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Utilization of Biomass Derived from Cyanobacteria-Based Agro-Industrial Wastewater Treatment and Raisin Residue Extract for Bioethanol Production

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Abstract: Biofuels produced from photosynthetic microorganisms such as microalgae and cyanobacteria could potentially replace fossil fuels as they offer several advantages over fuels produced from lignocellulosic biomass. In this study, energy production potential in the form of bioethanol was examined using different biomasses derived from the growth of a cyanobacteria-based microbial consortium on a chemical medium and on agro-industrial wastewaters (i.e., dairy wastewater, winery wastewater and mixed winery–raisin effluent) supplemented with a raisin residue extract. The possibility of recovering fermentable sugars from a microbial biomass dominated by the filamentous cyanobacterium *Leptolynbgya* sp. was demonstrated. Of the different acid hydrolysis conditions tested, the best results were obtained with sulfuric acid 2.5 N for 120 min using dried biomass from dairy wastewater and mixed winery–raisin wastewaters. After optimizing sugar release from the microbial biomass by applying acid hydrolysis, alcoholic fermentation was performed using the yeast *Saccharomyces cerevisiae*. Raisin residue extract was added to the treated biomass broth in all experiments to enhance ethanol production. Results showed that up to 85.9% of the theoretical ethanol yield was achieved, indicating the potential use of cyanobacteria-based biomass in combination with a raisin residue extract as feedstock for bioethanol production.

Keywords: bioethanol; *Saccharomyces cerevisiae*; cyanobacteria; *Leptolyngbya*-based microbial consortium; agro-industrial wastewater

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1. Introduction

Biomass is a promising renewable resource that can be used to generate different types of biofuels, including bioethanol. However, the use of biomass obtained from solid agricultural wastes increases fuel production cost due to its high lignin content that affects the saccharification process [1]. In contrast, many photosynthetic microorganisms (microalgae and cyanobacteria) have high a content of starch and cellulose and therefore constitute excellent substrates for bioethanol production [2–4] (Table 1). Microalgae including different phyla such as Chlorophyta (green algae), Rhodophyta (red algae), Heterokontophyta and Cyanophyta (blue green algae, cyanobacteria) are gaining wide attention as alternative renewable sources of biomass as they offer a number of potential advantages compared to plants [5–7].

There has been a remarkable surge in research investigating the utilization of microalgae as an advanced energy feedstock for biofuel production [8–10]. Species belonging to the genera *Chlorella*, *Dunaliella*, *Scenedesmus*, *Spirulina* and *Chlamydomonas* contain large

amounts of starch, thus making them valuable for bioethanol production [11,12]. Specifically, some cyanobacteria like *Anabaena* and *Synechococcus* sp. have recorded values for ethanol yields up to 90% on sugar consumed (Table 1). According to Chen et al. [13], several other microalgae species contain sugars that can also be fermented to bioethanol provided that each species is appropriately pretreated according to its particular cell wall structure and carbohydrate composition. Different growth conditions and/or genetic modifications are often applied in an attempt to maximize cellular carbohydrate content [14–17].

Table 1. Biomass types, pretreatment methods, initial sugar concentrations, ethanol production and yields recorded in the literature.

Substrate	Pretreatment Method	Initial Sugar Concentration (g L^{-1})	Maximum EtOH Concentration (g L^{-1})	EtOH Yield on Sugars Consumed (%)/(g/g)	References
Wheat straw	0.2% H_2SO_4, 300 bar, 190 °C, 10 min	43.0–46.0	38.0	69.0/0.35	[18]
Corn stover	Steam 50 °C 24 h, pH 4.8	~160.8	-	72.3/-	[19]
Molasses Molasses with 20% olive mill wastewaters in a batch bioreactor	1 M HCl, 100 °C, 30 min KOH 1 M	~100.0 ~100.0 ~135.0 ~150.0	37.3 44.4 52.4 50.6	-/0.44 -/0.49 -/0.48 -/0.45	[20]
Glucose-enriched olive mill wastewaters		~75.0 ~75.0 ~115.0	26.1 33.9 52.0	-/0.36 -/0.45 -/0.46	[21]
Chlorococcum sp.	Lipid-extracted microalgae	~100.0	3.58	-	[22]
Spirogyra sp.	Untreated	-	8 g/100 g substrate	-	[23]
Chlamydomonas reinhardtii	Enzymatic	-	11.73	-	[24]
Dunaliella sp.	1% H_2SO_4 121 °C, 15 min	-	7.26	-	[25]
Scenedesmus sp.	0.3 N H_2SO_4 121 °C, 15 psi, 20 min	15.0	6.6	86.0/-	[26]
Mixed algal biomass	10% H_2SO_4 121 °C 15 psi, 120 min	-	-	61.0/-	[27]
Chlamydomonas reinhardtii	3% H_2SO_4 110 °C, 30 min	28.5	14.6	-	[28]
Chlorella vulgaris FSP-E	1% H_2SO_4 121 °C, 20 min	22.0–24.0	11.7 *Zymomonas mobilis*	-	[4]
Scenedesmus obliquus	2 N H_2SO_4 120 °C, 30 min	14.4 (28.6% g/g DW)	-	-	[29]
Chlorococcum sp.	1% H_2SO_4 140 °C, 0 min, 3% H_2SO_4 160 °C, 15 min	-	7.20 6.0	-	[30]
Scenedesmus obliquus YSW15 in swine wastewater effluent	Ultra-sonication 15–60 min	10.5–10.8	6.7	-	[31]

Table 1. *Cont.*

Substrate	Pretreatment Method	Initial Sugar Concentration (g L^{-1})	Maximum EtOH Concentration (g L^{-1})	EtOH Yield on Sugars Consumed (%)/(g/g)	References
Microalgae biomass from wastewater	1 M H_2SO_4 90 °C, 30 min and 5 M NaOH 90 °C, 30 min	~35.0	0.53 *Clostridium saccharo-perbutylacetonicum*	-	[32]
Microalgae biomass from wastewater	1 M H_2SO_4 80–90 °C, 120 min	166.1 g/kg dry algae	0.53 *Clostridium saccharo-perbutylacetonicum*	-	[33]
Microalgae biomass from wastewater	1 M H_2SO_4 autoclaved	33.8	4.6 *Clostridium phytofermentans*	-	[34]
Chlorella sorokiniana, *Nannochloropsis gaditana*, *Scenedesmus almeriensis*	4% H_2SO_4 121 °C, 90 min 5 M NaOH 90 °C, 30 min, acid and enzymatic	136 mg/g dry algae 15 mg/g dry algae 129 mg/g dry algae	- - -	- - -	[35]
Scenedesmus obliquus	5% H_2SO_4 120 °C, 30 min	63.2	11.7 *Kluyveromyces marxianus*	-	[36]
Chlorella vulgaris	Bead-beating and enzymatic	~1.15	~0.5	89.0/-	[37]
Marine brown algae	Acid and enzymatic	90.0	25.8 *E. coli* KO11	-/0.41	[38]
Molasses	Sterilized	110.0 120.0	46.6 50.6	- -	[39]
Anabaena sp.	Genetically modified	-	-	70.2/-	[17]
Synechococcus sp.	Freezing and enzymatic	~65.0	30.0	0.27 g EtOH/ g DW	[14]
Synechococcus elongatus (recombinant)	2% H_2SO_4 autoclaved	-	7.2 *Zymomonas mobilis*	91.0/0.45	[15]
Arthrospira platensis (Spirulina)	0.5 N H_2SO_4, 80 °C, 180 min 1 N H_2SO_4, 60 °C, 90 min	-	-	53.3/- 30.4/-	[40]
Arthrospira platensis (Spirulina)	Enzymatic	-	6.5	86.0/-	[41]
Anabaena variabilis, *Microcystis aeruginosa*	2 N H_2SO_4 autoclaved	342.4 283.2	152.5 121.8	28.2 g EtOH/g DW 23.9 g EtOH/g DW	[16]
Microalgae biomass and raisin extract	2.5 N H_2SO_4 (6.6%) 120 min autoclaved	258.6	111.1	85.9/0.43	This study
Microalgae biomass from mixed wastewater	2.5 N H_2SO_4 120 min autoclaved	87.2	32.2	73.8/0.37	This study
Microalgae biomass from mixed wastewater	2.5 N H_2SO_4 180 min autoclaved	85.3	32.7	76.5/0.38	This study

Table 1. *Cont.*

Substrate	Pretreatment Method	Initial Sugar Concentration (g L^{-1})	Maximum EtOH Concentration (g L^{-1})	EtOH Yield on Sugars Consumed (%)/(g/g)	References
Microalgae biomass from dairy wastewater	2.5 N H_2SO_4 120 min autoclaved	87.2	31.5	70.7/0.36	This study
Microalgae biomass from winery wastewater	2.5 N H_2SO_4 120 min autoclaved	76.0	21.0	55.2/0.28	This study

The cost of producing biofuel from microalgae is usually higher than from conventional crops due to various factors including the high cost of chemicals used during cultivation or high-cost harvesting and drying processes [42]. Thus, the utilization of biomass produced through wastewater treatment is considered a more viable strategy for cost reduction in the microbial-based biofuel industry [43]. Counterbalancing financial costs, agro-industrial wastewaters usually contain nutrients in high concentrations and can effectively replace microalgae or cyanobacteria culture media [44]. Specifically, a microbial consortium dominated by the cyanobacterium *Leptolynbgya* sp. was proved effective in the treatment of agro-industrial effluents, such as raisin, winery and dairy wastewaters as well as poplar sawdust and grass hydrolysates, both in suspended and attached cultivation systems [45–47]. The same consortium also contained remarkable percentages of carbohydrates that exceeded 40% of dry biomass, thus making it a promising candidate as a substrate for bioethanol production [48].

Biomass pretreatment is considered an important stage to improve substrate assimilability and overall efficiency of the bioethanol production process [49]. Most carbohydrates/potential substrates for fermentation are entrapped within cell walls (i.e., cellulose) or intracellularly (i.e., starch), necessitating cell wall disruption and hydrolysis stages to enhance their breaking down into simple sugars. The overall efficiency of the pretreatment is a good balance between inhibitor formation and substrate assimilability [50,51]. Optimizing cell disruption and sugar extraction methods is essential for cost-effective and environmentally sustainable bioethanol production. Several studies on optimizing sugar release yields also examine various pretreatment methods including chemical, thermal, mechanical, biological and combinations of these [52]. Selection of the most suitable pretreatment method depends on the morphology (i.e., cell wall composition) of the algae species used [53]. For this reason a thorough economic assessment of microalgae biofuel that focuses on biomass pretreatment has not been made. Pretreatment of algal biomass for fermentation is mostly performed using chemical methods such as acid/alkaline treatment and the two significant goals that should be achieved are: (i) optimal saccharification yield under benign conditions, and (ii) the minimum formation of inhibitors [53]. The alkaline hydrolysis process produces lower sugar yields than acid hydrolysis [35]. Additionally, acid pretreatment shows higher disruption/sugar extraction efficiency than alkaline pretreatment or other physical methods (sonication, homogenization, beat-beating) in microalgae biomass [26,29,36]. On the other hand, the formation of inhibitors is avoided using enzymatic hydrolysis [54]. Nevertheless, acid hydrolysis is faster and cheaper than enzymatic hydrolysis and thus acid pretreatment is preferable for industrial applications [52,54]. In the next step of alcoholic fermentation, the microorganism most frequently used in industrial processes is the ethanol-tolerant yeast *Saccharomyces cerevisiae*. However, few studies focus on the use of *S. cerevisiae* strains for the valorization of microalgal biomass [26] and none refer to *Leptolynbgya*-based feedstock for the production of ethanol.

As bioethanol production is increasing worldwide, it is imperative to use sustainable biomass substrates in order to decrease the use of arable land and valuable water resources. To alleviate these problems, one alternative source of biomass could be the (blue–green) algae growing in wastes. The present study was undertaken to evaluate for the first time the use of biomass resulting from a *Leptolyngbya*-based treatment of a synthetic medium, as well as of dairy, winery and raisin wastewaters, for the production of bioethanol via fermentation with *Saccharomyces cerevisiae*. Cyanobacterial biomass was pretreated with dilute sulfuric acid to release fermentable sugars, while sulfuric acid concentrations and hydrolysis time were examined with the aim of increasing sugar yields. Additionally, an extract obtained from raisin waste streams was added into the biomass hydrolysate to enhance initial sugar concentrations.

2. Materials and Methods

2.1. Biomass Origin and Harvesting

A microbial population taken from the municipal wastewater treatment plant of Agrinio city (Greece) was cultivated under steady conditions for microalgae/cyanobacteria enrichment to establish stock cultures. The photosynthetic consortium was cultivated autotrophically in 10 L total volume (5 L working volume) lab-made photobioreactors (i.e., aquarium-like rectangular glass tanks) containing a mineral medium consisting of (in g L^{-1}): KNO_3 0.2; $MgSO_4 \cdot 7H_2O$ 0.1; $CaCl_2 \cdot 2H_2O$ 0.05; K_2HPO_4 0.108 and KH_2PO_4 0.056 at pH 7.2 $\pm$ 0.3 [45]. The reactors were placed under continuous illumination from fluorescent lamps (200 µmol m^{-2} s^{-1}, 25–29 W m^{-2}) at T = 28 $\pm$ 2 °C, and mixing was ensured by a centrifugal pump working at a flow rate of 380 L h^{-1}.

Identification of the microbial species was reported in Tsolcha et al. [47] where a microbial consortium dominated by the filamentous cyanobacterium *Leptolyngbya* sp. was observed in all types of wastewater tested. The established photosynthetic culture was used for inoculation and treatment of a synthetic medium (of chemical composition as described above), dairy wastewater, winery wastewater and mixed (winery and raisin) wastewater under similar environmental conditions. The *Leptolyngbya*-based microbial consortium was cultivated for 12 days and the produced biomass was harvested by centrifugation for 20 min at 4200 rpm. The biomass was then dried at 108 °C until constant weight which was gravimetrically determined [55].

2.2. Biomass Pretreatment

Biomass pellets slurred at a 5% solid to liquid ratio (w/v) were mixed with sulfuric acid at a final concentration 1.5 N or 2.5 N and pretreated in an autoclave vessel (116 °C, 0.8 bar) for durations ranging from 30 to 180 min. The hydrolyzate was collected and analyzed for reducing sugar content extracted under the different experimental conditions. Total sugars and reducing sugars content were determined according to the DuBois and dinitrosalicylic acid (DNS) methods, respectively [56,57]. The hydrolysates were neutralized with NaOH until pH 4.5 prior to the fermentation process.

2.3. Raisin Residue Extract Production

Raisin packaging facilities produce solid waste streams consisting of nucleate raisins that are often used for energy (bioethanol) production. The raisin residue used in this study was obtained from a local raisin processing factory and was treated as follows: 70 g raisin residue was crushed and boiled at 100 °C with 250 mL of distilled water for 20 min. The extract was filtered through a cheesecloth filter and used in the yeast fermentation experiments as a sugar enhancer. The initial total sugar concentration of the raisin residue extract was 414.9 $\pm$ 53 g L^{-1}, determined as above.

2.4. Yeast Strain and Bioethanol Analysis

Fermentation of the biomass hydrolysates supplemented with raisin residue extract was performed using *Saccharomyces cerevisiae* AXAZ-1, an ethanol-tolerant and psychrotolerant yeast strain [58]. The strain was kept on potato dextrose agar at T = 7 ± 1 °C and for long-term storage at −80 °C in a glycerol 30% solution. Pre-culture was carried out at 28 °C for 48 h in 50 mL potato dextrose broth medium enhanced with $(NH_4)_2SO_4$ (0.5 g L^{-1}) and HK_2PO_4 (1 g L^{-1}) in 250 mL Erlenmeyer flasks. Each medium of biomass hydrolysate and raisin extract was inoculated with 1 mL of a 48 h *S. cerevisiae* culture. Fermentations were performed under anaerobic conditions in Duran bottles (250 mL) with periodic stirring at 30 °C. At the beginning of the fermentation (i.e., the first 4 h) the flasks were aerated by stirring at 150 rpm to induce cell growth. Yeast cell growth was measured using a Neubauer type hemocytometer (Neubauer improved, Poly-Oprik, Bad Blankenburg, Germany) where the initial concentration for all experimental sets of 11.7×10^6 cells mL^{-1} was recorded. During fermentation, the temperature was constant at 28 °C and pH values ranged from 4.4–4.6, since optimal growth conditions for yeast range between 28–30 °C and pH 4–5 [59]. All fermentation experiments were performed in duplicate under non-aseptic conditions.

The bioethanol concentration was determined with an HPLC (Ultimate 3000, Dionex, Germany) system equipped with a reflective index detector (RI-101, Shodex, Kawasaki, Japan) (in which the detection of ethanol occurred) and Aminex HPX-87H column (300 mm × 7.8 mm, Bio-Rad, Hercules, CA, USA). The volume of samples analyzed was 20 μL of culture medium, previously filtered through Whatman membranes of 0.2 μm pore diameter. As eluent, H_2SO_4 (Fluka) 0.005 N was used at a flow rate of 0.6 mL min^{-1} and the column temperature was 65 °C. The concentration of ethanol in the sample was calculated using a calibration curve of different standard ethanol concentrations.

3. Results and Discussion

3.1. Biomass Pretreatment

The agro-industrial sector generates considerable amounts of wastewater, most of which are rich with inorganic and organic pollutants [46,47]. Using these pollutants as nutrient material for a microalgae-based cultivation system may minimize their discharge into the natural environment and further reduce a CO_2 footprint by utilizing the resulting biomass in energy production processes [2]. Due to the large amounts of carbohydrates contained in their cells, using microalgal/cyanobacterial biomass as feedstock for bioethanol production appears a very promising solution [3,30]. The bioethanol production procedure requires four major unit operations including pretreatment, hydrolysis, fermentation, and distillation [60]. To produce sugars from the microbial biomass, pretreatment is designed to convert complex carbohydrates (starch) into their constituent simple sugars, which can be fermented into ethanol by ethanol-producing microorganisms, which is then recovered and purified to meet fuel specifications [9].

In the present study, acid hydrolysis in an autoclave condition was used to obtain higher sugar yields than those produced by alkaline hydrolysis [35]. Cell disruption is an essential initial step of the biomass treatment process and methods vary usually in acid concentration, temperature and reaction time. Sulfuric acid was used in this research as it produces higher sugar production yields than other acids such as HNO_3, HCl and H_3PO_4 [40]. Lee et al. [61] hydrolyzed microalgae (i.e., *Chlorella vulgaris* and *Chlamydomonas reinhardtii*) with dilute H_2SO_4 (1–5% on 5% (*w*/*v*) dry solid basis (*v*/*v*)) and autoclaved at 100–120 °C for 120 min. Miranda et al. [36] reported that of the physical and physicochemical methods tested, the best results were obtained with acid hydrolysis by H_2SO_4 (2 N), at 120 °C for 30 min. Sivaramakrishnan et al. [26] obtained maximum sugar release with an autoclave pretreatment (120 °C) and H_2SO_4 (0.3 N) for 20 min. It appears that a combination of high temperature and pressure enhances hydrolysis efficiency by increasing the solubility of carbohydrates and exposing them to acid molecules. It is worth mentioning that a test hydrolysis for 120 min applied with 0% H_2SO_4 led to

3.0 g L^{-1} total sugar concentration and 20.5 ± 3.8% reducing/total sugars. Hence, the biomass pretreatment method selected for this study was designed at steady autoclave conditions with reaction times ranging from 30 to 180 min and acid concentrations of 1.5 N and 2.5 N H_2SO_4 [62]. It should be mentioned that these acid concentrations are the most commonly found in the literature for microalgae and cyanobacteria biomass pretreatment [4,15,16,28,30,35] (Table 1).

The dry biomass from the autotrophic culture of a *Leptolyngbya*-based microbial consortium was produced in the synthetic medium on day 12 and was pretreated with 1.5 N and 2.5 N H_2SO_4 for four different reaction times (Table 2). Sugar yields (% reduction of total sugars) were slightly higher when using 2.5 N H_2SO_4 and reached up to 94.4% of reducible sugars. Specifically, in the 30 min hydrolysis, the acid concentration did not affect sugar yields, while in the 180 min hydrolysis sugar yields were observed to decrease, probably due to sugar degradation [63]. The 120 min hydrolysis seemed to lead to relatively higher sugar yields than the 60 min hydrolysis for the specific biomass. Total sugar concentrations calculated in all the hydrolysis conditions tested ranged between 9.7 and 24.8 g L^{-1} corresponding to 15.4–31.8% *w*/*w* on dry biomass. These values are higher than those reported by Hernandez et al. [35] who recorded 13.6% when using dried *Chlorella* species biomass treated with 4% H_2SO_4 at 121 °C and 90 min. The values obtained in this study are within the range of those reported by John et al. [3] for dry microalgae biomass (i.e., 12–50% *w*/*w*).

Table 2. Sugar yields obtained from acid pretreatment of biomass for autotrophic microbial culture.

Cyanobacterial Growth Substrate	Percentage Reducing/Total Sugars (%) per Acid Pretreatment Duration				Sulfuric Acid Concentration
	30 min	60 min	120 min	180 min	
Synthetic medium	86.8 ± 4.0	80.0 ± 6.8	89.8 ± 4.2	51.8 ± 2.5	1.5 N
	86.7 ± 0.9	91.3 ± 4.3	94.4 ± 4.1	47.0 ± 5.4	2.5 N

The dried biomass obtained from the mixotrophic culture of a *Leptolyngbya*-based microbial consortium on dairy wastewater, winery wastewater and mixed (winery and raisin) wastewater was treated using the same pretreatment procedure described above (Table 3). Dried biomass obtained from the dairy wastewater yielded the highest amount of reducing sugars using 2.5 N H_2SO_4 for 120 min. In addition, reducing sugar concentrations recorded using 2.5 N H_2SO_4 ranged between 9.0 and 40.4 g L^{-1}, while when using 1.5 N H_2SO_4 the concentrations ranged between 9.0 and 30.7 g L^{-1}. Dried biomass obtained after cultivation on the winery wastewater substrate yielded the highest amount of reducing sugars when treated with 2.5 N H_2SO_4 for 120 min, while reducing sugar concentrations for all experimental conditions ranged between 10.3 and 14.4 g L^{-1}. Finally, biomass obtained from a mixed (winery–raisin) wastewater substrate, yielded the highest amount of sugars when treated with 2.5 N H_2SO_4 at hydrolysis time of 120 min and the reducing sugar concentrations ranged between 8.0 and 22.3 g L^{-1}. Similar reducing sugar concentrations ranging between 10.9–22.4 g L^{-1} were recorded using 1.5 N H_2SO_4 at hydrolysis time of 120 min. In summary, the 120 min hydrolysis time resulted in higher yields of extracted sugars (up to 40.4 g L^{-1}), especially for the mixed (winery and raisin) wastewater and the dairy wastewater. Castro et al. [33] also used microalgae biomass cultivated in wastewater as substrate for ethanol fermentation and recorded up to 16.6% *w*/*w*/ sugars per dry biomass at 120 min hydrolysis time, which is lower than the yields obtained in the present study.

Table 3. Sugar yields obtained from acid pretreatment of biomass harvested from cyanobacterial cultures grown on different wastewater substrates (ND: not determined).

Cyanobacterial Growth Substrate	Percentage Reducing/Total Sugars (%) per Acid Pretreatment Duration				Sulfuric Acid Concentration
	30 min	**60 min**	**120 min**	**180 min**	
Dairy wastewater	ND ND	ND 85.3 ± 2.0	83.0 ± 6.0 93.0 ± 7.0	ND 50.3 ± 2.5	1.5 N 2.5 N
Winery wastewater	91.0 ± 0.3 87.5 ± 2.0	ND 91.5 ± 1.5	ND 98.0 ± 1.0	ND 51.8 ± 3.0	1.5 N 2.5 N
Mixed wastewater (winery and raisin)	ND 84.3 ± 2.9	ND 89.5 ± 2.2	46.6 ± 3.0 98.1 ± 1.9	ND 60.7 ± 1.0	1.5 N 2.5 N

3.2. Yeast Growth Conditions and Bioethanol Production

Following pretreatment, alcoholic fermentation was performed applying the most frequently used microorganism in the industrial process, i.e., *S. cerevisiae* strain AXAZ-1, to investigate the potential of bioethanol production. All biomass hydrolysates were supplemented with raisin residue extract. The yeast converts only simple sugars to ethanol but has the ability to grow rapidly under anaerobic conditions [64,65]. Yeast cell growth and concentrations of ethanol and reducing sugars were measured during alcoholic fermentation, where yeast cell increase was associated with decrease in sugar concentration. Based on Tables 2 and 3, the following substrates were examined and the experimental results are presented in Figures 1 and 2: Dried biomass from synthetic medium treated using 2.5 N H_2SO_4 at hydrolysis times of 60 (Figure 1a) and 120 min (Figure 1b); biomass from the dairy wastewater treated in 2.5 N H_2SO_4 at 120 min (Figure 2a); biomass from the winery wastewater treated in 2.5 N H_2SO_4 at 120 min (Figure 2b); biomass from the mixed wastewater (winery and raisin) treated in 2.5 N H_2SO_4 at 120 (Figure 2c) and 180 min (Figure 2d). Measurements of initial sugar concentrations in all biomass hydrolysates prior to fermentation indicated low values (under 41 g L^{-1}), as the highest fermentation capacity of *S. cerevisiae* was observed at initial sugar concentrations above 50 g L^{-1} [66]. Hence, all biomass hydrolysates were strengthened by the addition of a raisin residue extract containing 318.0 g L^{-1} of reducing sugars.

As shown in Figure 1a,b, for similar initial reducing sugar concentrations (approximately 83 and 89 g L^{-1} for 60 and 120 min hydrolysis time, respectively), although the maximum ethanol concentrations achieved were almost the same in both experiments (around 22 g L^{-1}), the final yeast concentration was about 22.3 × 10^6 cells mL^{-1} and 68.0 × 10^6 cells mL^{-1}, respectively, indicating a change in yeast behavior in the substrates treated for different time intervals. The results of fermentation of hydrolyzed biomass originating from the various wastewaters (Figure 2a–d) showed significant differences in both fermentation time and duration of lag phase, even though the same initial yeast cell density was used as inoculum. It is probable that the substrates originating from cyanobacterial biomass cultivated in the various wastewaters contained inhibitors that were released in different concentrations during hydrolysis pretreatment, however this requires further investigation.

According to the literature, a number of inhibitory compounds form during the hydrolysis pretreatment and these can greatly inhibit the subsequent fermentation process [67,68]. The accumulation of sugar degradation products such as acetic acid, formic acid and furfural has damaging effects on the fermentation process by delaying or even completely inhibiting it [27,69]. The formation of furfural depends on the retention time and acid concentration, and phenolic compounds present in the hydrolysate can minimize the ethanol yield [8,70]. It is well known that the acid pretreatment method using H_2SO_4, generates

not only soluble sugars but also chemical compounds, such as furfural and hydroxymethylfurfural (HMF), that may have an inhibitory or toxic effect on microorganisms [47]. The formation of these inhibitors induces a general issue in bioenergy production. In the experiments presented in Figure 2a–d, the initial sugar concentrations ranged from 76 to 87 g L^{-1} and the final ethanol and biomass concentrations achieved were between 20 to 33 g L^{-1} and 12 to 55 $\times$ 10^6 cells mL^{-1}, while the fermentation time ranged from 120 to 347 h. It is worth mentioning that sugar consumption in the substrate consisting of biomass derived from the dairy wastewater treated with 2.5 N H_2SO_4 for 120 min (Figure 2a), occurred in a shorter time period (i.e., 90 h) than the other substrates. This could be attributed to the fact that dairy wastewaters contain high quantities of hexoses, such as glucose and galactose [71], which can be easily metabolized by *S. cerevisiae*. However, in addition to ethanol concentration, it is important to consider ethanol yield. The maximum ethanol yield (EtOH/sugars % *w*/*w*) was recorded with the use of hydrolysate biomass from mixed (winery–raisin) wastewater ranged between 73.0–76.5% of the theoretical ethanol yield (Table 4). This value is higher than the 61% recorded by Kumar et al. [27] who used mixed algal biomass as bioethanol substrate, and also higher than that recorded by Smachetti et al. [17] (i.e., 70.2%) who applied a genetically modified strain of the cyanobacterium *Anabaena*.

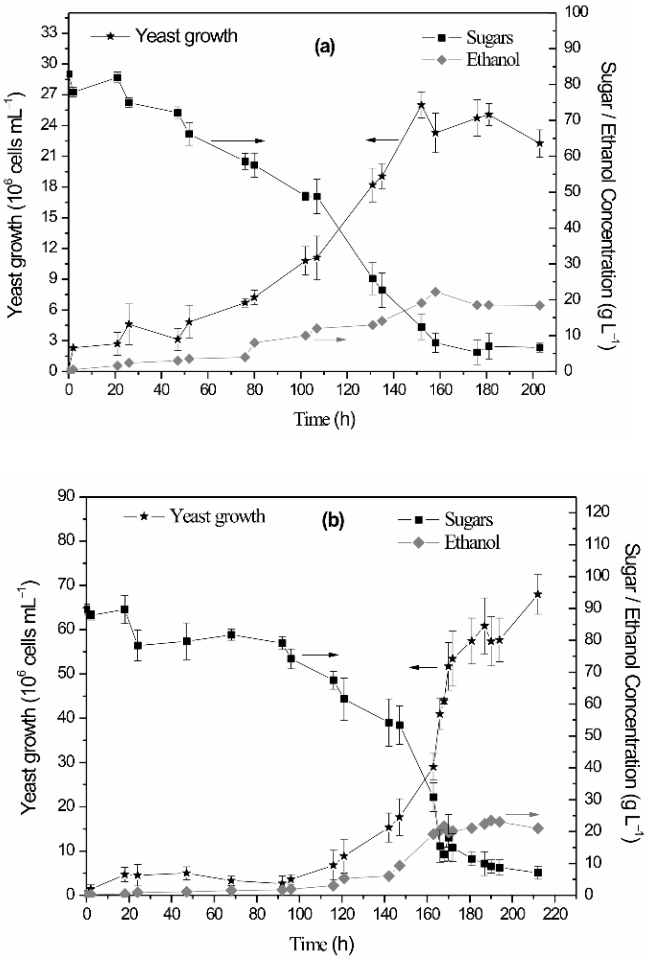

Figure 1. Profiles of yeast growth, sugar depletion and ethanol production through time using synthetic medium cyanobacterial biomass substrates treated with H_2SO_4 at: (**a**) 60 min and (**b**) 120 min.

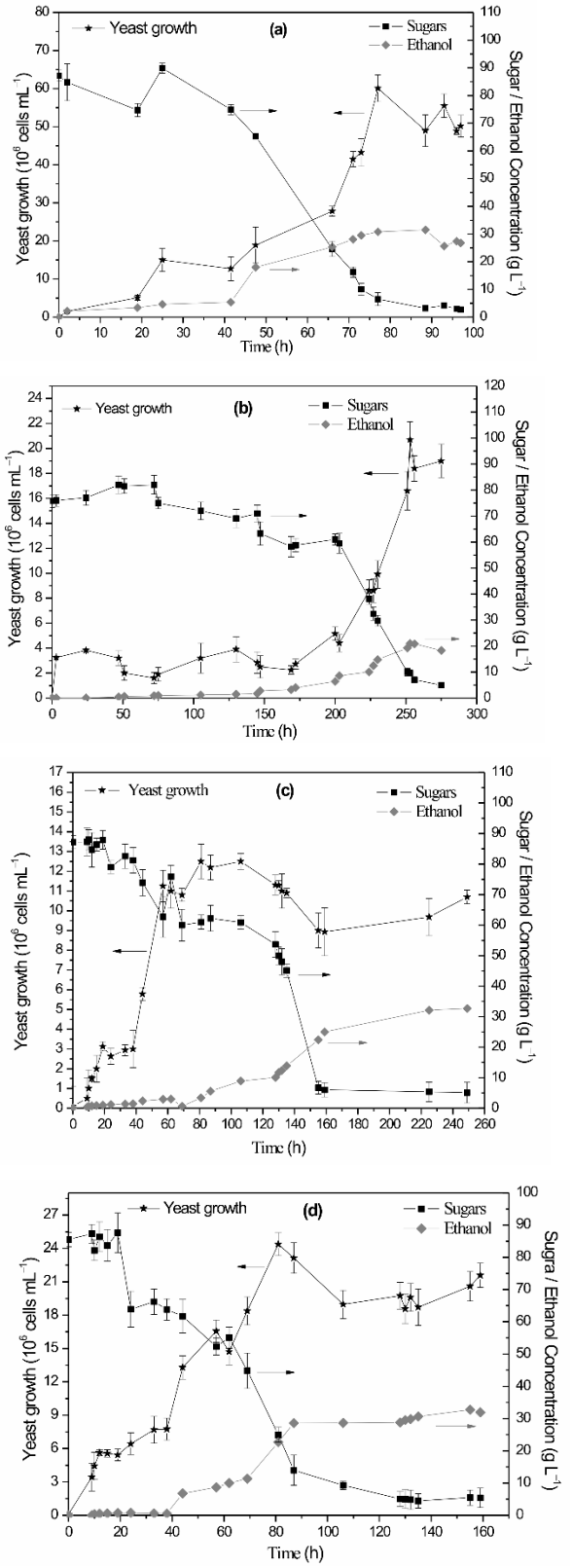

Figure 2. Profiles of yeast growth, sugar depletion and ethanol production through time using cyanobacterial-based biomass grown on: (**a**) dairy wastewater treated with H_2SO_4 at 120 min hydrolysis time, (**b**) winery wastewater treated with H_2SO_4 at 120 min hydrolysis time, (**c**) mixed wastewater (winery and raisin) treated with H_2SO_4 at 120 min hydrolysis time, and (**d**) mixed wastewater treated with H_2SO_4 at 180 min hydrolysis time.

Table 4. Ethanol production and yields from acid pretreatment of biomass from cyanobacteria-based cultures grown on synthetic medium treated for 60 min (SM-60) and 120 min (SM-120), dairy wastewater treated for 120 min (DW-120), winery wastewater treated for 120 min (WW-120), and mixed wastewater (winery and raisin effluents) treated for 120 min (MW-120) and 180 min (MW-180). RR: raisin residue extract.

Substrate	g Sugars/g Dry Biomass (%)	Initial Sugar Concentration (g L^{-1})	Ethanol Concentration (g L^{-1})	Ethanol Yield in Sugars (%)
SM-60	21.5	83.0	22.2	53.5
SM-120	31.8	89.7	22.8	62.0
DW-120	26.1	87.2	31.5	70.7
WW-120	20.6	75.8	21.0	55.2
MW-120	19.0	87.2	32.2	73.8
MW-180	-	85.3	32.7	76.5
RR; SM-120	-	258.6	111.1	85.9

Finally, a set of experiments was performed using as substrate hydrolyzed biomass from synthetic medium pretreated with 2.5 N H_2SO_4 for 120 min with the addition of raisin residue extract to test the ability of this specific yeast to grow in a higher initial sugar concentration (Figure 3). In this experiment, the initial reducing sugar concentration was 258.6 g L^{-1}, while the final concentrations of ethanol and number of cells recorded were about 111.0 g L^{-1} and 180 $\times$ 10^6 cells mL^{-1}, respectively. A plethora of research studies deal with the resistance of *S. cerevisiae* by performing fermentation in high initial sugar concentrations ranging between 120–350 g L^{-1} [72–74]. Bely et al. [75] studied fermentation of must with *S. cerevisiae* in co-culture, having an initial sugar concentration of 360 g L^{-1}, where the fermentation was completed in 11 days and yielded 0.48 EtOH/sugars (*w*/*w*). Similar studies with high initial sugar concentrations (250 g L^{-1}) were conducted by Sarris et al. [76] where grape must was used to achieve ethanol production of 106.4–119.2 g L^{-1} using the strain MAK-1 of *S. cerevisiae*. Chang et al. [74] tested the strain BCRC 21812 of *S. cerevisiae*, and employed a feed batch system to enhance the fermentative substrate (up to 260 g L^{-1} of glucose). They recorded a maximum ethanol production of 130.1 g L^{-1} corresponding to 51% of the theoretical ethanol yield. However, Kopsahelis et al. [77] used the same yeast strain as in this study and achieved less ethanol production (71.3 g L^{-1}) with initial sugar concentrations of about 216 g L^{-1}. Ellis et al. [32] used microalgae biomass derived from wastewater treatment for the production of ethanol and recorded an ethanol concentration of 0.53 g L^{-1} with *Clostridium saccharoperbutylacetonicum* N1–4 (Table 1). In the present study, the addition of the raisin residue extract improved ethanol concentration which reached up to 111 g L^{-1} when applying *S. cerevisiae* AXAZ-1 from cyanobacterial biomass derived by synthetic medium culture growth, which is among the highest ethanol concentration values in the current literature [78].

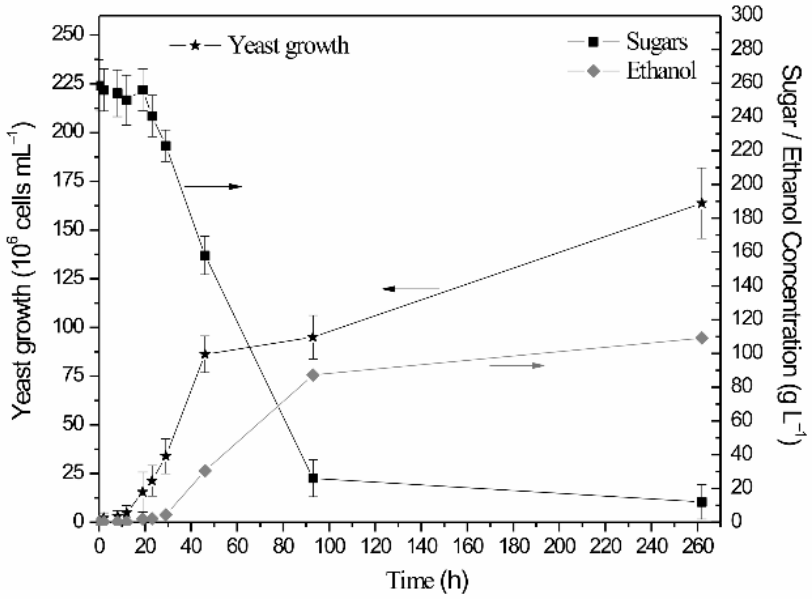

Figure 3. Profile of yeast growth, sugar depletion and ethanol production through time using a high sugar concentration substrate (derived from synthetic medium cyanobacterial-based biomass treated with H_2SO_4 at 120 min hydrolysis time supplemented with raisin residue extract).

It is noteworthy that the variations observed in sugar and ethanol concentrations in Table 1 are due to the use of a wide variety of substrates and pretreatment conditions that impede direct comparison of the results. It has been observed that in most cases about 50% of the initial sugar concentration is consumed during fermentation, a figure also observed in the present study. The results showed that cyanobacteria-based microbial cultures derived from wastewater treatment processes are feedstocks suitable of supporting high ethanol yields—up to 76.5% of the theoretical ethanol yield. The production of biofuels from cyanobacterial biomass derived from waste treatment plants is an interesting alternative method that with future improvements could potentially contribute to the production of clean energy.

4. Conclusions

Due to environmental pollution, climate change and the depletion of natural resources, bioethanol has attracted attention as an octane booster, fuel additive, a neat fuel and a means to reduce SO_2 and CO_2 emissions. Furthermore, to abate industrial pollution and enhance profitability and sustainability, bioremediation technologies require reconsideration and innovation. Low-cost microalgal/cyanobacterial biomass used as bioenergy feedstock could form part of an integrated system that uses wastewater as a nutrient substrate. The present study reveals the prospect of using cyanobacteria-based microbial biomass from wastewater treatment processes as feedstock for bioethanol production using the yeast *S. cerevisiae* AXAZ-1. All the substrates tested demonstrated an ethanol yield of over 50% (up to 85.9% of the theoretical ethanol yield) when a high initial sugar concentration was applied. However, research is required to improve pretreatment methods and enhance biomass and sugar production rates to levels sufficient for economic and sustainable biofuel production. The combination of wastewater treatment with microbial biomass production could be a promising way to break the bottleneck of feedstock availability for microbial bioethanol.

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writing—review and editing, A.G.T., G.A., C.N.E., M.D., V.P. and O.N.T.; supervision, A.G.T. All authors have read and agreed to the published version of the manuscript.

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