBA (University of Guadalajara, Jalisco, Mex) according to the AOAC International methods (AOAC Official Method 4.6.03 and 4.6.04)[60] with 44.45 ± 2.35, 19.23 ± 4.20 and 5.78 ± 0.57 of cellulose, hemicellulose and lignin, respectively (% w/w dry weight basis). The particle size used in pretreatment experiments was 40 US Mesh (425 µm). A dilute acid pretreatment of straw was carried out according to the method proposed by Rojas-Rejon and Sanchez[61]. Wheat straw was slurried in dilute H_2SO_4 (0.75 % v/v) at 4 % (w/v) and pretreated at 121°C for 1 h in a steam sterilizer with heating and cooling ramps of 30 min each. The liquid fraction was recovered and the samples were taken, it was centrifuged at 10,000 rpm and concentrated by evaporation at 70°C until reaching the carbohydrate concentrations established by the experiment design as indicated in section 2.2. Supernatants were stored at -20°C for their further analysis.

3.1.2 Biohydrogen production using analytical grade carbohydrates as substrate

The ability of *Escherichia coli* WDHL [17] strain to produce biohydrogen from pentoses as xylose and arabinose were found in hydrolysates of lignocellulosic material [3] was determined by using analytical grade carbohydrates as substrate (glucose, galactose, xylose, arabinose) at a concentration of 20 g dm^{-3}. The experiments were done in anaerobic serological bottles containing 110 cm^3 of HP medium (0.8 g dm^{-3} NaCl, 0.2 g dm^{-3} KCl, 1.43 g dm^{-3} Na_2HPO_4, 0.2 g dm^{-3} KH_2PO_4), 1 cm^3 dm^{-3} trace elements solution[17], 0.01 g dm^{-3} $MgSO_4$ and 2.75 g dm^{-3} yeast extract (Difco). The cultures were started with 1.0 OD_{600nm}, pH 7.5 and they were incubated at 37°C and 175 rpm. All the experiments were carried out in triplicate. Biohydrogen production was measured as it was indicated in section 2.5.

3.1.3 Experimental design

A Central Composite experimental design with six centrals point (Table 3.1) was used to establish the optimal conditions for the biohydrogen and bioethanol production, by using wheat straw hydrolysate (WSH) as a carbon source. In which, temperature, pH, and WSH were the independent variables. The response variables were bioethanol production (EtOH), bioethanol production rate (r_{EtOH}), bioethanol yield (Y_{EtOH}), biohydrogen production (H_2), biohydrogen production rate (r_{H_2}), and biohydrogen yield (Y_{H_2}). The experiments were performed in 120 mL anaerobic serological bottles containing medium B[41], 1 mL dm^{-3} trace elements solution[17], 1 g dm^{-3} yeast extract, and WSH concentrations of experimental

Table 3.1 Central Composite experimental design and corresponding results by *E. coli* WDHL using Wheat Straw Hydrolysate as substrate.

n	WSH (g TRS dm^{-3})	T (°C)	pH (-)	EtOH[a] (g EtOH dm^{-3})	r_{EtOH}[b] (g EtOH dm^{-3} h^{-1})	Y_{EtOH}[c] (g EtOH g TRS^{-1})	H_2[d] (cm^3 H_2)	r_{H_2}[e] (cm^3 H_2 dm^{-3} h^{-1})	Y_{H_2}[f] (cm^3 H_2 g TRS^{-1})
1	10	28	5.5	0.0	0.000	0.00	2.1	0.08	1.9
2	15	37	6.5	7.3	0.015	0.48	444.2	8.87	269.2
3	15	37	6.5	6.7	0.015	0.44	315.5	6.05	191.2
4	10	46	7.5	0.0	0.000	0.00	0.6	0.82	0.5
5	15	37	6.5	6.8	0.014	0.45	287.5	5.54	174.3
6	20	28	7.5	8.2	0.028	0.41	387.8	11.01	176.3
7	10	28	7.5	5.1	0.021	0.51	263.3	13.14	239.3
8	15	37	8.2	6.4	0.054	0.43	356.0	25.27	215.7
9	20	46	5.5	0.0	0.000	0.00	5.3	7.38	2.4
10	15	37	6.5	6.6	0.018	0.44	342.3	7.63	207.5
11	10	46	5.5	0.0	0.000	0.00	3.5	4.92	3.2
12	15	52.1	6.5	0.0	0.000	0.00	0.0	0.00	0.0
13	15	21.9	6.5	0.0	0.000	0.00	16.5	0.63	10.0
14	15	37	6.5	6.6	0.016	0.44	333.1	7.86	201.9
15	20	46	7.5	0.0	0.000	0.00	0.6	0.82	0.3
16	6.6	37	6.5	3.1	0.014	0.46	103.7	2.78	143.1
17	20	28	5.5	0.0	0.000	0.00	8.0	0.18	3.7
18	23.4	37	6.5	8.0	0.021	0.34	509.2	10.44	197.7
19	15	37	6.5	7.1	0.019	0.47	320.4	8.33	194.2
20	15	37	4.8	6.5	0.017	0.43	354.5	8.05	214.9

[a] Bioethanol production. [b] Bioethanol production rate. [c] Bioethanol yield. [d] Biohydrogen production. [e] Biohydrogen production rate. [f] Biohydrogen yield.

design. The volume to obtain the total reducing sugars (TRS) concentration for each experiment was calculated by using the following formula: $V_1 = WSH_2V_2/WSH_1$, where, WSH_1 is concentration of total reducing sugars in wheat straw hydrolysate in g TRS L^{-1}; WSH_2 is the concentration of total reducing sugars in each experiment; V_1 is the volume of WSH to obtain the concentration of TRS for each experiment of the experimental design in mL; V_2 is volume of work (110 cm^3). To determine $OD_{600nm\ initial}$ in the experiments with WSH as the substrate; 1, 1.5 and 2 $OD_{600nm\ initial}$ were tested in an experiment (n = 3) and the obtained results were 229 ± 29, 313 ± 10 and 223 ± 25 cm^3 H_2, respectively. Using an analysis of *t*-student significant difference ($p < 0.05$) was found between 1 and 1.5 OD_{600nm}, also for 1.5 and 2 OD_{600nm} but not for 1 and 2 OD_{600nm}. Therefore, cultures were adjusted to an initial OD_{600nm} of 1.5 and were shaken at 175 rpm; all the experiments were monitored during 650 hours. Consequently, the data was analysed by the response surface methodology (RSM). Analysis of variance (ANOVA), RSM and the optimum conditions were performed by using the Design-Expert® Version 7.0 software (Stat-Ease, Inc.). ANOVA *F* test was used to assess the adjusted models. The significance of each coefficient was determined with the *t* test with a *p*-value less than 0.05. Biohydrogen production was measured as it was indicated in section 3.1.5.

3.1.4 Batch culture on a bioreactor

A batch culture was performed by using the medium B [12] plus 23.4 g TRS dm^{-3} of WSH (0.50 g dm^{-3} glucose, 13 g dm^{-3} xylose and 0.62 g dm^{-3} arabinose), 1 mL dm^{-3} trace elements solution [17] and 1 g dm^{-3} yeast extract, in a 1 dm^3 bioreactor

(Applikon, Holland) equipped with two six-blade Rushton turbines flat. The cultures were performed at 31°C, initial pH 8.2, initial OD_{600nm} of 1.5 and stirred at 175 rpm. Redox potential, pH and dissolved CO_2 were monitored using autocleavable electrodes (Applikon) and connected to Bioconsole ADI 1035 (Applikon) controlled by the ADI 1030 Biocontroller (Applikon). BioXpert 1.3 software (Applikon) was used for data acquisition. Biohydrogen production was measured as it was indicated in section 3.1.5.

3.1.5 Analytical methods

Total reducing sugars (TRS) estimation was performed by the dinitro salicylic acid (DNS) method[62], with some modifications as follow: 0.25 cm^3 of WSH with 0.75 cm^3 of DNS reagent (10 g dm^{-3} NaOH, 200 g dm^{-3} $KNaC_4H_4O_6 \cdot 4H_2O$, 0.5 g dm^{-3} $Na_2S_2O_5$, 2 g dm^{-3} C_6H_6O, 10 g dm^{-3} 3,5-Dinitrosalicylic acid) were heated for 15 minutes in a boiling water bath and then cooled to room temperature. For the calibration curve, glucose (0.1 to 1.0 g dm^{-3}) was used as the reference standard. The absorbance was measured at 550 nm (Varian's Cary® 50 Bio UV-Visible Spectrophotometer).

Carbohydrates and metabolites concentration was quantified by a high-performance liquid chromatography (HPLC) in an Agilent chromatograph equipped with a refractive index (Agilent Technologies 1220 Infinity LC). A Phenomenex® - organic acid analysis column operated at 60°C with H_2SO_4 0.0025 M as a mobile phase (0.550 cm^3 min^{-1}) it was used for its separation.

Furfural was determined spectrophotometrically by the method established by the Mexican standard NMX-V-004-1970[63], with some modifications. One cm^3 of

WSH was accurate to 5 cm^3 with 50% aqueous ethanol solution, 0.1 cm^3 of aniline and 0.5 cm^3 of hydrochloric acid concentrate were added. The mixture was cooled in a cold-water bath (15°C) by 30 minutes. For the calibration curve (1 to 5 mg dm^{-3}), furfural was used as the reference standard. The absorbance was measured at 520 nm (Varian's Cary® 50 Bio UV-Visible Spectrophotometer).

Biohydrogen production was measured by NaOH 1 N displacement in an inverted burette connected to the bioreactor or to serological bottles with rubber tubing and a needle. Analysis of biohydrogen by gas chromatography was performed with a thermal conductivity detector (Agilent Technologies 6890N Network GC Systems) and using Agilent J&W HP-PLOT Molesieve (0.32 mm ID, 30 m length, 12 µm film) under the following conditions: 200 °C, injector temperature; 280 °C, detector temperature; 300 °C, oven temperature. Biohydrogen volume was corrected to standard conditions of temperature and pressure (298.15K and 10^5 Pa).

3.2 Results and discussion

3.2.1 Composition of Wheat Straw Hydrolysate

Dilute-acid pretreatments have received great attention in the past years due to their high sugar yields and are currently employed in biorefineries at demonstration and commercial-scale [64]. The chosen method can provide a high xylose yield (*i.e*., almost 80 % of the theoretical yield) with production of alcoholic fermentation inhibitors (i.e., HMF, furfural, acetic acid acetovanillone) below inhibition concentrations.

WSH used here is a typical hemicellulosic hydrolysate containing xylose, arabinose and glucose (Table 3.2) [65]. Total reducing sugars were detected with a value of

21 g dm^{-3}, the remaining carbohydrates are probably dimers or others produced in wheat straw hydrolysis that were not detected in the HPLC analysis. In addition, 1.01 g dm^{-3} formic acid and 3.59 g dm^{-3} acetic acid were detected. These compounds are commonly produced from the lignocellulosic materials pretreatment and saccharification. Furfural concentration was 0.12 g dm^{-3}, this compound is produced from the dehydration of pentoses during the pretreatment and it is typically in a range of 0 to 5 g dm^{-3} [66].

Table 3.2 Composition of Wheat Straw Hydrolysate.

Component	Concentration (g dm^{-3})
Reducing sugars	21
Glucose	1.54
Xylose	13.96
Arabinose	1.93
Formic acid	1.01
Acetic acid	3.59
Furfural	0.12

The lignocellulosic biomass is processed into component sugars, lignin solids, and inhibitory compounds. These inhibitors can affect microbial growth in various ways, including DNA mutation, membrane disruption, intracellular pH drop, and other cellular targets. Moreover, organic acids have been shown to primarily inhibit the production of cell mass, but not the fermentation itself. Furfural has been identified as a key inhibitor in lignocellulosic hydrolysate because it is toxic by itself and also acts synergistically with other inhibitors. Hydrophobicity is a marker of organic compounds toxicity. Highly hydrophobic compounds have been shown to compromise membrane integrity. Interestingly, perceptible membrane damage in

E. coli resulting from furfural exposure has not been observed in works in that the microorganism was exposing to furfural [66–69].

3.2.2 Simultaneous production of bioethanol and biohydrogen using wheat straw hydrolysate as substrate

Analytical grade carbohydrates were tested to determinate if *E. coli* WDHL had the capacity to produce biofuels since pentoses like xylose and arabinose, are present in WSH. Production of biohydrogen using analytical grade carbohydrates is shown in Figure 3.1. *E. coli* WDHL metabolized all of them and the highest biohydrogen production of 83.1 ± 5.6 cm^3 H_2 was obtained using xylose as a substrate, which is the main pentose in the lignocellulosic hydrolysate. The OD_{600nm} was measured as the same time as the biohydrogen production and the end of the experiment was approximately three times the OD_{600nm} initial.

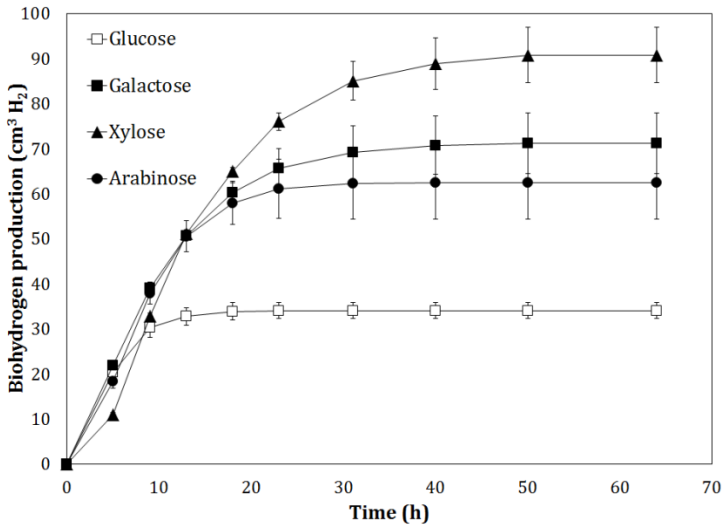

Figure 3.1 Biohydrogen production kinetics by *E. coli* WDHL using 20 g dm^{-3} of glucose, galactose, xylose, arabinose, fructose or maltose as substrate.

The effect of substrate concentration, temperature and pH in production, production rate and yield of bioethanol and biohydrogen was evaluated with a Central Composite experimental design (Table 1). Consequently, the experiment 18 showed the highest production of bioethanol and biohydrogen, with 8.0 g EtOH dm^{-3} and 509.2 cm^3 H_2, respectively. 0.054 g EtOH dm^{-3} h^{-1} and 25.27 cm^3 H_2 dm^{-3} h^{-1}, were the highest production rates, and occurred when the working conditions were 15 g TRS dm^{-3}, 37°C and pH 8.2. Experiment 7 exhibited the highest yields, for bioethanol 0.51 g EtOH g TRS^{-1} and for biohydrogen 239.3 cm^3 H_2 g TRS^{-1}. Biohydrogen production obtained by using WSH is higher than the one obtained with carbohydrates analytical grade; this phenomenon could be explained because WSH contains a mixture of carbohydrates and acid organics that *E. coli* can metabolize. Table 3.3 shows the mathematical models for every response variables. The models of the RSM were examined by using ANOVA.

Table 3.3 Mathematical models for production, production rate and yield of bioethanol and biohydrogen.

Term	EtOH	r_{EtOH}	Y_{EtOH}	H_2	r_{H_2}	Y_{H_2}
C*	-121.640665	-0.15403472	-8.19709454	-5788.59424	-82.5579803	-3533.08653
WSH	1.08063025	0.0022369	0.06240457	54.1108964	1.60657756	38.2195981
T	3.85151039	0.01178107	0.2469315	191.212543	5.71728982	110.386296
pH	14.4104808	-0.02624782	1.09899091	631.241898	-11.553335	423.990573
WSH•T	-0.00861111	-1.9444×10^{-5}	0.00027778	-0.35722222	0.01247222	0.16722222
WSH•pH	0.0775	0.000175	-0.0025	2.92	-0.11725	-1.605
T•pH	-0.18472222	-0.00068056	-0.01277778	-9.00833333	-0.47986111	-5.76111111
WSH^2	-0.03665624	-7.9335×10^{-5}	-0.00202841	-1.59834915	-0.03703224	-1.11801531
T^2	-0.03553861	-0.00010087	-0.002372	-1.7949179	-0.03890648	-1.06701375
pH^2	-0.59820794	0.0043806	-0.04010367	-22.7053234	2.62740554	-12.0758355
R^2	0.8264	0.8088	0.8273	0.7652	0.8552	0.7983

*C: constant

Table 3.4 shows the *p*-values for each terms of mathematical models obtained for all the response variables. Terms with significant effect on the response variables had a *p*-value less than 0.05.

Table 3.4 *P*-values for production, production rate and yield of bioethanol and biohydrogen.

Source	***P*-value**					
	EtOH	r_{EtOH}	Y_{EtOH}	H_2	r_{H_2}	Y_{H_2}
WSH	0.1513	0.5473	0.5392	0.1004	0.2802	0.9026
T	0.0985	0.1350	0.0813	0.1621	0.3461	0.0974
pH	0.1022	0.0042	0.0813	0.1878	0.0047	0.1157
WSH•T	0.5912	0.7677	0.7887	0.7165	0.6258	0.7458
WSH•pH	0.5912	0.7677	0.7887	0.7414	0.6107	0.7297
T•pH	0.0386	0.0596	0.0298	0.0889	0.0031	0.0445
WSH^2	0.1087	0.3776	0.1648	0.2411	0.2914	0.1276
T^2	0.0003	0.0035	0.0002	0.0011	0.0035	0.0004
pH^2	0.2770	0.0687	0.2633	0.4950	0.0101	0.4893

The results indicated that the concentration of the substrate did not have an effect on the response variables. Similar results were reported using cheese whey and sweet sorghum syrup as substrate by *E. coli* and a mixture culture, respectively [17, 70], but in both cases, the substrate concentration had an effect on the biofuel production. In Table 3.1 it is possible to observe that if the concentration of substrate increases, the biofuels productions increases as well, this can be seen by comparing experiments 6 and 16 in which the WSH concentration was 20 and 6.6 g dm^{-3}, respectively. Nevertheless, it can also be noted that temperature and pH have a more important role on production biofuels because this can be inhibited if these variables do not have an adequate value. Notice the results of experiments

6 and 17, both have the same concentration of WSH, but the temperature and pH in the experiment 17 does not allow the biofuels production. Something similar is observed contrasting experiments 7 and 13; the highest biofuels production was obtained in the experiment 7 in which the WSH concentration was lower than experiment 13. The response surface plots were used to identify the interaction between the independent variables (substrate concentration, temperature and pH) with the dependent variables (bioethanol and biohydrogen production). As a result, production of both biofuels increased as WSH concentration increased. According to the RSM the optimal conditions are: pH 8.2, 31°C and 23.4 g dm^{-3} WSH (Figure 3.2). These conditions were confirmed by an additional experiment carried out by triplicate (Figure 3.3). The model predicted 8.4 g EtOH dm^{-3} and 465.8 cm^3 H_2, whilst the experimental values were 8.9 ± 0.7 g EtOH dm^{-3} and 513.4 ± 15.4 cm^3 H_2. Other response variables (r_{EtOH}, Y_{EtOH}, r_{H_2} and Y_{H_2}) are shown in Table 3.5; results indicated that production and yield of both biofuels are better than the predicted value. However, in the case of production rate, the experimental value is the half of the value predicted by the mathematical model. Bioethanol production in this study was about 127 times greater than that reported by Kongjan and Angelidaki [71], which was 0.07 g EtOH dm^{-3}. Bioethanol yield obtained by *E. coli* WDHL was 0.37 ± 0.03 g EtOH g TRS^{-1}, whereas values of 0.50 g EtOH g $glucose^{-1}$[72], 0.48 and 0.50 g EtOH g^{-1} of available mixture sugars[73, 74], and 0.40 g EtOH g $carbohydrate^{-1}$[75] were attained using alcohologenic *E. coli* strains that contain the genes for pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase

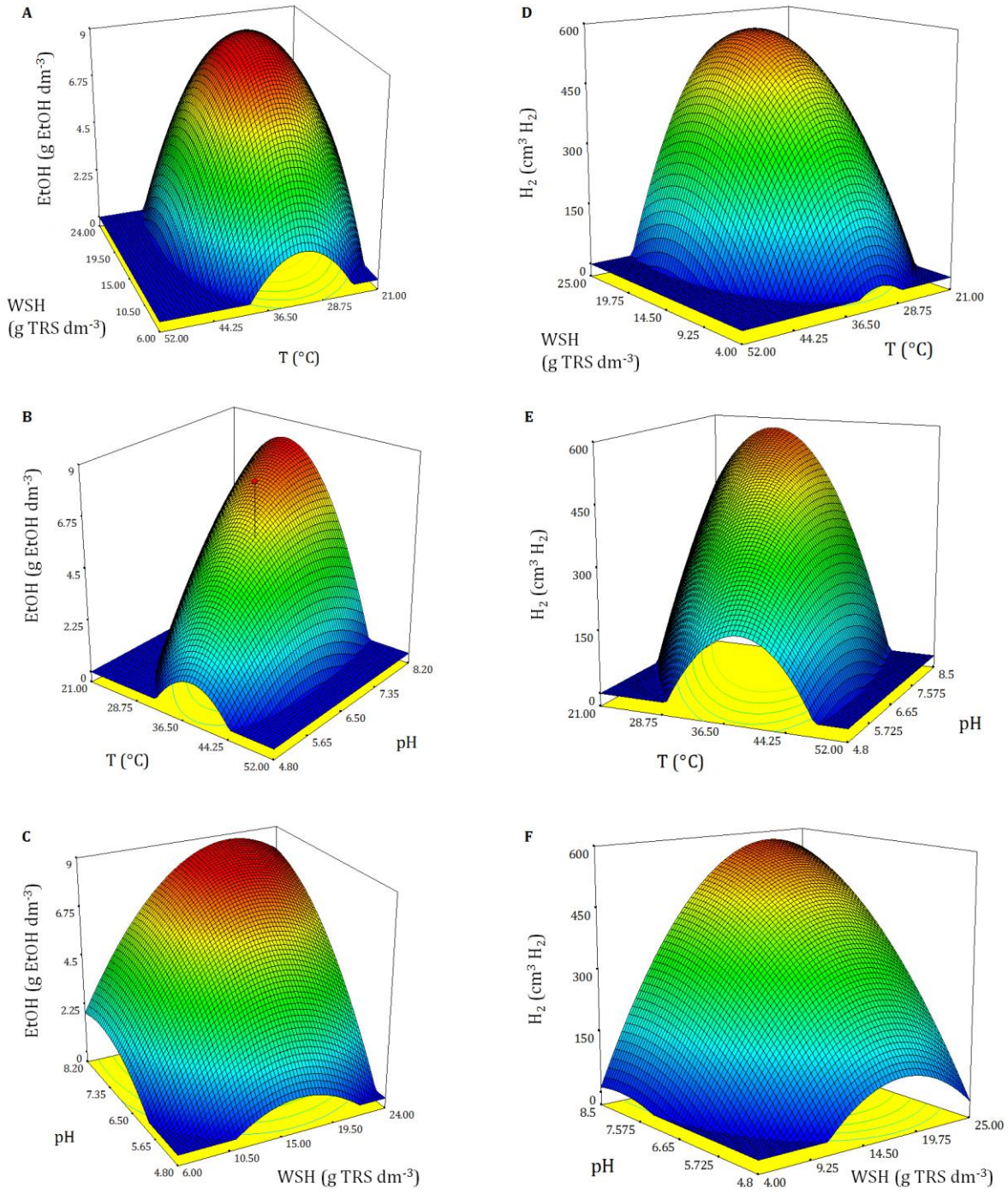

Figure 3.2 Response surface for bioethanol (A, B, C) and biohydrogen production (D, E, F) by *E. coli* WDHL in optimal conditions. pH adjusted to 8.2 in A and D, temperature fixed at 31°C in B and E, concentration of WSH fixed at 23.4 g dm^{-3} in C and F.

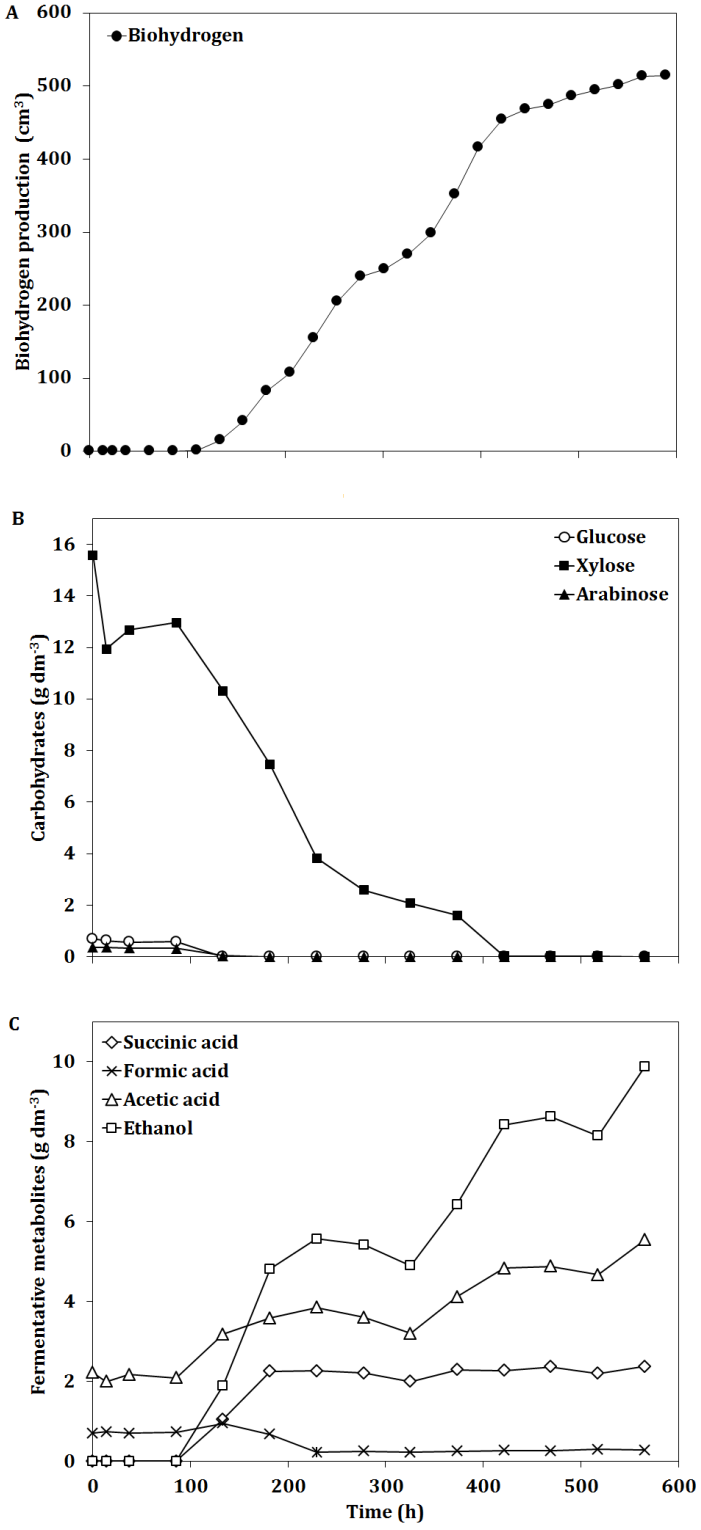

Figure 3.3 Batch culture (110 mL anaerobic serological bottles) of *E. coli* WDHL in optimal conditions: 23.4 g dm^{-3} WSH, 31°C and pH 8.2. (A) Biohydrogen production kinetic. (B) Consumption sugars. (C) Fermentative metabolites.

Table 3.5 Comparison between predicted and experimental values of production, production rate and yield of bioethanol and biohydrogen in optimal conditions (pH 8.2, 31°C and 23.4 g TRS dm^{-3}).

Response variable	Predicted value	Experimental value
EtOH (g EtOH dm^{-3})	8.429	8.9 ± 0.7
r_{EtOH} (g EtOH dm^{-3} h^{-1})	0.049	0.023 ± 0.001
Y_{EtOH} (g EtOH g TRS^{-1})	0.317	0.37 ± 0.03
H_2 (cm^3 H_2)	465.801	513.4 ± 15.4
r_{H_2} (cm^3 H_2 dm^{-3} h^{-1})	21.104	9.8 ± 0.9
Y_{H_2} (cm^3 H_2 g TRS^{-1})	159.251	199.5 ± 7.3

(*adh*) from *Z. mobilis*. In the case of r_{EtOH} that was obtained from this study (0.023 ± 0.001 g EtOH dm^{-3} h^{-1}) was lower than those attained using the recombinant strain described above. Where values of 0.13-0.90 g EtOH dm^{-3} h-1 were attained[72–74]. For Y_{H_2}, Kongjan *et al*.[55], Kongjan and Angelidaki[71] and Lo *et al*.[54] reported values less than 180 dm^{-3} H_2 g $hexose^{-1}$, whereas we obtained 199.5 ± 7.3 cm^3 H_2 g TRS^{-1}. But, Kongjan *et al*. [55] worked with a thermophilic mixed culture and WSH as substrate, making the fermentation at 70°C and initial pH of 5.5. Kongjan and Angelidaki [71] carried out the fermentation at 70°C with anaerobic sludge and WSH.

We obtained 9.8 ± 0.9 cm^3 H_2 dm^{-3} h^{-1} for r_{H_2} and observed that this value is higher than the ones obtained by Fangkum and Reungsang [76] and Kongjan *et al*. [55], because they obtained results between 4.2-7.9 cm^3 H_2 dm^{-3} h^{-1} in batch cultures. Whereas, Kongjan and Angelidaki [71] and Saraphirom and Reungsang [70]

reported up 800 cm^3 H_2 dm^{-3} h^{-1} but using extreme thermophile microorganisms in continuous reactors. Fangkum and Reungsang [76] worked with mixed cultures that were isolated from elephant dung and sugarcane bagasse hydrolysate as substrate, cultured at 55°C and initial pH of 5.5. Saraphirom and Reungsang [70] used anaerobic seed sludge and sweet sorghum syrup as substrate, carried out the fermentation at 30-32°C and initial pH of 4.78. The strain used in the present study has deleted *hycA* and *lacI* genes [17] for the purpose of overproduce hydrogen and increase the lactose consumption rate. Therefore to increase r_{EtOH}, Y_{EtOH}, r_{H_2} and Y_{H_2}, genetic modifications into the ethanol production pathway and to increase the xylose consumption rate as well as a hydrolysate with higher content of carbohydrates are recommended.

The optimal conditions were tested in the bioreactor 1 dm^3 (Figure 3.4) to 200 h of fermentation, the results were: 8.9 g EtOH dm^{-3}, 0.051 g EtOH dm^{-3} h^{-1}, 0.38 g EtOH g TRS^{-1}, 3,277.7 cm^3 H_2, 18 cm^3 H_2 dm^{-3} h^{-1} and 140.1 cm^3 H_2 g TRS^{-1}. Production and yield of biohydrogen obtained in 1 dm^3 bioreactor were lower than the ones observed in serological bottles (4,667.4 ± 171.2 cm^3 H_2 dm^{-3} and 199.5 ± 7.3 cm^3 H_2 g TRS^{-1}, respectively). However, other response variables (EtOH, r_{EtOH}, Y_{EtOH} and r_{H_2}) were similar or higher. According to the results, biofuels production started at 50 hours in bioreactors (1 dm^3) whereas in the serological bottles (110 cm^3) occurred after 100 hours (Figures 3.3 and 3.4). The use of WSH as substrate to biofuels production implicated a diauxic shift, in serological bottles this happened at 277 hours and in bioreactor at 117 hours. The fermentation behavior (1 dm^3-bioreactor) with respect to change of pH over time is visible in Figure 4.4D when

the pH is between 7 and 6.5 the production of bioethanol and biohydrogen began, the diauxic shift occurred below the 5.5, and the biofuels production finished at 4.5. Besides, in the bioreactors, the succinic acid was the second main metabolite whereas in serological bottles was acetic acid. We previously reported the effect of different carbon sources on the metabolite production by WDHL, for instance using lactose; the main metabolite was lactate, whereas using galactose was ethanol [18]. Koskinen *et al*. [23], Barros and Silva [77], Reungsang *et al*. [78], and Varrone *et al*. [79] obtained values of ethanol production of 1.46 ± 0.10, 1.359, 5.53 and 7.92 g EtOH dm^{-3}, respectively, which are lower than those attained in our study. Koskinen *et al*. [23] used a thermophilic bacteria cultured at 60°C and glucose as substrate. Reungsang *et al*. [78] and Varrone *et al*. [79] used glycerol as substrate, whilst in our study fermentation is performed at 31°C and the carbohydrates conversion was 99% using WSH as substrate. Kaparaju *et al*. [38] used WSH as substrate to produce bioethanol and biohydrogen but their process is carried out in two stages, one for bioethanol production (32°C) and another to biohydrogen production (70°C). Production rates (r_{EtOH}, r_{H_2}) obtained by Han *et al*. [80] and Karadag and Puhukka [81] are higher (Table 3.6) than those obtained in this study. Karadag and Puhukka [81] reported that by increasing biohydrogen yield decreases bioethanol production, which is consistent with the results obtained in our research (Table 1.1; compare Y_{H_2} with EtOH in experiments 7, 8 and 18). Lay *et al*. [82] reported 0.19 g EtOH g $hexose^{-1}$ and 126.70 cm^3 H_2 g $hexose^{-1}$, these results were lower than those obtained in our study. Zhao *et al*. [83] reported an ethanol yield of 0.23 g EtOH g $glucose^{-1}$, which is lower than the ones obtained by

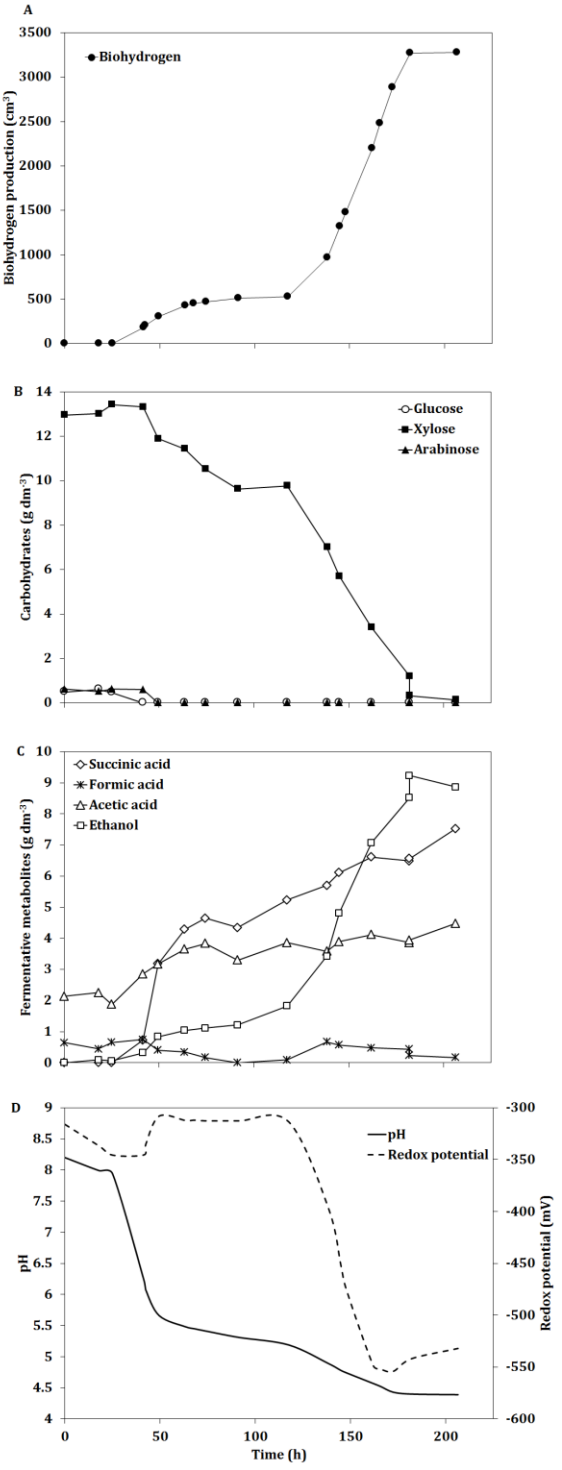

Figure 3.4 Batch culture (1 L bioreactor) of *E. coli* WDHL in optimal conditions: 23.4 g dm^{-3} WSH, 31°C and pH 8.2. (A) Kinetics of biohydrogen production. (B) Consumption carbohydrates. (C) Fermentative metabolites. (D) pH and redox potential.

Table 3.6 Comparison of production, yield and production rate of bioethanol and biohydrogen.

Microorganism	EtOH (g EtOH dm^{-3})	Y_{EtOH} (g EtOH g^{-1})	r_{EtOH} (g EtOH dm^{-3} h^{-1})	Y_{H_2} (cm^3 H_2 g^{-1})	r_{H_2} (cm^3 H_2 dm^{-3} h^{-1})	Reference
E. coli WDHL	8.9	0.38	0.051	140.1	18	This study
S. cerevisiae, extreme thermophilic mixed-culture	NR	0.41 (32° C)	NR	178 (70° C)	NR	[38]
Wastewater sludge	NR	NR	0.93* (20.27 mmol dm^{-3} h^{-1})	NR	313.55* (12.4 mmol H_2 dm^{-3} h^{-1})	[80]
Mixed culture	NR	0.31* (1.2 mol EtOH mol $glucose^{-1}$, 37° C, pH 6)	616.80* (289.2 mol EtOH d^{-1}, pH 6)	176.54* (1.3 mol H_2 mol $glucose^{-1}$, 37° C, pH 4.9)	7,894.25* (6.7 mol H_2 d^{-1}, 37° C, pH 4.9)	[81]
Thermophilic Bacteria	1.464* (31.77 mM)	0.345* (1.35 mol EtOH mol $glucose^{-1}$)	NR	108.64* (0.8 mol H_2 mol $glucose^{-1}$)	166.76* (6.1 mmol H_2 dm^{-3} h^{-1})	[23]
Thermophilic mixed-culture	NR	0.230* (0.90 mol EtOH mol $glucose^{-1}$)	NR	214.56* (1.58 mol H_2 mol $glucose^{-1}$)	NR	[83]
Anaerobic Sludge	1.359 (AFBR containing PET)	0.614* (2.4 mol EtOH mol $glucose^{-1}$, AFBR containing PET)	NR	308.645* (2.11 mol H_2 mol $glucose^{-1}$, AFBR containing grounded tire)	520 (AFBR containing polystyrene)	[77]
Mixed culture	NR	0.192* (0.75 mol EtOH mol $hexose^{-1}$, pH 6)	NR	126.703* (0.83 mol H_2 mol $hexose^{-1}$, pH 7)	130.96* (123.5 mmol H_2 dm^{-3} d^{-1}, pH 7)	[82]
E. aerogenes KKU-S1	5.528* (120 mM)	0.415 g EtOH g-$glycerol^{-1*}$ (0.83 mol EtOH mol $glycerol^{-1}$)	NR	35.835 cm^3 H_2 g $glycerol^{-1*}$ (0.12 mol H_2 mol $glycerol^{-1}$)	6.11* (0.24 mmol H_2 dm^{-3} h^{-1})	[78]
Mixed culture	7.92	0.500 g EtOH g $glycerol^{-1*}$ (1 mol EtOH mol $glycerol^{-1}$)	NR	286.680 cm^3 H_2 g $glycerol^{-1*}$ (0.96 mol H_2 mol $glycerol^{-1}$)	2,191	[79]

* Converted units from the original data. NR: Not reported.

us, but the hydrogen yield reported by them was 214.56 cm^3 H_2 g $glucose^{-1}$, which it was higher than attained in our study. Organic acids production and consumption caused reduction on pH from 8.2 to 4.31 and a drop of redox potential (E_h) from -316 to -520 mV was mainly related with the cell growth [17] (Figure 3.4D). *Escherichia coli* grow well under anaerobic conditions upon a decrease in external E_h. At bacterial growth under the indicated conditions, a shift in E_h from positive to negative values is observed. It is worthy of note that the positive values, resulting from dissolved oxygen, inhibit the growth, whereas the positive values, created by the presence of other chemicals, are not able to affect the growth [84, 85]. In the E_h profile (Figure 3.4D) it is possible to observe three changes: a) since -316 to -350 mV, this shift coincide with the total consumption of glucose; b) -350 to -315 mV, when the production of biofuels and metabolites began; c) -315 to -520 mV, when the diauxic shift occur. The bacteria are abundant under highly reducing conditions (E_h < 0 mV) [86]. A positive E_h provides evidence that a culture medium may be in an oxidized state and can to be the limiting factor in the growth of anaerobic bacteria [87, 88]. Thus the drop of E_h can be explained as an effect of the bacterial growth because this always remains with values lower than zero mV, additionally a decrease of pH probably entails the dropping of redox potential [89].

3.3 Summary

We demonstrate the simultaneous production of bioethanol and biohydrogen from wheat straw hydrolysate by *E. coli* WDHL. The variables affecting the process are temperature and pH, and the optimal conditions are 23.4 g TRS dm^{-3}, 31°C and pH 8.2. This strategy could be used in the conceptual design of 2G biorefineries.

Improvement of production rate and yields are still necessary, and they could be attained through genetic modifications in the microorganism as well as to use hydrolysates with higher content of fermentable carbohydrates.

4 Scaling-up of the simultaneous production of ethanol and hydrogen by *Escherichia coli ΔhycA Δfrd ΔldhA* from wheat straw hydrolysate

The interest in the genetic transformation of microorganisms increases due to the development of a society toward a bioeconomy. Chemical production routines using fossil fuels as substrates are more and more being replaced by sustainable processes that work with biological catalysis and renewable substrates. Strains developed several years ago are still the starting points for the development of new strains that either broaden the spectrum of producible substances or increase the efficiency of an applied process [90, 91]. ‘Fermentation’ is a term used to describe a biological process in which a substrate is converted into a product of interest by a microbial strain. Fermentation processes are advantageous for the production of such molecules, in comparison to chemical processes, because they are generally considered to be more sustainable due to lower temperature processing, lower pressure, and no requirements for harsh chemicals [92]. The fermentation products of the microorganisms include organic acids, such as lactic, acetic, succinic, and butyric; as well as, neutral products, such as, ethanol, butanol, acetone, and butadienol. *Escherichia coli* is capable to produce a variety of substances in the absence of oxygen as electron acceptors. Under these conditions, acetate is the main product. Under fermentative conditions, a mixture of succinate, formate, acetate, lactate and ethanol is produced [91, 93, 94].

The strong dependence on fossil fuels comes from the intensive use and consumption of petroleum derivatives which, combined with diminishing petroleum resources, causes environmental and political concerns. There is clear scientific

evidence that emissions of greenhouse gases, such as carbon dioxide, methane and nitrous oxide, arising from fossil fuel combustion and land-use change as a result of human activities, are perturbing the Earth's climate. Recently, society began to recognize the opportunities offered by a future sustainable economy based on renewable sources and has been starting to finance R&D activities for its implementation. It is increasingly acknowledged globally that plant-based raw materials have the potential to replace a large fraction of fossil resources as feedstocks for industrial productions, addressing both the energy and non-energy sectors [53, 95, 32].

The aim of this work was scaling up the simultaneous production of ethanol and hydrogen from wheat straw hydrolysate by recombinant *E. coli* strain. To improve the simultaneous production of ethanol and hydrogen the complete deletion of *frd* and *ldhA* genes was performed in *E. coli* W3110 *ΔhycA*. The production of lactate is catalysed by the soluble lactate dehydrogenase (*ldhA*) via reduction of pyruvate. Succinate formation stars with the carboxylation of phosphoenolpyruvate to oxaloacetate by PEP-carboxylase, and is subsequently achieved via the activity of malate dehydrogenase, fumarase, and fumarate reductase (*frd*). Strains with defective *frd* and *ldhA* genes overproduce ethanol.

4.1 Material and methods

4.1.1 Construction of mutant strains

Strains, plasmids and primers used for the construction of the mutant strains are shown in Table 4.1. Strains W3110 *frd*$^{-}$ and W3110 *ldhA*$^{-}$ from Keio collection were

used as donor. The complete deletion of *frd* and *ldhA* genes was achieved using P1 transduction method [96], with some modification as follows:

(A) Preparation of liquid P1 lysate. An overnight culture of *E. coli* donor strain (W3110 *frd*$^-$ or W3110 *ldhA*$^-$) in LB broth ($kan_{30mg/L}$) was washed and suspended in MC media (10 mM $MgSO_4$ and 5 mM $CaCl_2$). Phage P1 was added with multiplicity of infection between 0.1 and 1.0, and the culture was incubated for 30 min at room temperature. After incubation the culture was added into soft agar and finally plated in agar plates (LB media) and incubated overnight at 37°C. After lysis the culture was treated with chloroform and the debris was removed by centrifugation. The phage were stored at 4°C until it application.

(B) Transduction. An overnight culture of *E. coli* recipient strain (W3110 *ΔhycA*) in LB media was washed and suspended in MC media (10 mM $MgSO_4$ and 5 mM $CaCl_2$). Phage P1 lysate with the donor strain (W3110 *frd*$^-$ or W3110 *ldhA*$^-$) was added with multiplicity of infection between 0.1 and 1.0. The phage were allowed to absorb for 30 min at room temperature, and then 1 M sodium citrate was added. Finally, all the mixture was plated on selective plates (LB $kan_{30mg/L}$) and incubated at 37°C until colonies appeared.

The deletion of *frd* and *ldhA* genes was verified by colony PCR with OGF-F, OGF-R, OG-L-F and OG-L-R primers. The Δ*frd,* Δ*ldhA,* Δ*ldhA* Δ*frd* strains were transformed electroporation with the pCP20 plasmid, and selected by both kanamycin and ampicillin resistant at 28 °C. Transforming cells were incubated overnight in LB media without antibiotic at 37 °C, and then they were submitted at water bath at 42 °C during 1 h. After water bath, the cells were tested for sensitivity

for both antibiotics. Sensible colonies were tested by PCR to confirm the loss of *kan* gene.

Table 4.1 Strains, plasmid and primers used in this work

Strains	**Relevant genotype**	**Source**
W3110 *frd*$^-$	lac$^+$, gal$^+$, F$^-$, λ^- IN (rrnD-rrnE)1, rph-1, Δ*frd*	Laboratory stock
W3110 *ldhA*$^-$	lac$^+$, gal$^+$, F$^-$, λ^- IN (rrnD-rrnE)1, rph-1, Δ*ldhA*	Laboratory stock
Δ*hycA*	W3110 Δ*hycA*	[17]
Δ*hycA* Δ*frd*	W3110 Δ*hycA* Δ*frd*	This work
Δ*hycA* Δ*ldhA*	W3110 Δ*hycA* Δ*ldhA*	This work
Δ*hycA* Δ*ldhA* Δ*frd*	W3110 Δ*hycA* Δ*ldhA* Δ*frd*	This work
Plasmid		
pCP20	FLP recombinase expression plasmid (*bla*, *cat*) λp_R FLP$^+$, λ cI857$^+$, pSC101 ori TS	[97]
Primers	**Sequence**	
OGF-F	GAGGGGCAGCAAATGTGGAG	This work
OGF-R	TGAACTGGCACCGAAAGCGG	This work
OG-L-F	CGCGGCTACTTTCTTCATTG	This work
OG-L-R	GGTTGCGCCTACACTAAGCAT	This work

4.1.2 Effect of the complete deletion of *frd* and *ldhA* genes in hydrogen production

The effect of the complete deletion of *frd* and *ldhA* genes in hydrogen production by recombinant *Escherichia coli* strains (Table 4.1) was determined by using analytical grade carbohydrates as substrate (glucose) at a concentration of 20 g/L. The experiments were done in anaerobic serological bottles containing 110 cm^3 of B medium [41], 1 cm^3 dm^{-3} trace elements solution [17], 0.01 g dm^{-3} $MgSO_4$ and 2.75 g dm^{-3} yeast extract (Difco). The cultures were started with 0.2 OD_{600nm}, pH

7.5 and they were incubated at 31 °C and 175 rpm. All the experiments were carried out in triplicate. Ethanol and hydrogen production were measured as it was indicated in section 4.1.4.

4.1.3 Scaling up of the simultaneous productions of ethanol and hydrogen by *E. coli ΔhycA ΔldhA Δfrd* using wheat straw hydrolysate as substrate

The scaling up of the simultaneous production of ethanol and hydrogen by *E. coli ΔhycA ΔldhA Δfrd* from wheat straw hydrolysate at 16.4 g/L of total reducing sugars was performed using 0.01, 0.1, 1 and 10 L as working volumes. The experiments were done in anaerobic serological bottles and anaerobic bioreactor containing B medium [41], 1 cm^3 dm^{-3} trace elements solution [17], 0.01 g dm^{-3} $MgSO_4$ and 2.75 g dm^{-3} yeast extract (Difco). The cultures were started with 0.2 OD_{600nm}, pH 8.2 and they were incubated at 31 °C and 175 rpm. The experiments in anaerobic serological bottles (0.001 and 0.1 L) were carried out in quadruplicate. Ethanol and hydrogen production were measured as it was indicated in section 4.1.4.

4.1.4 Analytical Methods

Total reducing sugars (TRS) estimation was performed by the dinitro salicylic acid (DNS) method[62], with some modifications as follow: 0.25 cm^3 of WSH with 0.75 cm^3 of DNS reagent (10 g dm^{-3} NaOH, 200 g dm^{-3} $KNaC_4H_4O_6 \cdot 4H_2O$, 0.5 g dm^{-3} $Na_2S_2O_5$, 2 g dm^{-3} C_6H_6O, 10 g dm^{-3} 3,5-Dinitrosalicylic acid) were heated for 15 minutes in a boiling water bath and then cooled to room temperature. For the

calibration curve, glucose (0.1 to 1.0 g dm^{-3}) was used as the reference standard. The absorbance was measured at 550 nm (Varian's Cary® 50 Bio UV-Visible Spectrophotometer).

Ethanol concentration was quantified by a high-performance liquid chromatography (HPLC) in an Agilent chromatograph equipped with a refractive index (Agilent Technologies 1220 Infinity LC). A Phenomenex® - organic acid analysis column operated at 60°C with H_2SO_4 0.0025 M as a mobile phase (0.550 cm^3 min^{-1}) it was used for its separation.

Hydrogen production was measured by NaOH 1 N displacement in an inverted burette connected to the bioreactor or to serological bottles with rubber tubing and a needle. Analysis of biohydrogen by gas chromatography was performed with a thermal conductivity detector (Agilent Technologies 6890N Network GC Systems) and using Agilent J&W HP-PLOT Molesieve (0.32 mm ID, 30 m length, 12 µm film) under the following conditions: 200 °C, injector temperature; 280 °C, detector temperature; 300 °C, oven temperature. Hydrogen volume was corrected to standard conditions of temperature and pressure (298.15K and 10^5 Pa).

4.2 Results and discussion

4.2.1 Improvement of the hydrogen production by the complete deletion of *frd* and *ldhA* genes in *Escherichia coli*

The production of hydrogen via dark fermentation is an active field of research, because hydrogen is a promising fuel as it has higher energy content than oil and its combustion results only in water and energy. Given its ease of manipulation, *Escherichia coli* has become a workhorse for enhanced hydrogen production

through metabolic engineering, heterologous gene expression, adaptive evolution, and protein engineering. This study reports the fermentative hydrogen production by recombinant *Escherichia coli* strains using glucose as substrate (Figure 4.1). To increase the hydrogen production, the complete deletions of *frd* and *ldhA* genes was performed in *E. coli ΔhycA*. To evaluate the effect of the complete deletion of *frd* and *ldhA* genes on the hydrogen production by recombinant strains, they were cultured in 120 mL anaerobic serological bottles contained 100 mL of medium B with 20 g/L of glucose. The cultures were started with 0.2 OD_{600nm}, pH of 7.5 and were incubated at 31°C and 150 rpm. After 205 h of fermentation, the hydrogen production by the wild-type strain was 1,744.5 ± 44.6 mL H_2/L, whereas, recombinant strains *E. coli ΔhycA*, *E. coli ΔhycA ΔldhA*, *E. coli ΔhycA Δfrd* and *E. coli ΔhycA Δfrd ΔldhA* produced 1,987.6 ± 83.2, 2,523.0 ± 93.5, 1,649.1 ± 228.1 and 3,015.1 ± 317.6 mL H_2/L, respectively.

The hydrogen production by *E. coli ΔhycA Δfrd ΔldhA* was almost two times that obtained by the wild-type strain. As we know, *frd* and *ldhA* genes are involved in the succinate and lactate metabolisms, respectively. Succinate and lactate production was reduced dramatically by the completed deletion of *frd* and *ldhA* genes, whereas the ethanol production was improved (Table 4.2). The results showed that absence of *frd* and *ldhA* genes improve hydrogen and ethanol production.

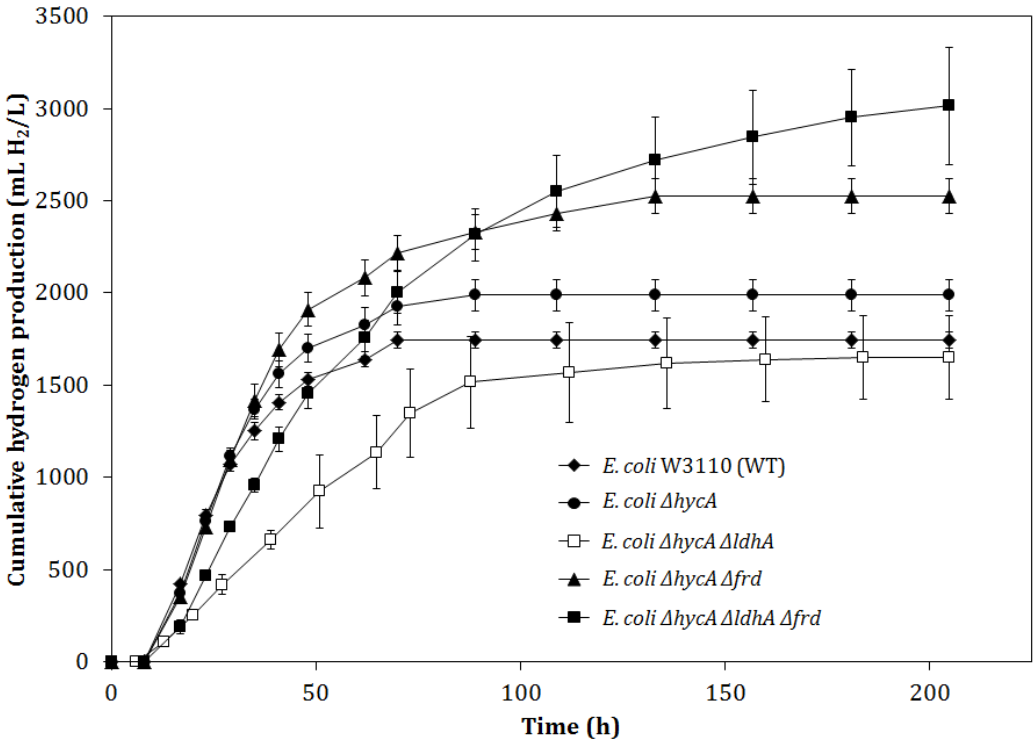

Figure 4.1 Batch culture (0.01 L bioreactor) of recombinant *E. coli* strains (31°C and $pH_{initial}$ 7.5) using glucose as substrate

Table 4.2 Soluble metabolite concentrations accumulated during hydrogen production process via dark fermentation by recombinant *E. coli* strains

	Metabolites (g/L)			
Strain	**Succinate**	**Lactate**	**Acetate**	**Ethanol**
***E. coli* W3110**	1.8	2.7	2.4	2.7
E. coli ΔhycA	1	4.4	2.1	1.9
E. coli ΔhycA ΔldhA	1.2	0.9	3.8	5.9
E. coli ΔhycA Δfrd	0	7.2	1.7	1.3
E. coli ΔhycA ΔldhA Δfrd	0	1.3	2.5	6.7

4.2.2 Scaling-up of the simultaneous production of ethanol and hydrogen by *E. coli ΔhycA Δfrd ΔldhA* using wheat straw hydrolysate as substrate

The scaling up is a vital tool to development bioprocesses, since it can reduce errors in the designs or lack of information. For this reason, after obtain *E. coli ΔhycA ΔldhA Δfrd*, the scaling up of the simultaneous production of ethanol and hydrogen was carried out used as a hydrolyzed substrate of wheat straw. The working volumes used to scaling up were 0.01, 0.1, 1 and 10 L in batch culture. The wheat straw hydrolysate used as substrate and it content 10 g/L xylose, 0.5 g/L glucose and 2 g/L arabinose. The optimal pH and temperature used in this study were obtained previously by our group [98]. Figure 4.2 shown production and yield of ethanol obtained by *E. coli ΔhycA ΔldhA Δfrd.* At the end of fermentation the total reducing sugars were complete consumed, and the production and yield of ethanol were 8.22 ± 0.61 g EtOH/L (Figure 3.2 A) and 0.51 ± 0.04 g EtOH/g TRS (Figure 4.2 B), respectively. A not statistically significant difference (Table 4.2, $p < 0.001$) was found between each level of the scaling up.

The kinetics of hydrogen production shows the profile of the hydrogen production to 0.01, 0.1 and 1 L working volumes (Figure 4.3). Hydrogen production started in the first 15 h of fermentation and it keeping similar in each level of the scaling up. The pH at the end of fermentation was 5.84 ± 0.18, it decrease approximately two units due to the low organic acids production, which in turn is consequence of the deficiency *frd* and *ldhA* genes, involved in the succinate and lactate production, respectively.

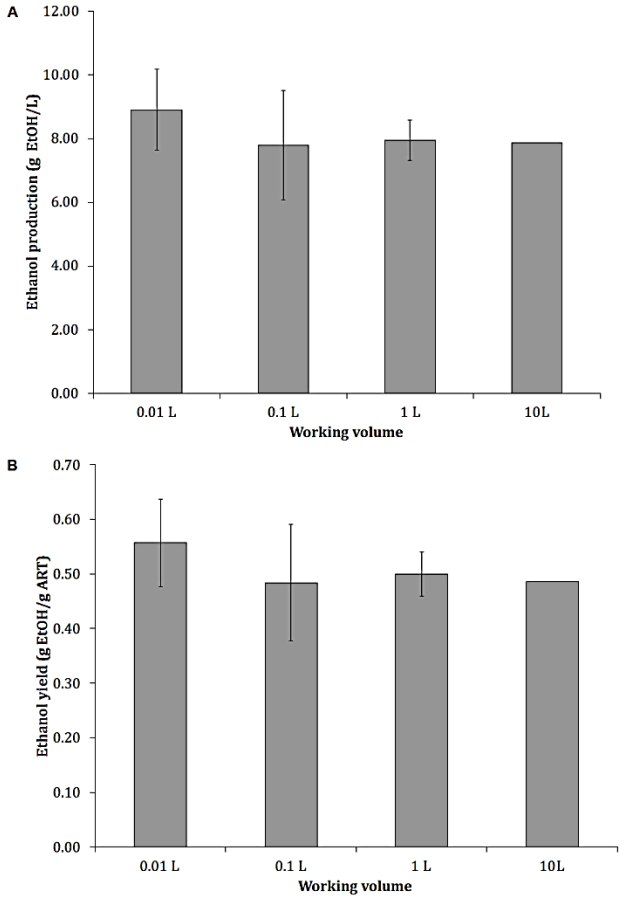

Figure 4.2 Production (A) and (B) yield of ethanol by *E. coli ΔhycA ΔldhA Δfrd* strain from wheat straw hydrolysate. Substrate concentration: 16.4 g TRS/L. Initial pH: 8.2. Incubation temperature: 31°C

Table 4.3 Analysis of variance for production and yield of ethanol ($p < 0.001$)

Ethanol production

	DF*	Sum of Squares	Mean Square	*F* Value	*p*-value
Model	3	2.92292	9.74E-01	0.48102	0.70574
Error	7	14.17839	2.02548		
Total	10	17.10131			

Ethanol yield

	DF*	Sum of Squares	Mean Square	*F* Value	*p*-value
Model	3	0.01248	0.00416	0.52963	0.6761
Error	7	0.05497	0.00785		
Total	10	0.06745			

*Degrees of freedom

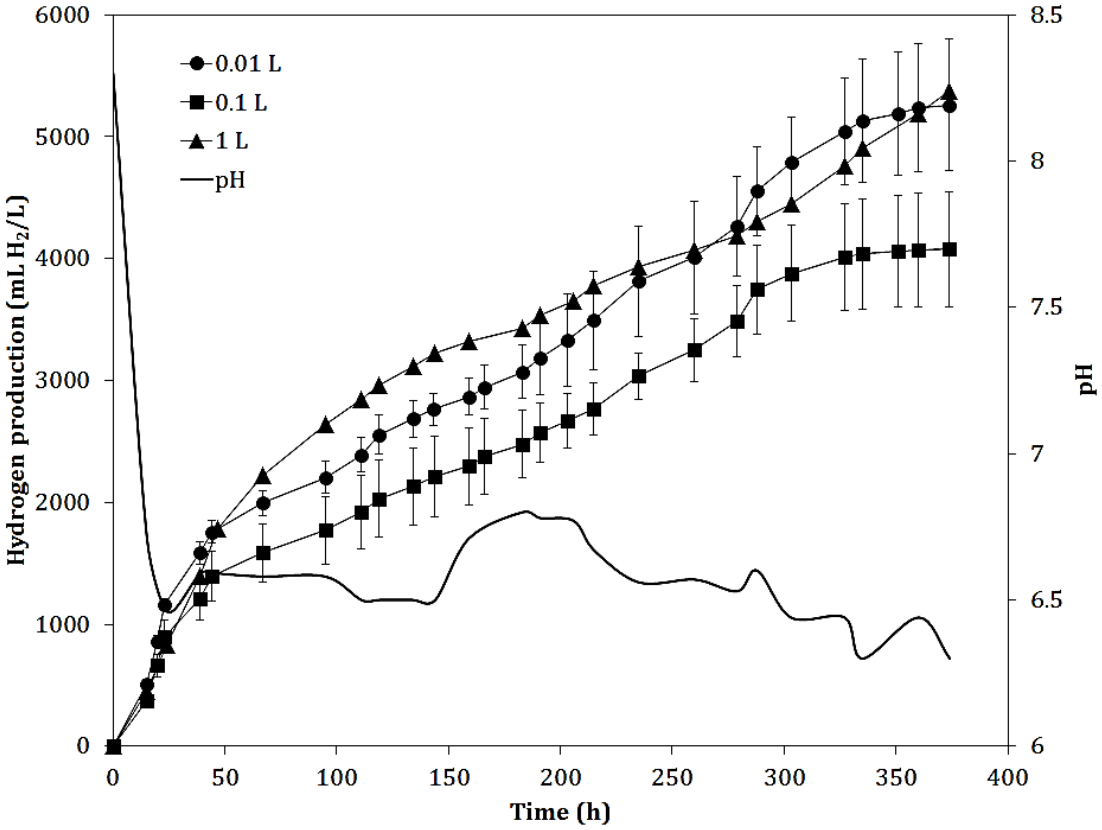

Figure 4.3 Kinetics of hydrogen production by *E. coli ΔhycA ΔldhA Δfrd* strain from wheat straw hydrolysate. Substrate concentration: 16.4 g TRS/L. Initial pH: 8.2. Incubation temperature: 31°C. pH profile is corresponding to 1 L bioreactor

After 380 h of fermentation were obtained 4902.41 ± 717.76 mL H_2/L, 35.28 ± 4.08 mL H_2/L/h y 306.58 ± 46.47 mL H_2/g TRS. The production (Figure 4.4A), production rate (Figure 4.4B) and yield (Figure 4.4C) of hydrogen were not affect by the scaling up due to were not found statistically significant difference (Table 4.4, $p < 0.001$) between each level of the scaling up.

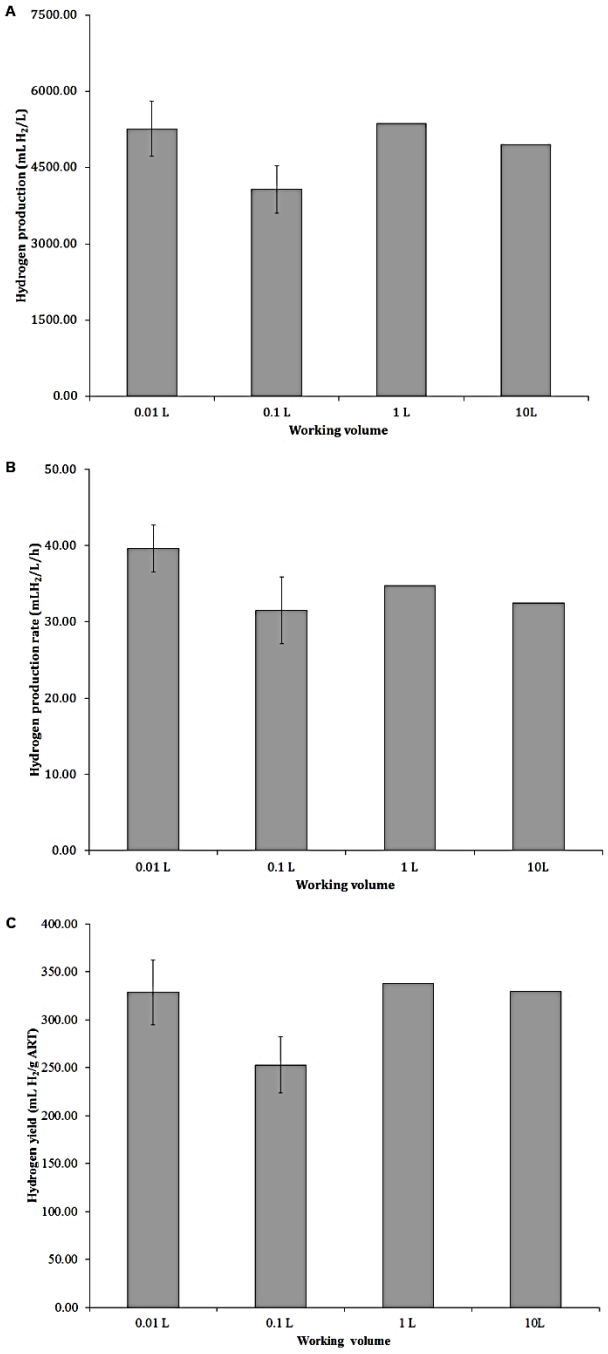

Figure 4.4 Production (A), (B) production rate and (C) yield of hydrogen by *E. coli ΔhycA ΔldhA Δfrd* strain from wheat straw hydrolysate. Substrate concentration: 16.4 g TRS/L. Initial pH: 8.2. Incubation temperature: 31°C

Table 4.4 Analysis of variance for production, production rate and yield of hydrogen ($p < 0.001$)

Hydrogen production

	DF*	Sum of Squares	Mean Square	*F* Value	*p*-value
Model	3	3279640	1.09E+06	4.27656	0.06169
Error	6	1533780	255629.174		
Total	9	4813420			

Hydrogen production rate

	DF*	Sum of Squares	Mean Square	*F* Value	*p*-value
Model	3	138.25406	46.08469	3.16278	0.10697
Error	6	87.42579	14.57097		
Total	9	225.67985			

Hydrogen yield

	DF*	Sum of Squares	Mean Square	*F* Value	*p*-value
Model	3	14120.8834	4706.96114	4.73898	0.05038
Error	6	5959.46523	993.24421		
Total	9	20080.3487			

*Degrees of freedom

From the comparison of the ethanol yield, hydrogen production rate and hydrogen yield obtained by *E. coli ΔhycA ΔldhA Δfrd* in this work with the obtained previously by *E. coli ΔhycA ΔlacI* [98] in experiments related with the optimization of simultaneous production of ethanol and hydrogen (Table 4.5), we can notice the following:

a) The *E. coli ΔhycA ΔldhA Δfrd* strain increase 38% the ethanol yield.
b) The hydrogen production rate obtained by *E. coli ΔhycA ΔldhA Δfrd* was approximately two times that the achieved by *E. coli ΔhycA ΔlacI*.
c) The hydrogen yield was increased 53% by *E. coli ΔhycA ΔldhA Δfrd*.

This demonstrate that the complete deletion of *frd* and *ldhA* genes improve the simultaneous ethanol and hydrogen production.

Table 4.5 Comparison of ethanol yield, hydrogen production rate and hydrogen yield obtained by *E. coli ΔlacI ΔhycA* and *E. coli ΔhycA ΔldhA Δfrd* strains in optimal conditions (Initial pH: 8.2, Incubation temperature: 31°C) using wheat straw hydrolysate

	Strains			
	E. coli ΔhycA ΔlacI		***E. coli ΔhycA ΔldhA Δfrd***	
Working volume (L)	**0.1**	**1**	**0.1**	**1**
Ethanol yield (g EtOH/g TRS)	0.37 ± 0.03	0.38	0.48 ± 0.11	0.50 ± 0.04
Hydrogen production rate (mL H_2/L/h)	9.8 ± 0.9	18	31.50 ± 4.40	34.75
Hydrogen yield (mL H_2/ g TRS)	199.5 ± 7.3	140.1	253.17 ± 29.09	337.81

4.3 Summary

The results obtained by us in this study demonstrated that the complete deletion of *frd* and *ldhA* genes improve the simultaneous ethanol and hydrogen production using wheat straw hydrolysate as substrate under optimal operation conditions. Also, was proved that the scaling up of the simultaneous production of ethanol and hydrogen from wheat straw hydrolysate is feasible due to the working volume does not affected the production and yield of ethanol, as well as, production, production rate and yield of hydrogen.

5 Antioxidants from seaweeds: optimization of solvent mixture and extraction conditions for ultrasonic assisted extraction

Free radicals are atoms, molecules and/or ions that contain one or more unpaired electrons. Free radicals (*e.g.* reactive oxygen species, ROS) are produced as normal part of metabolism and external factors, such as smoking, environmental pollutants, radiation, drugs, pesticides, industrial solvents and ozone [99]. ROS can (1) cause structural alterations in DNA, (2) affect cytoplasmic and nuclear signal transduction pathways, and (3) modulate the activity of the proteins and genes that respond to stress and which act to regulate the genes that are related to cell proliferation, differentiation and apoptosis [99, 100]. Compounds with properties to scavenge and prevent the formation of free radicals are known as antioxidants and basically they act as electron donors to neutralize the free radicals [101].

Plants have the capacity to produce a vast and diverse array of secondary metabolites. Land plants have developed a sophisticated antioxidant system for controlling ROS levels [102]. Natural antioxidants can be divided into six major groups: vitamins, carotenoids, polyphenols, small antioxidant molecules, enzymes, and trace elements [103]. However, not only land plants contain antioxidants. Seaweeds are frequently exposed to extreme and varying conditions along with exposure to strong light and high oxygen concentrations that leads to formation of ROS and other strong oxidizing agents. Nevertheless, damage into the structural components or any serious photodynamic damage of the seaweed rarely occurs. In order to survive these harsh marine environments, seaweeds have developed strong protective antioxidant defence systems consisting of an array of antioxidant

compounds that may or may not work synergistically to limit oxidation [104]. The bioactive compounds, *e.g.* phenolic compounds and carotenoids, produced by seaweed have different physiological effects on human health. Polyphenols have anti-oxidative and anti-mutagenic activities, and cholesterol-lowering effects. Fucoxanthin, the dominant carotenoid in brown seaweeds, has strong antioxidant properties with significant anti-cancer, anti-obesity and anti-inflammatory effects [105–107].

Antioxidants are of interest for the food industry and have medicinal and pharmacological applications due to their properties in providing general wellness and delaying aging, as well as the ability to retard the progress of chronic diseases, and lipid peroxidation in food and pharmaceutical products [99]. Recovery of these bioactive compounds from plant material is usually performed by means of an extraction process. Ultrasound-assisted is widely used for extracting plant compounds for reducing extraction times, improving yields and quality of the extracts. When solid–liquid extraction is assisted by ultrasound the mass transfer is intensified, the solvent penetration and capillary effects are improved. The concomitant collapse of cavitation bubbles near the cell walls is expected to produce the cell disruption together with a good penetration of the solvent into the cells, through the ultrasonic jet [108–110]. In related works dealing with extraction of pigments and/or phenolic compounds, typical solvents used are acetone, ethanol, methanol, dichloromethane and water; and the extract obtained by each solvent had different content of pigments or phenolic compounds as well as antioxidant activity [104, 111]. In response to environmental problems and the need to reduce waste generation, the appropriate selection of the solvent is

indisputably necessary to guarantee the sustainability of a chemical production process [112]. According to a survey of solvent selection guides for green solvents published by the Innovative Medicines Initiative (IMI)-Chem21 [113]one of the green solvents often recommended is ethanol and therefore selected in this work.

The main objective of this study was to extract and quantify the content of fucoxanthin and phenolic compounds and measure their antioxidant activity in seaweed species native to Nordic conditions. Moreover, it was aimed at maximizing the overall antioxidant activity and the content on fucoxanthin and phenolic compounds in seaweed extracts using ultrasound-assisted extraction. Among the solvents used for TPC and fucoxanthin extraction, ethanol and acetone have shown to have a good performance regards to extraction yield compared to aqueous solvents and water [105], respectively. However, minor attention has been placed in the “design” of solvent mixtures for extraction of TPC. In the present study, this is addressed through a “mixture problem” approach and a D-Optimal experimental design. The effect of sample concentration, number of extractions and extraction time were evaluated using a Box-Benhken experimental design.

5.1 Materials and Methods

5.1.1 Chemicals

All the chemicals and standards used in this study were of analytical grade and purchased from Sigma-Aldrich ApS (Brøndby, Denmark), except ethanol absolute, which was purchased VWR – Bie & Berntsen A/S chemicals (Søborg, Denmark).

5.1.2 Seaweed samples

Samples of *Saccharina latissima* (SL), *Laminaria digitata* (LD), *Fucus evanescens* (FE), *Fucus vesiculosus* (FV) and *Fucus serratus* (FS) used in this work were collected from Hanstholm, Nivå Bugt and Øresund, Denmark, between June 2013 and March 2016. The seaweed samples were rinsed carefully in fresh seawater and then frozen. The frozen samples were lyophilized (CoolSafe 100-9Pro, SCANVAC, Denmark), pulverized into powder by a vibratory disc mill (SIEBTECHNIK GmbH, Germany) and kept in the dark at -20 ºC.

5.1.3 Optimization of solvent mixture extraction

The “mixture problem” approach was used to find the optimal solvent mixture, in terms of composition, to maximize the extraction yield (Y) of total phenolic compounds (TPC), through a D-optimal experimental design (Table 5.1). A binary mixture of water-ethanol was used as an extraction solvent. The triplicates of mixtures 100% H_2O:0% EtOH (experiments 5, 6, and 11) and 20% H_2O:80% EtOH (experiments 2, 4, and 9) provide the statistical validity. All experiments were performed using 10 cm^3 of solvent mixture as a working volume and 0.1 g/dm^3 of dried matter (DM). The mixture solvent-dried seaweed was sonicated (Ultrasonic Bath UR1 Retsh®, Haan, Germany) twice for 30 minutes and centrifuged between each sonication for 15 minutes at 4600 rpm. The supernatants were collected together in a separate vial. The solvent was evaporated in a rotary evaporator (Heidolph, Schwabach, Germany) at 35 ºC and the extract was freeze dried and weighed for calculation of extraction yield. The freeze dried extracts were re-

dissolved in water and used for subsequent analyses. The algal material used to obtain the optimal solvent mixture extraction was *Saccharina latissima*.

Table 5.1 D-optimal experimental design and its corresponding results for each solvent mixture

Experiment	% (v/v) EtOH	% (v/v) H_2O	Y (%, g extract/100 g DM)	TPC (mg GAE/100 g DM)
1	10	90	14.3	80.5
2	80	20	12.6	86.5
3	40	60	15.5	90
4	80	20	11.1	86.6
5	0	100	17.3	74.2
6	0	100	15.9	78.4
7	70	30	14.5	103.3
8	50	50	16.6	116.0
9	80	20	13.0	86.6
10	60	40	14.6	120.2
11	0	100	17.0	73.3
12	40	60	14.9	95.2
13	20	80	16.5	91.5

5.1.4 Optimization of ultrasounds-assisted extraction

Response surface methodology (RSM) was used to find the sample concentration (g/L), number of extractions and extraction time that maximize the extraction of bioactive compounds and antioxidant activity through Box-Behnken experimental design (Table 5.2). The antioxidant activity was reported as 2, 2-diphenyl-1-picrylhydrazyl radical scavenging activity (DPPH) at 2.5 g extract/dm^3. Three levels for each independent variable were included and five central points (experiments 1, 10, 15, 16 and 17). All experiments were performed using 60% (v/v) aqueous EtOH. After ultrasound-assisted extraction and subsequent centrifugation, the solvent was evaporated in a rotary evaporator at 35 ºC and the extract was freeze

dried and weighed for calculation of extraction yield. The freeze dried extracts were re-dissolved in water and used for further analyses. The algal material used for obtain the optimal operating conditions of the ultrasounds-assisted extraction was *Saccharina latissima*.

5.1.5 Crude extracts partitioning

The crude extracts obtained at optimal extraction conditions were dissolved in water for quantification of total phenolic compounds and pigments content to decide which bioactive compounds to extract from each kind of seaweed. Initially, the crude extract from 7 g DM of *Fucus evanescens* (FE), *Fucus vesiculosus* (FV) and *Fucus serratus* (FS) were dissolved in 60% (v/v) EtOH (80 cm^3) and then partitioned with 40 cm^3 of butyl acetate. After separation, the aqueous phase was mixed with 30 cm^3 of distilled water and washed with 40 cm^3 of ethyl acetate. The aqueous phase obtained was washed with n-butanol and finally collected in a separate vial. The crude extract from 7 g of *S. latissima* was dissolved in 40 cm^3 of methanol and 20 cm^3 of distilled water, and washed with 40 cm^3 of n-hexane. The aqueous phase was mixed with 60 cm^3 of distilled water and partitioned with 60 cm^3 of ethyl acetate to obtain a fraction rich in fucoxanthin and chlorophyll c_2. The solvent of each fraction was evaporated in a rotary evaporator at 35 ºC and the extract-fractions were freeze dried and weighed. The procedure aforementioned is described in Figure 5.1.

Table 5.2 Box-Behnken experimental design and its corresponding results of total phenolic compounds content, fucoxanthin content and DPPH radical scavenging activity

Experiment	Sample (g/L)	Time(min)	Number of extractions	Y (%, g extract/100 g DM)	TPC (mg GAE/100 g DM)	Fxn (mg/100 g DM)	DPPH (%)
1	0.275	20	2	21.3	170.2	19.2	50.4
2	0.5	20	1	6.8	54.4	4.5	43.6
3	0.275	30	3	25.3	244.1	32.3	56.3
4	0.275	30	1	13.3	108.0	9.8	44.9
5	0.5	30	2	17.7	169.3	22.0	57.1
6	0.05	30	2	27.8	274.7	30.6	52.5
7	0.275	10	1	11.5	90.1	6.1	37.9
8	0.275	10	3	23.7	172.4	15.5	44.0
9	0.5	20	3	20.9	177.7	12.3	45.2
10	0.275	20	2	19.7	153.5	13.1	41.7
11	0.05	20	3	82.1	281.5	21.0	22.8
12	0.5	10	2	16.8	136.1	11.5	44.7
13	0.05	10	2	25.6	203.7	11.5	38.3
14	0.05	20	1	20.7	142.9	5.4	29.3
15	0.275	20	2	20.0	162.0	11.9	40.8
16	0.275	20	2	20.0	153.1	11.8	41.2
17	0.275	20	2	20.1	151.9	10.9	44.4

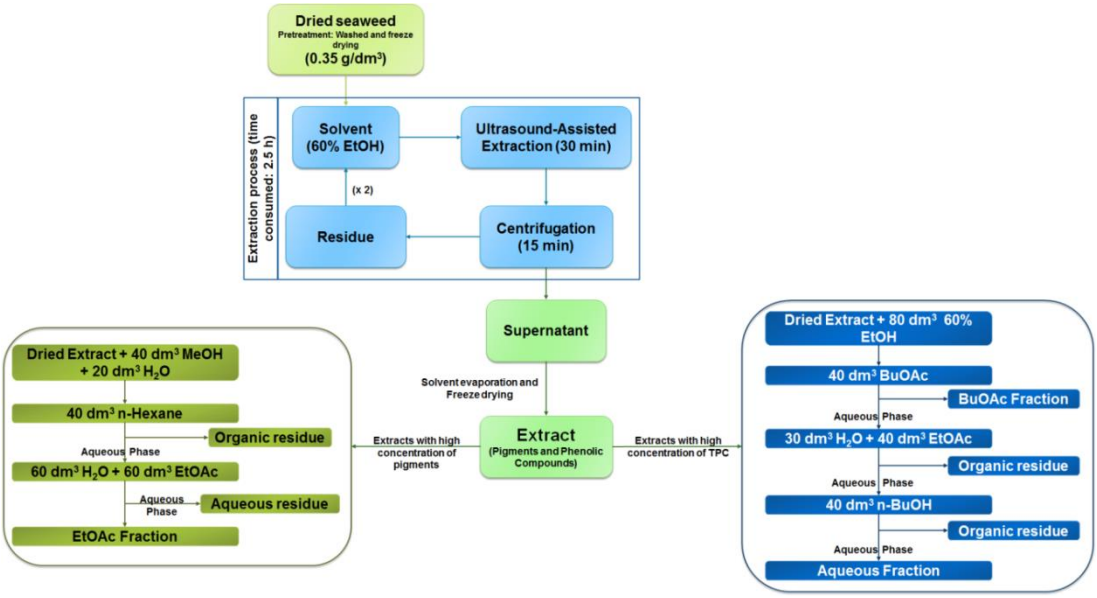

Figure 5.1 Diagram of extraction process of phenolic compounds and fucoxanthin from seaweed

5.1.6 Total Phenolic compounds (TPC) quantification

TPC content in the crude extracts was determined by the colorimetric method with Folin- Ciocalteu reagent [114]. 0.1 cm^3 of sample was mixed with 0.5 cm^3 Folin-Ciocalteu reagent (10% in distilled water, v/v) and after 5 min with 0.4 cm^3 sodium carbonate (7.5% in distilled water, w/v) was added. The absorbance was measured at 725 nm (Jenway 6405 UV/vis spectrophotometer) after 2 h incubation at room temperature in the darkness. Results were expressed as g of gallic acid equivalents (GAE) per 100 g DM.

5.1.7 Pigments quantification

Pigments were determined by HPLC analysis using a HPLC (Thermo Scientific Dionex UltiMate 3000, United States) with DAD and an Eclipse Plus C8 column

(3.0 x 150 mm, 1.8 µm; Agilent Technologies) at 60 ºC, using two eluents: (A) methanol/TBAA pH 6.5 (70/30) and (B) methanol. Chromatographic elution was carried out at a flow rate of 0.30 cm^3/min with the following elution profile of eluent B: 0 – 45 min, 5%; 45 – 60 min, 95%; 60 min. Standards and extracts were dissolved in 90% acetone and filtered through 0.22 µm syringe filters. 28 mM TBAA pH 6.5 is used as injection buffer and added to all samples and standards at a 1:3 sample:buffer ratio. Injection volume was 0.090 cm^3. Fucoxanthin (Fxn) and chlorophyll c_2 (Chl c_2) were identified and confirmed based on comparing retention times with standards (Figure 5.2) and on-line ultraviolet absorption spectrum data. The relative concentrations at different wavelengths (450 and 665 nm) were calculated from the calibration curves. Results are expressed as mg of pigment per 100 g of DM.

5.1.8 Screening of seaweed extracts for antioxidant activities

The antioxidant activity of the seaweed extracts obtained was tested using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and Iron (Fe^{2+}) chelating assays [105]. For the DPPH radical scavenging activity, 0.350 cm^3 of extract was mixed with 0.350 cm^3 of DPPH solution (0.1 mM in 96% ethanol). The absorbance (A_s) was measured at 517 nm (Jenway 6405 UV/vis spectrophotometer) after 30 min incubation at room temperature in the darkness. A blank (A_b) was treated in the same way using distilled water instead of sample. For each extract a sample control (A_c) was run using ethanol instead of DPPH. BTH (200 µg/cm^3) and used as the reference standard.

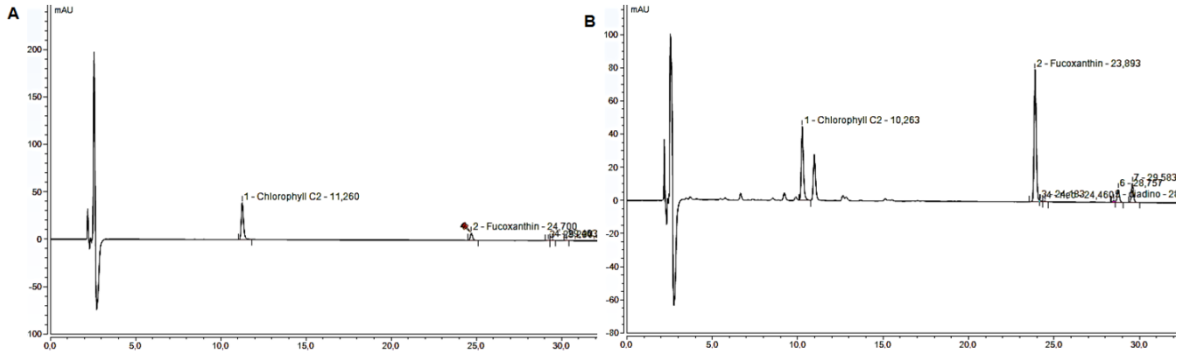

Figure 5.2 HPLC chromatogram: (A) standard pigments, (B) extraction under optimized conditions (60% EtOH aqueous, 0.35g/dm3 DM, three extractions, and 30 min of extraction time)

In the case of chelating activity, 0.250 cm^3 of the extract were added to 0.675 cm^3 of deionized water and 0.025 cm^3 of 2 mM ferrous chloride. After 3 min, 0.100 cm^3 of 5 mM ferrozine was added; the mixture was left at room temperature in the darkness for 10 min. The absorbance (A_s) of the resulting solution was measured at 562 nm using a spectrophotometer (Jenway 6405 UV/vis spectrophotometer). A blank (A_b) was treated in the same way using distilled water instead of sample. For each extract a sample control (A_c) was made without adding ferrozine. EDTA (200 µg/cm^3) was used as the reference standard. The antioxidant activities were calculated as follows,

$$\text{Antioxidant activity } (\%) = \left[1 - \frac{A_s - A_c}{A_b}\right] \times 100$$

The concentration of extracts required to get 50% antioxidant activity, the so-called half maximal effective concentration (EC_{50}) was also calculated.

5.1.9 Statistical analysis

Pearson correlation coefficient (Origin® 9) was used to determine the correlation between Y and TPC from each solvent mixture (D-optimal experimental design), using a confidence level of 95%.

An analysis of *t*-student (Origin® 9) was used to determine if the EC_{50} obtained by each solvent mixture (D-optimal experimental design) had significance difference ($p < 0.05$) from each other.

Analysis of variance (ANOVA), the optimum solvent mixture, RSM and the optimum conditions were performed using Design-Expert® Version 7.0 (Stat-Ease, Inc.). ANOVA F test was used to assess the adjusted models. The significance of each coefficient was determined with the *t*-test with a *p*-value less than 0.05.

5.2 Results and Discussion

5.2.1 Optimization of solvent mixture for total polyphenolic compounds (TPC) extraction and antioxidant activity

From Table 5.1 is observed that the solvent mixture giving the highest TPC content (120.2 ± 0.6 mg GAE/100 g DM) corresponded to 60 EtOH:40 H_2O% (v/v) with an extraction yield (%, g extract/100 g DM) of 14.6%. According to Pearson correlation coefficient, no correlation between yield (Y) and TPC content (mg GAE/100 g DM) was established. This means that the yield and TPC are not proportional. In Table 5.3 are presented the mathematical models proposed for Y and TPC content as well as its respective *p*-values for each model and mathematical terms.

Table 5.3 Mathematical models, *p*-values, predictions and experimental values with optimum solvent mixture found with D-optimal experimental design.

Mathematical model

Response variable	Equation
Y (%, g extract/100 g DM)	$Y = 0.16624\times H_2O + 0.041159\times EtOH + 2.12120E\text{-}003\times H_2O\times EtOH - 2.31396E\text{-}005\times H_2O\times EtOH\times(H_2O-EtOH)$
TPC (mg GAE/100 g DM)	$TPC = 0.76587\times H_2O + 0.089935\times EtOH + 0.026705\times H_2O\times EtOH - 2.34640E\text{-}004\times H_2O\times EtOH\times(H_2O-EtOH)$

Statistics

Source	*p*-value Response variable	
	Y	TPC
Model	0.0017	0.0008
Linear Mixture	0.0005	0.0041
$H_2O\times EtOH$	0.1037	0.0008
$H_2O\times EtOH\times(H_2O-EtOH)$	0.0732	0.0189

Optimization

Response variable	Prediction	95% CI low	95% CI high	Experimental value
Y (%, g extract/100 g DM)	15.3	14.1	16.6	19.3 ± 0.4
TPC (mg GAE/100 g DM)	111.5	102.4	120.6	130.0 ± 3.6

CI: Confidence interval

As observed, extraction yield was only influenced ($p < 0.05$) by the linear mixture and the TPC by all terms of the mathematical model. This implies that, (1) the extraction yield increases with the water percentage in the mixture solvent, and (2) concentration of phenolic compounds have a maximum value at specific conditions (see experiment 10, Table 5.1). According to the ANOVA (data not shown) the optimal solvent mixture extraction is 60% EtOH:40% H_2O (v/v). Figure 5.3 shows the half-maximal effective concentration (EC_{50}) as a function of the solvent mixture. The highest DPPH EC_{50} and Iron (Fe^{2+}) chelating activity EC_{50} (4.3 ± 0.2 g/dm^3 and of 3.5 ± 0.1 g/dm^3, respectively) were observed when a mixture of 60% EtOH:40% H_2O (v/v) was used as extraction solvent. To validate the predicted results further experiments with *S. latissima* and using the optimal solvent

extraction mixture were conducted in triplicates obtaining 19.3 ± 0.4% of extraction yield, 130.0 ± 3.6 mg GAE/100 g DM, and DPPH EC_{50} of 4.4 ± 0.1 g/dm^3. The results indicated that the response variables were optimized correctly due to that the experimental values obtained of each variable were within the confidence interval.

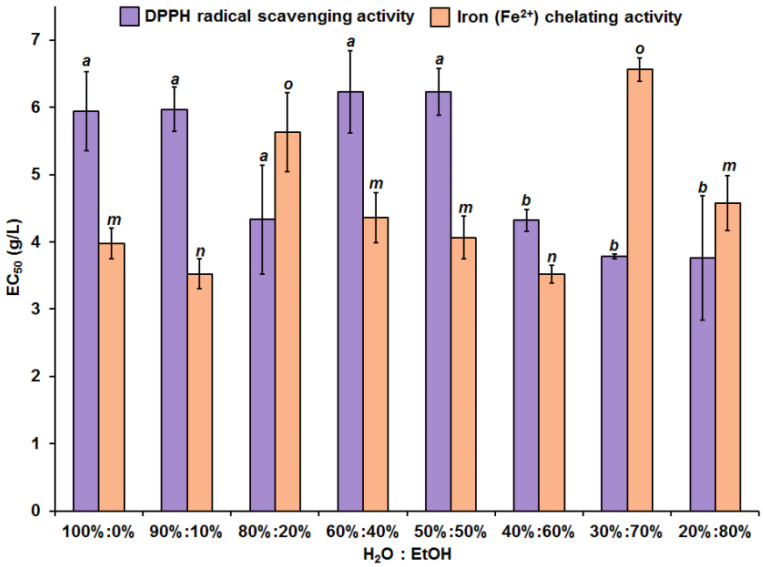

Figure 5.3 Half maximal effective concentration (EC_{50}) obtained using D-optimal experimental design. Letters *a*, and *b* indicates if there is significant difference ($p < 0.05$) in DPPH radical scavenging activity EC_{50} in terms of the solvent mixture. Letters *m*, *n* and *o* indicates if there is significant difference ($p < 0.05$) in Iron (Fe^{2+}) chelating activity EC_{50} in terms of the solvent mixture.

Extraction yield and TPC reported in this work were similar compared to other studies. For instance, the extraction solvent (or solvent mixture) affected the extraction yield, TPC extraction and antioxidant activity. In the extraction of TPC from *Stypocaulon scoparium* using water, water/methanol, methanol and ethanol [115] were obtained extraction yields between 2% and 17%. TPC and the

antioxidant activity were greater in the order of water>water/methanol>methanol>ethanol. The extraction of phenolic compounds from *Himanthalia elongate* was performed using water, methanol and mixtures (20–80%) [116], the highest extraction yield, TPC content and antioxidant activity were achieved with 60% methanol aqueous. Phenolic compounds from four kinds of seaweed were extracted [117] with water, ethanol/water (80:20% (v/v)) and acetone/water (80:20% (v/v)). The highest extraction yield was obtained with water, whereas the highest TPC and antioxidant activity were achieved with acetone/water. In the TPC extraction from *S. latissima* using water and ethanol [105] were obtained the highest extraction yield and antioxidant activity with water, whereas the highest TPC was achieved with ethanol. The content of TPC reported by them was lower (35.4 mg GAE/100 g DM) than the one obtained in this work, but the antioxidant activity (DPPH EC_{50}, 1.5 g/dm^3) was higher than the one observed in the present study. Although some authors mention that the higher the TPC concentration the higher the antioxidant activity of the extract [108, 114, 115]; it is important to consider that in the extractions either with a solvent mixture or water, not only phenolic compounds are extracted; but also some other components such as pigments and some polysaccharides that might contribute to boost the antioxidant activity of the extracts [104, 118, 119].

5.2.2 Optimization of ultrasound-assisted extraction

The advantages of the ultrasound-assisted extraction, compared to the conventional extraction methods, are lower solvent consumption, shorter extraction time, lower energy input and simplified manipulation; and has been applied to

extract bioactive compounds from different materials owing to its high reproducibility [108, 120, 121]. For maximizing the antioxidant activity, total polyphenolic compounds (TPC) and fucoxanthin (Fxn) contents, the effects of the sample concentration, number of extractions and extraction time were evaluated (Table 5.2). Central points (experiments 1, 10, 15, 16 and 17) of the Box-Bennken experimental design correspond to 20.2 ± 0.6% of extraction yield, 158.1 ± 7.9 mg GAE/100 g DM, 13.4 ± 3.4 mg Fxn/100 g DM and 43.7 ± 4.0% of DPPH. In Table S2 is shown the mathematical models that represent the response variables as a function of the independent variables (sample concentration, number of extractions and extraction time) in the experimental region.

According to the ANOVA the terms of the mathematical models with effect on the response variables had a *p*-value < 0.05 (Table 5.4). With the RSM, contour and response surface plots for TPC, Fux and DPPH were obtained (Figures 5.4 – 5.6). From the plots it can be revealed that high concentrations of TPC (Figure 5.4) and Fxn (Figure 5.5) could be obtained using sample concentration between 0.05 and 0.275 g/dm^3, in two to three extractions with extraction time of at least 20 minutes. Maximum antioxidant activity (Figure 5.6) was found approximately to be in a range of 0.275 to 0.5 g/dm^3 of sample concentration in at least two extractions for an extraction time of 27.5 to 30 minutes. Increasing extraction time and number of extractions, as well as, keep sample concentration near to the central level leads to maximize DPPH, which proves that the evaluated parameters affect the antioxidant activity. Hence, from these results, TPC, Fxn and DPPH radical scavenging were optimized to get the optimum values of each independent variable that maximize the antioxidant activity. According to the mathematical models and the RSM (Table

5.4) the maximum concentrations of TPC and Fxn, and the highest DPPH; can be attained using 0.275 g/dm^3 of sample concentration in three extractions of 30 min each.

The maximum values predicted by the mathematical models were 226.0 mg GAE/100 g DM, 28.9 mg Fxn/100 g DM and 54.8% of DPPH. To verify the predicted results, additional experiments were performed in triplicates using the optimized conditions obtained with the “mixture problem” approach and the RMS. The results obtained (Table 5.4) were 209.6 ± 4.5 mg GAE/100 g DM, 31.7 ± 2.7 mg Fxn/100 g DM and 52.5 ± 5.7% of DPPH; and also Chl c_2 was detected (15.1 ± 0.3 mg/100 gDM). The experimental values of independent variables are within the confidence interval; indicating that the response variables were optimized successfully. Besides, DPPH EC_{50} was reduced from 4.5 ± 0.2 to 2.6 ± 0.2 g/dm^3, meaning that at the optimized operational conditions using 60% EtOH aqueous the antioxidant activity is enhanced.

Table 5.4 Mathematical models, p-values, predictions and experimental values with optimum solvent mixture found with Box-Behnken experimental design

Mathematical model

Response variable	Equation	R^2
TPC (mg GAE/100 g DM)	TPC = 84.81214 – 3.47647×Sample – 4.57227×Time + 110.50978×Extraction – 0.041990×Sample×Time – 0.16948×Sample×Extraction + 1.34498×Time×Extraction + 0.047753×Sample^2 + 0.13645×Time^2 – 18.17937×Extraction^2	0.990
Fucoxanthin (mg/100 g DM)	Fxn = 3.30309 + 0.24335×Sample – 1.89103×Time + 13.82992×Extraction – 9.55752E-003×Sample×Time – 0.086867×Sample×Extraction + 0.32482×Time×Extraction + 3.77460E-004×Sample^2 + 0.053266×Time^2 – 2.75484×Extraction^2	0.937
DPPH radical scavenging (%)	DPPH = 27.60947 + 0.26437×Sample – 2.36486×Time + 23.86447×Extraction + 0.073429Time^2 – 5.57163×Extraction^2	0.764

Statistics

Source	*p*-value Response variable		
	TPC	Fxn	DPPH
Model	< 0.0001	0.0020	0.0034
Sample	< 0.0001	0.0723	0.0066
Time	0.0001	0.0006	0.0083
Extraction	< 0.0001	0.0004	0.3950
Sample×Time	0.0716	0.1992	-
Sample×Extraction	0.4202	0.2385	-
Time×Extraction	0.0194	0.0695	-
Sample^2	0.0008	0.9008	-
Time^2	0.0163	0.0087	0.0122
Extraction^2	0.0041	0.1047	0.0443

Optimization

Response variable	Prediction	95% CI low	95% CI high	Experimental value
TPC (mg GAE/100 g DM)	226.0	206.2	245.9	209.6 ± 4.5
Fucoxanthin (mg/100 g DM)	28.9	22.1	35.6	31.7 ± 2.7
DPPH radical scavenging (%)	54.8	47.1	62.4	52.5 ± 5.7

CI: Confidence interval

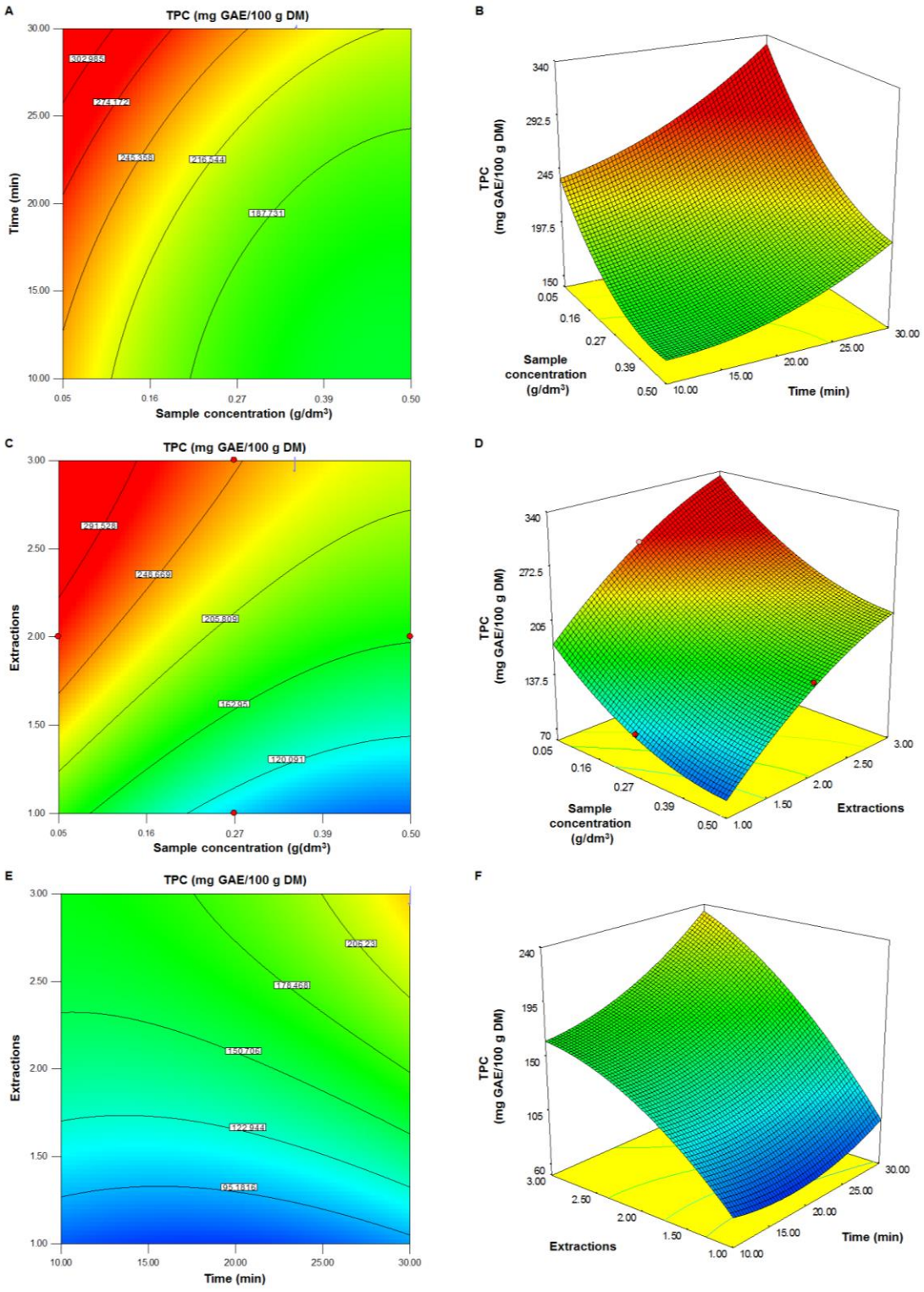

Figure 5.4 Contour and response surface plots of total phenolic compounds content under optimized conditions (60% EtOH aqueous, 0.35 g/dm^3 DM, three extractions, and 30 min of extraction time). Number of extractions was fixed at three in A and B, time was fixed at 30 min in C and D, sample was fixed at 0.35 g/dm^3 DM in E and F.

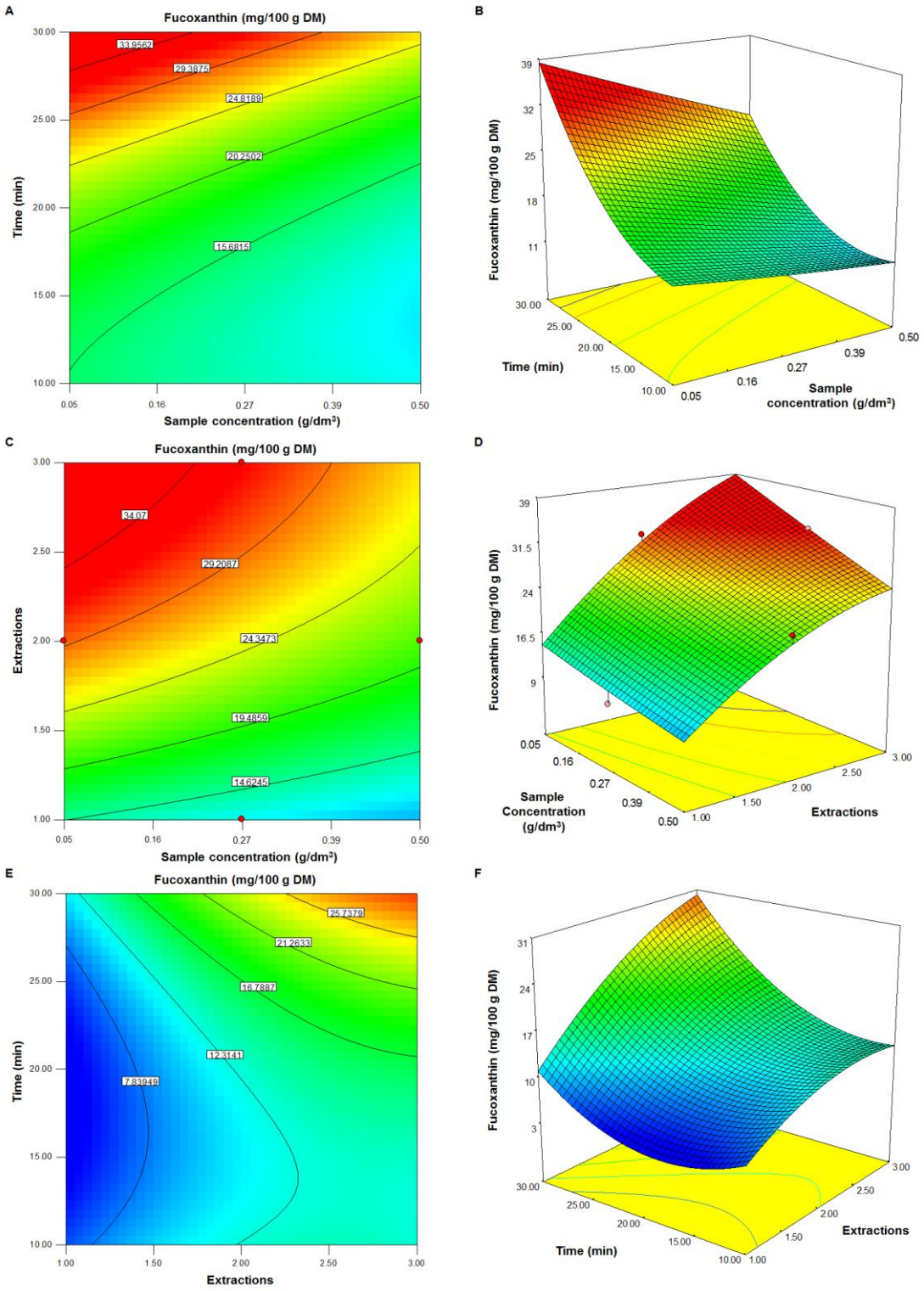

Figure 5.5 Contour and response surface plots of fucoxanthin content under optimized conditions (60% EtOH aqueous, 0.35 g/dm^3 DM, three extractions, and 30 min of extraction time). Number of extractions was fixed at three in A and B, time was fixed at 30 min in C and D, sample was fixed at 0.35 g/dm^3 DM in E and F.

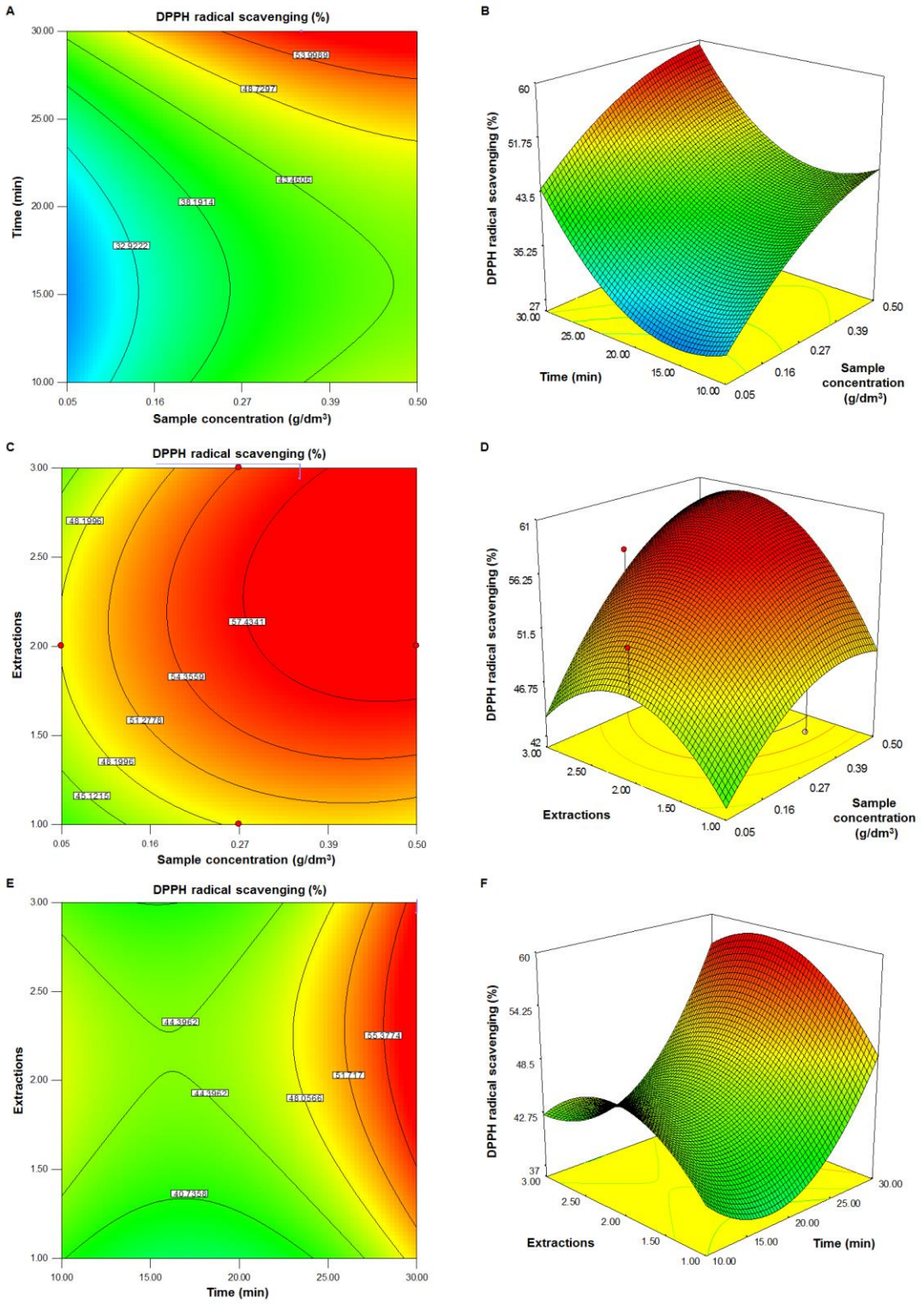

Figure 5.6 Contour and response surface plots of DPPH radical scavenging activity under optimized conditions (60% EtOH aqueous, 0.35 g/dm^3 DM, three extractions, and 30 min of extraction time). Number of extractions was fixed at three in A and B, time was fixed at 30 min in C and D, sample was fixed at 0.35 g/dm^3 DM in E and F.

5.2.3 Extraction of antioxidants in optimized conditions

Based on the optimized extraction process depicted in Figure 5.1, phenolic compounds, fucoxanthin and chlorophyll c_2 were extracted from *Saccharina latissima* (SL), *Laminaria digitata* (LD), *Fucus evanescens* (FE), *Fucus vesiculosus* (FV) and *Fucus serratus* (FS). The antioxidant activity of crude extract was found to increase in the order of FE, FV, FS > LD > SL. The crude extracts from *Fucus* species at 100 mg extract/dm^3 show a DPPH radical scavenging activity over the range of 66-84%, also these species had 2000 < TPC < 3050 mg GAE/100g DM. Extraction of bioactive compounds and their radical scavenging capacity have been reported for other seaweed species. For instance, phenolic compounds extracted from *H. siliquosa*, *F. serratus*, *A. bullosus* [122], *E. prolifera* [119], *F. vesiculosus* [123] and fucoxanthin extracted from *U. pinnatifida* [124] showed a DPPH EC_{50} between 7.9 µg/cm^3 and 20.2 g/dm^3. Also, works in which both phenolic compounds and fucoxhanthin were extracted from *S. horneri* [125] and *F. vesiculosus* [126] were reported with high antioxidant activities. Even when the antioxidant activity of the seaweed extracts obtained in this study was high (DPPH EC_{50} ranging from 100 mg/dm^3 to 2500 mg/dm^3; and Fe^{2+} EC_{50} ranging from 4 g/dm^3 to 7.6 g/dm^3), we decided to perform the fractionation of the crude extracts in order to study whether or not this might result in a positive effect on the antioxidant activity [104, 127, 128]. The crude extracts (CE) obtained at optimal extraction conditions were dissolved in water and characterized (TPC and pigments contents) to identify first the main bioactive compound(s). To select suitable alternative solvents for the liquid-liquid partitioning an approach for selection solvents was followed [129], using the polarity as search criteria (physicochemical properties

[130, 131]). As described earlier, butyl acetate, ethyl acetate, n-butanol and hexane were used to partitioning the crude extracts (Figure 5.1, Section 5.2.4).

Laminaria digitata-crude extract (LD-CE) was not considered for further fractioning because had the lowest content of TPC and pigments. From crude extracts of *F. evanescens*, *F. vesiculosus* and *Fucus serratus* were obtained two fractions rich in phenolic compounds, one organic and one aqueous, respectively; whilst a fraction rich in fucoxanthin and chlorophyll *c*2 was obtained from crude extract of *S. latissima* (Table 5.5). The highest DPPH radical scavenging was obtained by FV-butyl acetate fraction while FV-aqueous fraction showed the highest Iron (Fe^{2+}) chelating activity. *F. evanescens*, *F. vesiculosus* and *Fucus serratus* (crude extracts and fractions) showed higher antioxidant activity than SL-ethyl acetate fraction. The references (BTH and EDTA) used had higher antioxidant activity than the seaweed extracts (crudes and fractions).

Several studies have tested and documented the importance and benefits of the antioxidants properties of seaweed extracts. For example, tropical seaweeds from the Brazilian coast, *Amansia sp.*, *Bostrychia tenella*, *Cryptonemia seminervis*, *Hypnea musciformis*, *Plocamium brasiliense* (1), and *S. clavata*; showed more than 60% of inhibition of DPPH at 10 mg extract/dm^3 [132]; the methanol extract from another Brazilian seaweed (*Spatoglossum schroederi*) produced a DPPH radical scavenging activity close to 53% at 1500 mg extract/dm^3 [133]. In a study, in which the extraction of the bioactive compounds from *Gracilaria changii* (Santubong, Sarawak, Malaysia) was tested with different solvents [134], a DPPH EC_{50} of 51 mg extract/dm^3 for the ethyl acetate extract is reported. Besides, was reported acetone extracts with DPPH EC_{50} (16.9 g extract/dm^3) of *Padina durvillaei* from

Table 5.5 Antioxidant activity of seaweed extracts (crudes and fractions)

Seaweed	Standard or Extract*	Y (%, g extract/100 g DM)	Weigh (mg)	TPC (mg GAE/100 g DM)	Fxn (mg/100 g DM)	Chl-c_2 (mg/100g DM)	DPPH EC_{50} (mg extract/dm^3)	Fe^{2+} EC50 (g extract/dm^3)
-	BTH	-	-	-	-	-	11.6 ± 0.1	-
-	EDTA	-	-	-	-	-	-	0.1 ± 0.0
Saccharina latissima	SL-CE	24.1 ± 0.5	-	209.6 ± 4.5	31.7	15.1	2518.1 ± 50.7	7.4 ± 0.8
	SL-EtOAc	-	236.9	22.0 ± 0.2	14.0	13.0	236.5 ± 12.2	8.5 ± 0.4
Laminaria digitata	LD-CE	25.8 ± 0.6	-	41.6 ± 0.4	0.4	0.9	1523.7 ± 14.5	4.2 ± 0.1
Fucus evanescens	FE-CE	23.9 ± 0.8	-	2041.2 ± 13.2	2.7	2.3	< 100 (at 100 mg/dm^3 showed 66,4% of activity)	4.2 ± 0.1
	FE-BuOAc	-	858.7	599.7 ± 418.8	-	-	103.8 ± 3.2	8.3 ± 1.4
	FE-Aq	-	526.0	386.4 ± 2.0	-	-	< 50 (at 50 mg/dm^3 showed 52.3% of activity)	4.1 ± 0.1
Fucus vesiculosus	FV-CE	20.4 ± 0.3	-	3027.9 ± 24.7	3.0	2.5	< 100 (at 100 mg/dm^3 showed 84.1% of activity)	4.2 ± 0.1
	FV-BuOAc	-	472.9	607.6 ± 8.9	-	-	< 25 (at 25 mg/dm^3 showed 52.7% of activity)	7.5 ± 0.9
	FV-Aq	-	502.9	549.3 ± 4.9	-	-	< 50 (at 50 mg/dm^3 showed 57.6% of activity)	6.3 ± 0.2
Fucus serratus	FS-CE	24.1 ± 1.0	-	2661.7 ± 48.9	3.9	2.7	< 100 (at 100 mg/dm^3 showed 76.0% of activity)	7.6 ± 1.3
	FS-BuOAc	-	917.3	973.1 ± 26	-	-	67.8 ± 0.8	6.0 ± 0.5
	FS-Aq	-	669.7	1115.3 ± 12.4	-	-	< 50 (at 50 mg/dm^3 showed 55.1% of activity)	5.9 ± 0.3

*CE: crude extract; EtOAc: ethyl acetate fraction; BuOAc: butyl acetate fraction; Aq: aqueous fraction.

Sinaloa, Mexico[135]. *Fucus serratus* (Iceland) extracts, at 150 mg extract/dm^3, showed a DPPH radical scavenging of 88.7% and iron chelating activity of 20.8% [136]. While in this work, crude extracts of FE, FV and FS exhibited a DPPH EC_{50}<100 mg/dm^3. SL-crude extract showed lower antioxidant activity (DPPH EC_{50}: 2518 mg/dm^3) than all kind of seaweeds tested in this study. After partitioning of the crude extracts the antioxidant activity increased. The DPPH EC_{50} of SL-ethyl acetate fraction was ten times lower than *S. latissima* crude extract but the chelating activity was almost similar. The DPPH EC_{50} of butyl acetate fractions of FE, FV and FS ranging from 25 mg/dm^3 and 105 mg/dm^3. Finally, aqueous fractions obtained from FE, FV and FS exhibited a DPPH EC_{50}<50 mg/dm^3.

It was mentioned that the solvent affect the extraction of bioactive compounds from seaweed and therefore the antioxidant activity, but not only the extraction conditions have an effect on the effectiveness of the extracted compounds. Seasonal and spatial variations in biochemical composition of *S. latissima* and *L. digitata* also affect the antioxidant activity. Marinho *et al.* [137] and Nielsen *et al.* [138] found the highest content of bioactive compounds in July-August; and the highest biomass production potential, the highest protein content, and the highest capacity for bioremediation of nitrogen at high salinities, whilst the highest concentrations of fermentable sugars and pigments at low salinities. Therefore the kind of seaweed, the TPC or Fxn content, harvesting time and the geographical location are factors that need to be considered to maximize the extraction of these bioactive compounds and consequently the antioxidant activity.

In general, we did not find any correlation between TPC or pigments concentration and the antioxidant activity. In our study, using the optimized extraction process the

content of the antioxidant compounds was increased, this was observed in the increase of the antioxidant activity. Even though the bioactivity of the extracts was maximized further work is necessary for a detailed identification and quantification of the different phenolic compounds in seaweed extracts thereby targeting the ones exhibiting high antioxidant activity.

5.3 Summary

The optimization of ultrasound-assisted extraction of phenolic compounds and fucoxanthin from macroalgae with low solvent consumption (60% EtOH aqueous), short extraction time, and low energy consumption is presented. Successfully the extraction of antioxidant compounds was increased. Under optimized conditions extracts rich in phenolic compounds and fucoxanthin from five kinds of seaweeds were obtained; its antioxidant activity increased in the order of *F. evanescens*, *F. vesiculosus*, *F. serratus* > *L. digitata* > *S. latissima*. Although the bioactivity of the extracts was maximized further work is necessary to test other effects like antimicrobial, anti-virulence or anticancer which has been attributed to seaweed extracts.

6 Antimicrobial, anti-virulence and anticancer activities of pigments and polyphenols from seaweed

Marine macroalgae (seaweed) is more than the wrap that keeps rice together in sushi. Seaweed biomass is already used for a wide range of other products in food, including stabilising agents. Seaweeds are rich in bioactive compounds that could potentially be exploited as functional ingredients for both human and animal health applications. Despite the intensive efforts that are being made to isolate and identify new compounds with potential medicinal, health or pharmaceutical activities, very few compounds with real potency are available. Bioactive compounds that are most extensively researched include polysaccharides, proteins, lipids and polyphenols. These compounds have been reported to possess strong anti-viral, anti-tumor and anti-cancer properties. At the same time, the prebiotic health potential of the polysaccharides from seaweeds is also increasingly being studied either by feeding whole seaweeds or purified polysaccharides to laboratory and farm animals [139, 140].

Many reports have been published about isolated compounds from algae with biological activity, demonstrating their ability to produce metabolites however a lot of research is needed before this vast untapped resource could be utilized for beneficial purposes. Thus, the investigation of new algal chemical compounds, a different source of natural products, can prove to be a promising area of pharmaceutical study. In the present study we report the antimicrobial, anti-virulence and anticancer activities of seaweed extracts with high content of pigments or polyphenols.

6.1 Material and methods

6.1.1 Seaweed extracts

Seaweed extracts were obtained by ultrasound-assisted extraction in optimal conditions (60% EtOH aqueous, 0.35 g/cm^3 dry material, three extractions, and 30 min of extraction time) and fractionation according to the Figure 5.1 (Section 5.1.5). In Table 6.1 shown the samples name and descriptions of seaweed extracts used in this study.

Table 6.1 Sample name and descriptions of the seaweed extracts

Sample name †	Description
SL-CE	*Saccharina latissima* crude extract
SL-EtOAC	Ethyl acetate fraction from *S. latissima* crude extract
LD-CE	*Laminaria digitata* crude extract
FE-CE	*Fucus evanescens* crude extract
FE-BuOAc	Butyl acetate fraction from *F. evanescens* crude extract
FE-Aq	Aqueous fraction from *F. evanescens* crude extract
FV-CE	*Fucus vesiculosus* crude extract
FV-BuOAc	Butyl acetate fraction from *F. vesiculosus* crude extract
FV-Aq	Aqueous fraction from *F. vesiculosus* crude extract
FS-CE	*Fucus serratus* crude extract
FS-BuOAc	Butyl acetate fraction from *F. serratus* crude extract
FS-Aq	Aqueous fraction from *F. serratus* crude extract

†CE: crude extract; EtOAc: ethyl acetate fraction; BuOAc: butyl acetate fraction; Aq: aqueous fraction.

6.1.2 Disk diffusion assay

Gram negative bacteria, *Pseudomonas aeruginosa* DSM 1117 and *Escherichia coli* DSM 498, and gram positive bacteria, *Staphylococcus aureus* DSM 2569, *Enterococcus faecalis* DSM 2570, were used as control microorganisms to antimicrobial susceptibility testing. The test was performed by applying a bacterial

inoculum (100 µL, 0.5 OD_{600nm} control microorganisms culture) in the surface of a Mueller-Hinton agar plate. Paper test disks with the bioactive compounds (2 mg extract/disk) are placed on the inoculated agar surface. Plates are incubated for 16–24 h at 37°C prior to determination of results. The zones of growth inhibition around each of the antibiotic disks are measured to the nearest millimetre. The diameter or area of the zone is related to the susceptibility of the isolate and to the diffusion rate of the bioactive compound through the agar medium.

6.1.3 Well plate assay

Staphylococcus aureus 8325-4-derived PC322 (*hla*::*lacZ*), PC203 (*spa*::*lacZ*) and SH101F7 (*rnaIII*::*lacZ)* fusion strains were incorporated in tryptic soy agar (TSA) plates containing erythromycin and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). 2 ml of 10^{-3}-diluted overnight culture (tryptone soya broth) were placed in petri dishes to which 50 mL of TSA (~40°C) containing 150 µg/mL X-gal and 5 µg/mL erythromycin was added and mixed by careful whirling. The plates were dried, and wells were formed with a sterile sharp iron tube/drill, 20 µL of seaweed extracts were added into the wells. The plates were incubated at 37°C until blue colour appeared on the agar.

6.1.4 Selection of chemoresistant colorectal cancer cell

Cell Lines HCT116 cells were obtained from NCI, and LoVo cells were purchased from the American Type Culture Collection. The cells were cultured in RPMI 1640 medium with glutamax and 10% fetal calf serum Invitrogen (Nærum, Denmark) at 37 °C and 5% CO2. Oxaliplatin was produced by Sanofi-Aventis (Paris, France),

and SN38 was purchased from Sigma-Aldrich (Copenhagen, Denmark). The drugs were prepared at appropriate concentrations immediately prior to use. The oxaliplatin- or SN38-resistant variants were generated by continuous exposure to gradually increasing drug concentrations over a period of 10 months. Prior to the subsequent experiments, the cells were maintained in drug-free growth medium for at least 1 week. The non- and drug-resistant cell lines were confirmed using short tandem repeat DNA analysis by IdentiCell (Authentication Service, Aarhus University Hospital, Aarhus, Denmark).

6.1.5 Seaweed extract sensitivity analysis and MTT assay

In vitro seaweed extract sensitivity was determined using a standard MTT (methylthiazolyldiphenyl-tetrazolium bromide) assay (Sigma–Aldrich) according to manufacturer's instructions. Briefly, cells were seeded in 96-well plates, and a range of seaweed extract concentrations was added the following day. Following 48 h of seaweed extracts exposure, the medium was discarded and the plates were incubated with medium containing MTT (0.5 mg/ml, Sigma–Aldrich) for 3 h. Acidified (0.02 M HCl) sodium dodecyl sulphate (20%, Sigma–Aldrich) was added to dissolve the formed formazan. Optical density at 570 nm (and 670 nm for background) was measured, and the cell viability was calculated in percent compared to untreated cells.

6.2 Results and discussion

6.2.1 Antimicrobial activity

Due to the emergence of antibiotic-resistant bacteria and the increased incidence of food-borne disease there is a demand for novel antimicrobials for clinical, veterinary and food applications. Unusual marine environments are associated with chemical diversity, leading to a resource of novel active substances for the development of bioactive products. Marine organisms are important sources of bioactive molecules that have been used to treat various diseases. Marine algae metabolites encompass a wide range of substances with diverse biological functions, including antibacterial, antiviral or antifungal activity, and as a result are often the focus of screening to identify novel antimicrobial agents [141, 142].

The antimicrobial activity of seaweed extracts was tested by the exposition of four control microorganism to seaweed extracts.

Table 6.2 show the inhibitory spectra of seaweed extracts (crude and fractions). According to the results the seaweed extracts show higher antimicrobial activity against gram positive bacteria. 58% of the seaweed extracts showed moderate and high antimicrobial effect in gram positive bacteria, whereas only the 42% inhibit growth of gram negative bacteria. Extracts from *S. Latissima* (SL-CE and SL-EtOAc) contain high concentrations of fucoxanthin and chlorophyll c_2, on the other hand, extracts from *F. evanescens* (FE-CE, FE-BuOAc and FE-Aq), *F. vesiculosus* (FV-CE, FV-BuOAc and FV-Aq) and *Fucus serratus* (FS-CE, FS-BuOAc and FS-Aq) show high concentrations of total phenolic compounds (See Table 5.5, section 5.2.3), indicating that pigments and polyphenols from macro algae have antimicrobial potential activity.

Table 6.2 Inhibitory spectra of bioactive compounds from seaweeds

Sample name	Control microorganism for antimicrobial susceptibility testing (disk diffusion assay)*			
	Pseudomonas aeruginosa DSM 1117	*Escherichia coli* DSM 498	*Staphylococcus aureus* DSM 2569	*Enterococcus faecalis* DSM 2570
SL-CE	-	-	-	-
SL-EtOAC	-	-	++++	++
LD-CE	-	+++	+/-	-
FE-CE	-	+/-	+	-
FE-BuOAc	-	+/-	+/-	-
FE-Aq	-	+	+	++
FV-CE	-	++	+	++
FV-BuOAc	-	-	-	-
FV-Aq	++	+++	++	++
FS-CE	-	+++	++	+++
FS-BuOAc	-	+/-	+	++
FS-Aq	-	++++	++++	++++

*Average area (mm^2) of zones of inhibition from triplicate assays. + = 3.1-10; ++ = 10.1-17; +++ = 17.1-24; ++++ = >24; - = no antimicrobial activity; +/- = variable activity

The ethyl acetate fraction from *S. latissima* (SL-EtOAc) inhibits growth *S. aureus* and *E. faecalis* effectively and moderately, respectively. Crude extract from *L. digitata* showed effect only against *E. coli*. Extracts from *F. evanescens* was the less active against control microorganisms. Aqueous fraction from *F. vesiculosus* (FV-Aq) showed moderate effect against all control microorganisms. The crude extract from *F. serratus* (FS-CE) has a moderate antimicrobial effect on *E. coli* and the two gram positive bacteria used in this study, whereas the aqueous fraction (FS-Aq) shown the biggest inhibitory spectra in cultures of *E. coli*, *S. aureus* and *E. feacalis*. Similarly as us, fucoxanthin extracted from brown seaweeds (*Saccharina japonica* and *Sargassum horneri*) inhibit growth gram-positive bacteria (*L. monocytogenes*, *B. cereus*, and *S. aureus*) [143]. Also, phenolic compounds from *Turbinaria ornata* and *Sargassum polycystum* extracts (500 mg/L) exhibited antimicrobial activity against gram-positive bacteria (*S. aureus*) but not against

gram-negative bacteria (*S. enteritidis* and *A. niger*) [144]. On the other hand, polyphenols from *Himanthalia elongata* showed antimicrobial activity against gram-positive (*L. monocytogenes* and *E. faecalis*) and gram-negative bacteria (*P. aeruginosa* and *S. abony*) [116]. Carotenoids and polyphenols from macroalgae have potential application as antimicrobial against gram-positive and gram-negative bacteria.

6.2.2 Anti-virulence activity

Staphylococcus aureus is a human and animal gram-positive pathogen with the capacity of adapted too many environments; is capable to adhere to each other and on surface, generating an extracellular polymeric matrix. In *S. aureus*, the ability to cause disease relies on the timely production of an impressive collection of virulence factors. During exponential growth, the cell surface-located virulence factors are expressed, including *spa*, encoding protein A, while upon entry into stationary phase, transcription of *hla*, encoding α-hemolysin and other genes encoding extracellular factors are induced. This regulation is mediated partly by the *agr* quorum sensing system, composed of a two-component system and a regulatory RNA molecule, RNAIII, that is synthesized in response to increasing concentrations of autoinducer peptide (AIP), also encoded by the *agr* locus [145–148].

The aim of anti-virulence therapy is to silence virulence gene expression, allowing the host immune system time to act and eradicate the pathogen. The anti-virulence activity of the seaweed extracts was tested using the strains PC203 (*spa::lacZ*), PC322 (*hla::lacZ*) and SH101F7 (*rnaIII::lacZ*). The first one when is incubated at

37°C for optimized periods of time, become light blue; the other ones become intensely blue. Figure 6.1 show the ability to the seaweed extracts to modulate *hla* and *spa* expression.

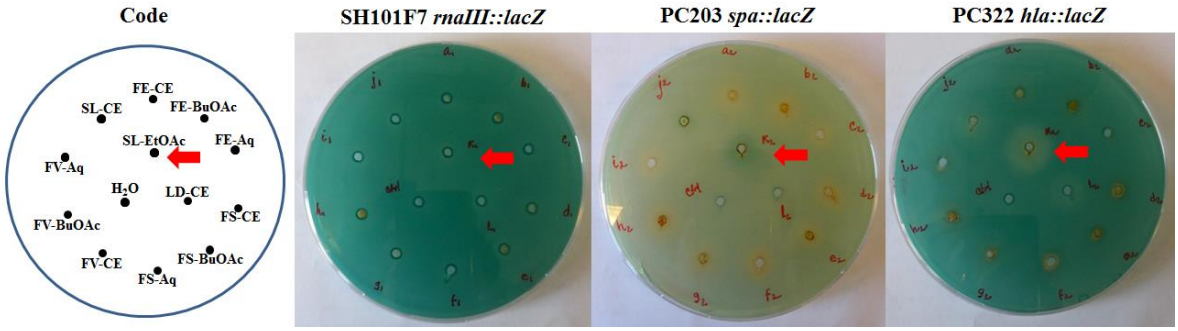

Figure 6.1 Effect of seaweed extracts on virulence gene expression

Ethyl acetate fraction (red arrow) from *S. latissima* crude extract (SL-EtOAc) showed pronounced *agr* inhibition which is visualized by enhanced *spa* expression and decreased *hla* expression as well as repressed expression of RNAIII. The effect on the expression of *spa*, *hla* and *rnaIII* by SL-EtOAc supports that affects virulence gene expression independently of *agr*.

6.2.3 Anticancer activity

Colorectal cancer (CRC), is the third most common cancer in the world affecting >1.36 million people every year arises due to complex interactions between genetic, lifestyle and environmental factors. Chemotherapeutic treatment of metastatic CRC is generally based on the anti-metabolite drug 5-fluorouracil combined with either the DNA-binding agent oxaliplatin or the topoisomerase I inhibitor irinotecan. Notwithstanding the efficacy of these combination regimens,

which have significantly increased the response rate and survival of metastatic CRC patients, only 30–50% of patients show an objective response to either of the combination therapies and progression of the cancer is a common outcome. Thus, resistance to chemotherapeutic drugs is a major clinical problem in the treatment of metastatic CRC [149–152]. For these reason, we decided evaluated the ability of the seaweed extracts to reduce the proliferation of colorectal cancer cells, sensitive and resistant.

The anticancer effect of the extracts seaweeds (crudes and fractions) on colorectal cancer was tested *in-vitro* using HCT116, HT29 and LoVo cell lines. For each of these we have chemotherapy sensitive cells (parental), as well as oxaliplatin resistant and SN-38 resistant derivatives. The graphs (Figures 6.2 – 6.10) show the percentage of proliferation in relation to control no treatment. In green were identified experiments with high standard deviation. In each plot were included the proliferation of solvent controls and control no treatment. The extracts were tested at 100, 500 and 1000 µg extract/mL. Low percentage of proliferation indicated high anticancer activity; extracts with proliferation <40% in all concentrations tested were considered with anticancer activity.

FS-Aq shows anticancer activity in all cell lines but with the highest standard deviation. FS-CE has antiproliferative effect in almost all cell lines except HT29 parental (Figures 6.4) and HT29 SN38 resistant (Figure 6.) with a proliferation (100 µg extract/mL) of 55 and 57 %, respectively. Similar results at 500 and 1000 µg extract/mL were observed in crude extract and fractions obtained from *Fucus serratus*. SL-EtOAc showed anticancer activity in all cell lines at 500 and 1000 µg extract/mL and is quite stable (low standard deviation).

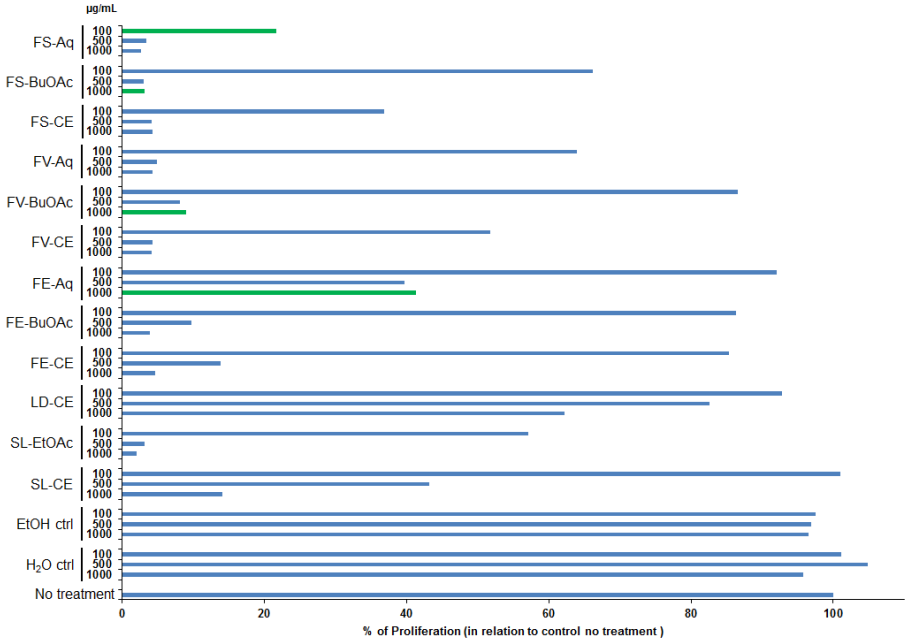

Figure 6.2 Influence of extracts (crudes and fractions) seaweeds on proliferation of HCT116 parental cell line

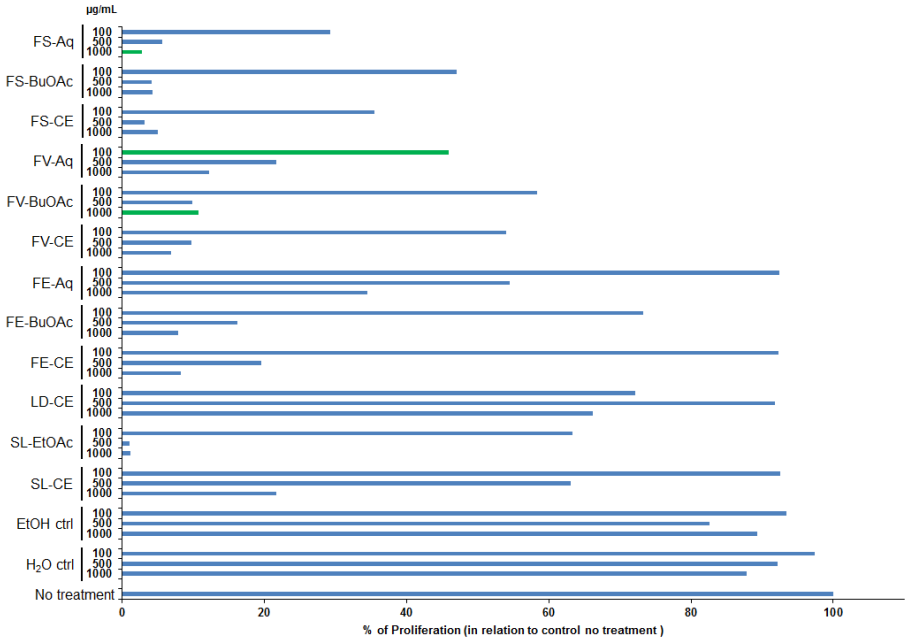

Figure 6.3 Influence of extracts (crudes and fractions) seaweeds on proliferation of HCT116 oxaliplatin resistant cell line

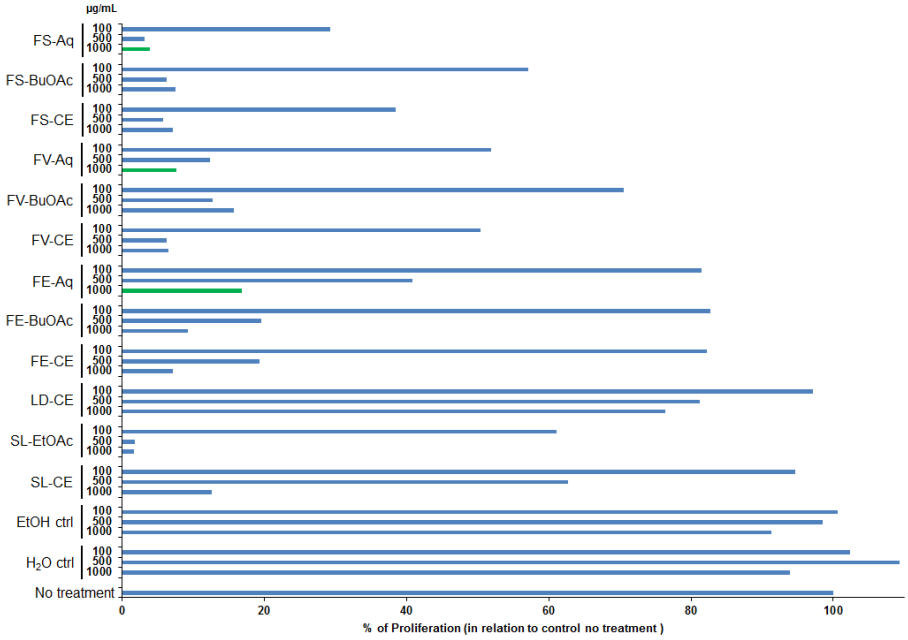

Figure 6.4 Influence of extracts (crudes and fractions) seaweeds on proliferation of HCT116 SN38 resistant cell line.

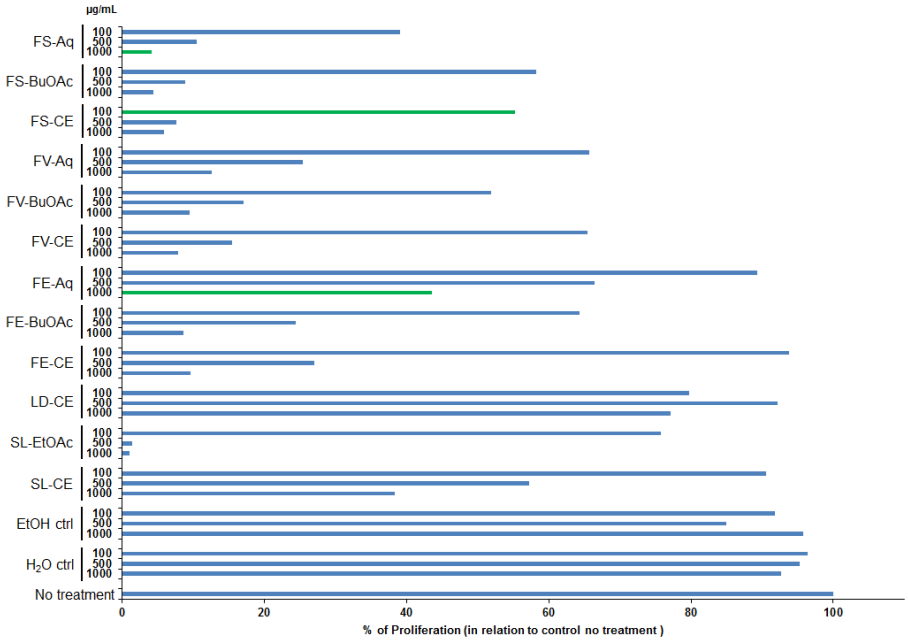

Figure 6.5 Influence of extracts (crudes and fractions) seaweeds on proliferation of HT29 parental cell line.

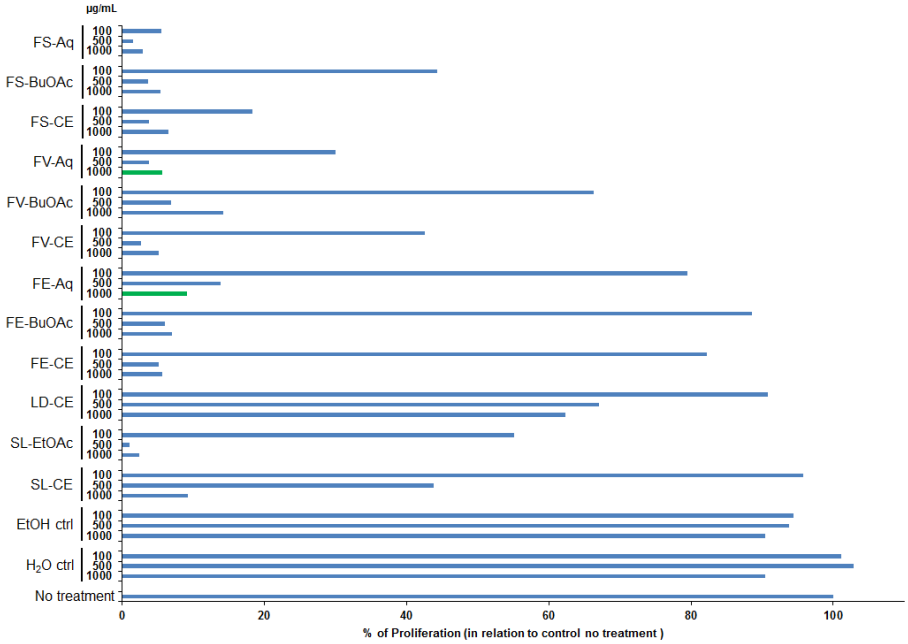

Figure 6.6 Influence of extracts (crudes and fractions) seaweeds on proliferation of HT29 oxaliplatin resistant cell line.

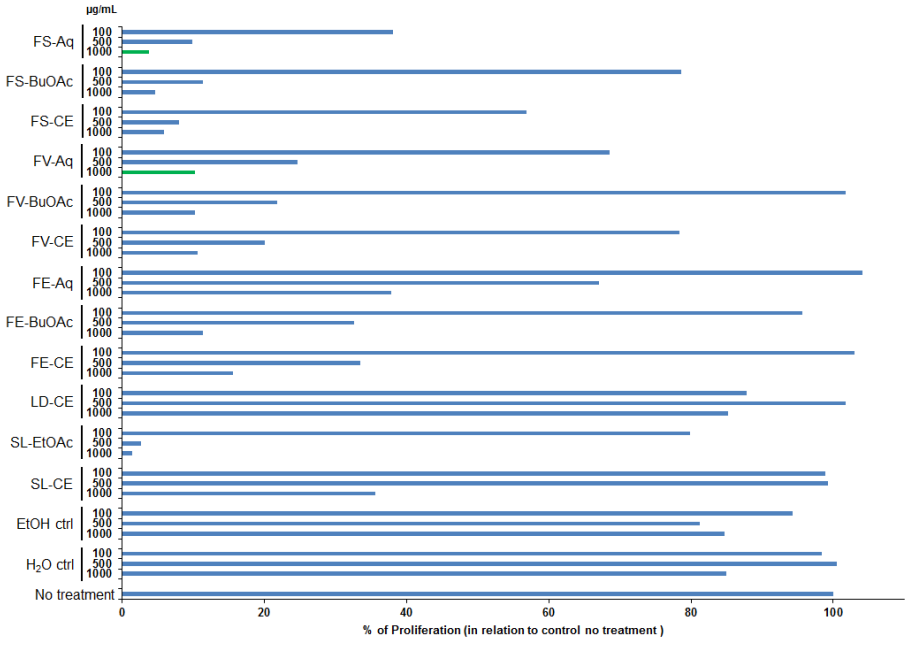

Figure 6.7 Influence of extracts (crudes and fractions) seaweeds on proliferation of HT29 SN38 resistant cell line.

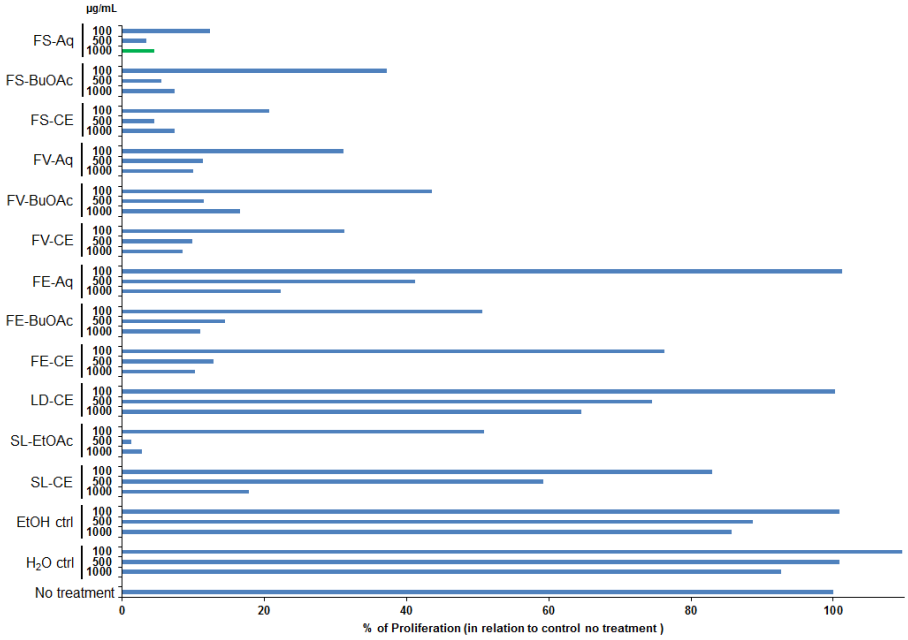

Figure 6.8 Influence of extracts (crudes and fractions) seaweeds on proliferation of LoVo parental cell line.

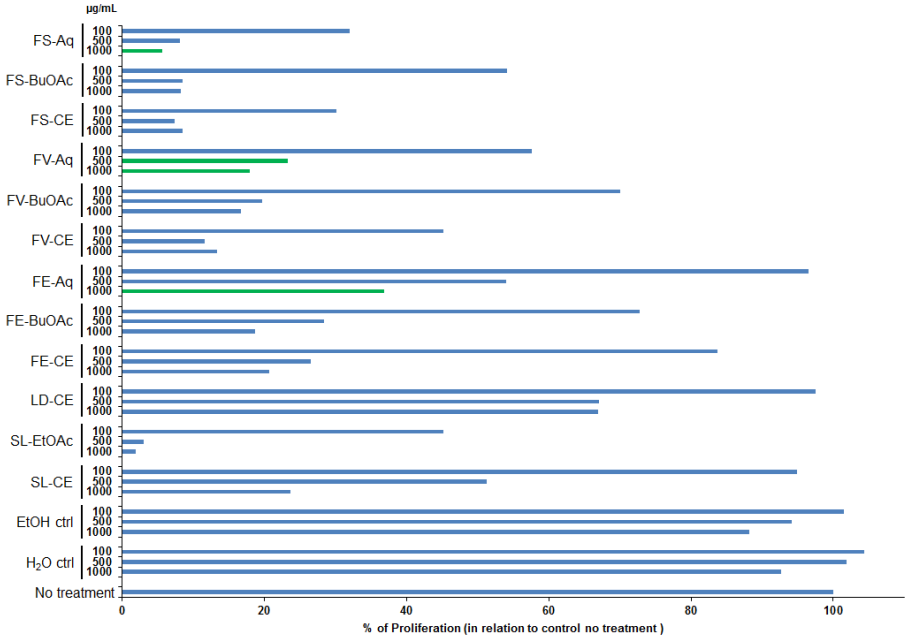

Figure 6.9 Influence of extracts (crudes and fractions) seaweeds on proliferation of LoVo oxaliplatin resistant cell line.

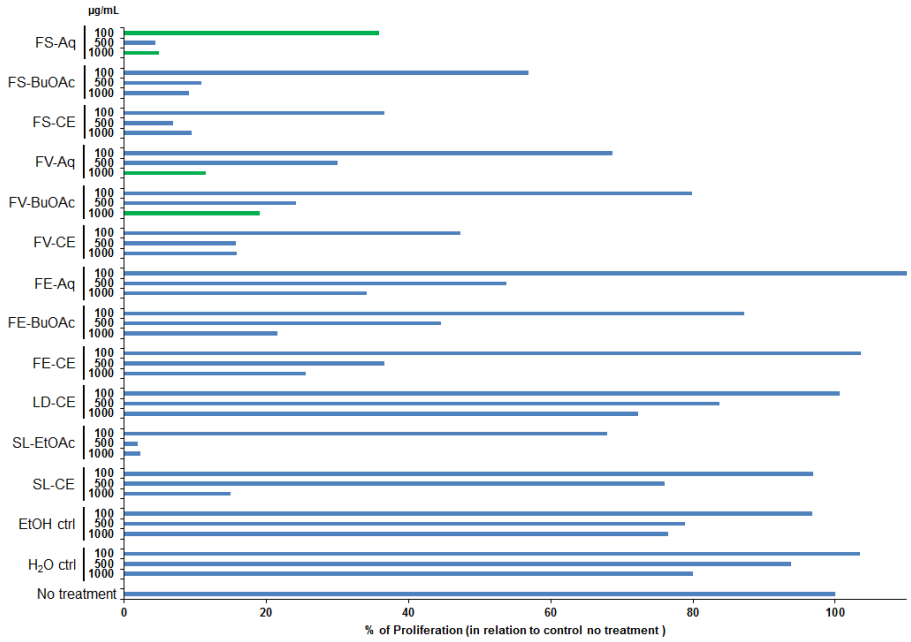

Figure 6.10 Influence of extracts (crudes and fractions) seaweeds on proliferation of LoVo SN38 resistant cell line.

6.3 Summary

The antimicrobial, anti-virulence and anticancer activities of seaweed extracts obtained by an optimized extraction method are presented. Organic and aqueous fractions from seaweeds rich in fucoxanthin and polyphenols could be use as new antimicrobial against gram-positive and gram-negative bacteria. The ethyl acetate fraction from *S. latissima* has potential in the anti-virulence therapy against *S. aureus* due to modulation in the expression of *spa, hla* and RNAIII. Extracts from *F. serratus* and *S. latissima* reduced the proliferation of chemoresistant colorectal cancer cell. The results obtained in this work demonstrate that the seaweed extracts could be use as antimicrobial, anti-virulence or anticancer compounds.

7 Concluding remarks

This thesis focused on the production of bioactive compounds and biofuels from macroalgae and lignocellulosic biomass. The use of different microorganisms and substrates to produce liquid and gaseous biofuels was investigated. Natural products were extracted from seaweed and their potential bioactivities were determinate. The major contributions resulting from these studies are summarized below:

- The demonstration of natural ability to produce hydrogen by psychrophilic microorganisms isolated from Antarctic samples was achieved. The psychrophilic bacteria also produce ethanol from glucose fermentation, which makes then attractive as a potential candidate for further investigation.
- The evaluation of the use mixtures of agro-industrial wastes to generate biofuels by anaerobic granular sludge was performed, and also was demonstrate that the process is industrially scalable.
- The optimization of the simultaneous production of bioethanol and biohydrogen from wheat straw hydrolysate by recombinant *E. coli* strain was achieved. Also, the effect of the working volume in the optimal conditions (31°C and pH 8.2) was demonstrated.
- The extraction of compounds with high added value from marine algae was optimized. Additionally, was determinate their possible use as antioxidants and antimicrobials, or in anti-virulence therapies against *Staphylococcus aureus* or colorectal cancer.

8 Perspectives

This project showed that the second generation biofuels could be produce and scale up using pure and mixed culture, moreover, that the macroalgae biomass could be use as feedstock for new antioxidants, antimicrobials and anticancer compounds. However to improve the utilization of biomass in a more efficient and profitable way further research has to be done. The major suggestions for additional research are reported below:

- Optimization of the fermentation operational conditions to produce biofuels by psychrophilic bacteria, as well as studies of assimilation of different carbon sources such as pentoses, complex carbohydrates, agro-industrial residue.
- Scale up to pilot plant (100 L) the simultaneous production of ethanol and hydrogen by recombinant *E. coli* strains.
- Identify and purify the bioactive compounds extracted from seaweed and identify their action mechanism to act as antioxidants, antimicrobial and anticancer agents.
- Utilization of the extraction residue to produce biofuels in a third generation biorefinery concept.

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