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Biomass and β-Glucosidase Production by the Cyanobacterium *Pseudanabaena* sp. under Heterotrophic Conditions

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Abstract: A cyanobacterium producing β-glucosidase was isolated from Lake Pamvotis located in Ioannina in Greece. This microorganism, named Pamv7, was identified as *Pseudanabaena* sp. using phylogenetic characterization. The high-throughput BiologMicroPlate™ method, used for the rapid assessment of heterotrophic potential, indicates that *Pseudanabaena* sp. metabolizes a wide range of organic substrates such as amino acids, carbohydrates, and carboxylic acids. When the strain grows in a culture medium containing cellobiose as a carbon source, it produces a significant amount of intracellular β-glucosidase. The effect of cellobiose concentration, nitrogen source, and nitrogen concentration of the growth medium, as well as the temperature of the culture, on biomass and β-glucosidase by *Pseudanabaena* sp., was studied. Biomass and β-glucosidase production by the strain in a lab-scale bioreactor at optimal conditions (10 g/L cellobiose, 1.5 g/L yeast, and 23 ± 1 °C) reached 2.8 g dry weight/L and 44 U/L, respectively. The protein and lipid content of the produced cyanobacterium biomass were 23% and 43 *w*/*w*, respectively. This study is the first report of β-glucosidase production by a cyanobacterial strain and concomitant high production of microalgae biomass, making *Pseudanabaena* sp. a promising microorganism in the field of enzyme biotechnology.

Keywords: β-glucosidase; cyanobacterium; biomass; *Pseudanabaena* sp.; Biolog ECO

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1. Introduction

Beta-glucosidase (β-D-glucoside glucohydrolase) hydrolyzes the β-glucosidic linkages of cellobiose, short-oligosaccharides, as well as alkyl- and aryl-glucosides into glucose units [1–4], in cooperation with endoglucase (1,4-β-D-glucan hydrolase) and exoglucanase (1,4-β-D-glucan glucohydrolase), which degrade cellulose into glucose units.

Cellulolytic enzymes and especially β-glucosidase have potential for many biotechnological applications based on both hydrolytic and synthetic activity [1,5]. Some of the applications of β-glucosidases are in ethanol and biofuel production; the decomposition of organic matter; and food, wine, juice, textile, and paper industry [3,5–7]. In most cases, β-glucosidase constitutes a bottleneck in the degradation of cellulose by commercial cellulolytic enzymatic products. This imbalance in cellulolytic enzymatic cocktails could be improved by adding appropriate β-glucosidase quantities [8]. Through reverse hydrolysis or transglycosylation, β-glucosidase catalyzes the synthesis of glucosidic bonds of oligosaccharides and alkyl glucosides [1,3]. Therefore, β-glucosidases find application as tools with diagnostic, therapeutic, or bactericidal purposes in medical, pharmaceutical, and cosmetic industries [9].

Cellulolytic enzymes have been found in many organisms such as plants, animals, fungi, and bacteria [10]. Bacteria and fungi are among the favorable microorganisms for the production of these enzymes as they grow faster with much lower cost [5]. Moreover,

some of them are able to produce enzymes with special characteristics, such as stability in harsh temperature and pH conditions, which constitute high-quality features for some applications [10,11]. One strategy for finding enzymes with desirable catalytic properties is to discover new strains of microorganisms as a small percentage of the existent biodiversity has been isolated and cultured in the laboratory. The potential of algal and cyanobacterium species to be among the candidates for enzyme production has been reported [12].

Cyanobacteria (formerly blue-green algae) are a ubiquitous and abundant component of phytoplankton. They first appeared approximately 3.5 billion years ago, triggering major ecological change through the photochemical release of molecular oxygen from water into the atmosphere. They occasionally form blooms and constitute a wide range of morphological groups. These microorganisms are able to grow under different conditions, and they possess the ability to grow under autotrophic, mixotrophic, and heterotrophic conditions [13,14]. Different cyanobacterial species and strains are thought to be promising as sources of proteins, exopolysaccharides, carbohydrates, and lipids, suitable for the production of highly valuable products [15,16], whereas they are also able to produce nutrients, hormones, and metabolites that are appropriate to enhance plant growth and their energy yield [17]. Microbial biomass, agricultural wastes, and forest residues are the most promising sustainable energy sources for biofuel production [18,19]. Some cyanobacteria can be used for hydrogen production, as they are able to accumulate a great amount of starch on their biomass [20]. The main energy storage components of cyanobacterial biomass, carbohydrates, lipids, and proteins form a potential feedstock that can be converted to bioenergy. The lipid fraction can be converted to biodiesel by transesterification, the carbohydrates to bioethanol by fermentation under dark, anoxic conditions, whereas, alternatively, using anaerobic digestion, all three fractions can be converted to biogas [21]. Moreover, filamentous cyanobacteria are able to use agro-industrial wastes and wastewaters as substrates to help them grow, while, at the same time, they lead to a reduction of the organic load of the wastes [22]. Recent techno-economic analyses and life cycle assessments of cyanobacterial biomass suggest a biorefinery approach as a promising zero-waste technology. In the biorefinery, the cyanobacterial biomass should be fully utilized using various enzymatic, chemical, and physical treatments, including heat and water flows [23].

More specifically, cyanobacteria of the *Pseudanabaena* family are known as important sources of metabolites with great biotechnological and pharmaceutical applications. For instance, *Pseudanabaena* cells are used for the production of AgNPs with great bactericidal properties [24] or for biodiesel production when cultivated at forest biomass hydrolysates [25]. *Pseudanabaena galeata* extracts show cytotoxic and antiviral properties [26]. Phycobiliproteins from *Pseudanabaena tenuis* show antioxidant properties against mercury chloride oxidation stress [27].

Lake Pamvotis is an ancient suburban Mediterranean lake, having been in continual existence throughout the Plio-Pleistocene period. It is characterized by rich biodiversity, and for this reason, it has been placed under European Community legislation [28]. Lake Pamvotis has suffered from eutrophication for the last three decades, with periodic blooms of cyanobacteria. Moreover, this lake is a habitat for a variety of filamentous cyanobacteria, which are divergent from other populations [29].

In the present study, we presented a cyanobacterium strain (*Pseudanabaena* sp.) as a novel β-glucosidase producer. *Pseudanabaena* sp. strain Pamv7, which belongs to filamentous cyanobacteria, has been isolated from a local lake (Lake Pamvotis) in Greece. The ability of the strain to grow in heterotrophic conditions, under a variety of different organic sources, was studied, whereasβ-glucosidase production was studied when the strain was grown using cellobiose as an organic source. Moreover, the cyanobacterium cells were cultivated at different substrate concentrations, different nitrogen sources, different concentrations of the optimal one, and different temperatures toward the optimization of culture conditions for β-glucosidase and biomass production. At the end of the above process, cyanobacterium cells were cultivated under the optimal conditions for the enzyme production in a bioreactor system of 2 L. The results of this work suggest the *Pseudan-*

abaena sp. as a new innovative candidate for the simultaneous cyanobacterial biomass and industrial enzyme production.

2. Materials and Methods

2.1. Field Sampling

Lake Pamvotis is an ancient shallow lake located in NW Greece (Ioannina, Greece (39°39′45″ N 20°53′06″ E)). In our analysis, we used surface water samples from a sample station (SS). Water samples were collected immediately below the surface in sterile bottles. Samples were kept cool until they were processed within 1 h from the collection.

2.2. Isolation

A volume of 100 µL water sample from Lake Pamvotis was interspersed on BG11 medium agar plates. A variety of cyanobacterial colonies considering the morphological features were picked from the plates and purified further on BG11 agar plates. To isolate and cultivate cyanobacteria, we used a BG11 medium (Fluka Analytical, Sigma-Aldrich, Burlington, MA, USA) under aerobic conditions, 23 ± 1 °C temperature, and 12:12 h light and dark periods with white fluorescent irradiation. The intensity of light during the light period was 80 µmol of photons m^{-2} s^{-1}.The purity of the strains was assessed by a microscopic examination and by plating onto LB/agar plates for 48 h to exclude the possibility of the presence of heterotrophic bacteria.

2.3. DNA Extraction, PCR Amplification, Cloning, and Sequencing

We performed DNA extraction using an Ultra Clean soil DNA isolation kit from MoBio Laboratories (Power Soil DNA Isolation kit, Carlsbad, CA 92010, USA) in accordance with the manufacturer's instructions.

PCR amplification was performed in a BioradiCycler in a 50µL reaction volume. For 16S rDNA amplification, we used the forward primer 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) [30] and the reverse primer 518R (5′-ATTACCGCGGCTGCTGG-3′) [31]. PCR conditions were as described earlier [31].

For internal transcribed spacer sequence (ITS) amplification, we used the forward CSIF (5′-GYCACGCCCGAAGTCRTTAC-3′) primer and the reverse ULR (5′-CCTCTGTGTGCCT-AGGTATC-3′) primerearlier [32]. PCR conditions were as described earlier [32]. PCR products were purified using a Macherey-Nagel DNA cleanup kit (NucleoSpin Gel and PCR Clean-up, Duren, Germany). Afterward, they were cloned using a TOPO TA cloning kit (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions. Inserts were fully determined by sequencing, which was performed by Eurofins Genomics/VBC Biotech (Vienna, Austria).

2.4. Nucleotide Sequences and Accession Numbers

The sequences were deposited at GenBank and were assigned accession numbers MN047231 (16S rRNA partial-ITS full length-23S rRNA partial) and MN047223 (16S rRNA partial).

2.5. Phylogenetic Characterization

All sequences were compared against each other for similarity using NCBI-BLAST, and the results were analyzed for obtaining their phylogenetic affiliation. Phylogenetic analysis was performed using MEGA 6.1 software (https://mega.software.informer.com/6.1/, accessed on 7 September 2022). Trees were constructed using the neighbor-joining method with Jukes–Cantor distance correction [33].

2.6. Screening of Substrates for Heterotrophic Growth of Pseudanabaena sp. *Cyanobacterium (BIOLOG)*

To achieve a first determination of the possible carbon sources that can be used as substrates for the heterotrophic growth of the cyanobacterium, we used a rapid method, the Biolog-ECO plate technique. The Biolog-ECO plate consists of 96 wells, which means

31 sole carbon sources and one water blank, while each one has three replicates. The inoculum for each well was prepared from a culture that was in exponential phase. The cyanobacterium solution was diluted in sterile water to Abs 0.100 at 600 nm; then 150 µL was inoculated into each well of the Biolog-ECO plate, and it was incubated in the dark in 23 ± 1 °C. The growth of the cyanobacterium was determined by periodically taking readings at 600 nm.

The carbon sources of the Biolog-ECO plate can be classified into six different biochemical categories (carbohydrates, carboxylic acids, amino acids, amines, phenolic compounds, and polymers) based on their compounds [34]. To determine which organic source is preferred by the cyanobacteria, the average absorbance at the endpoint in each category was calculated, as described by Choi K.H. et al. [35] and Tian-Yuan et al. [34], and compared among these categories.

2.7. Culture Growth Conditions of Pseudanabaena sp. *Cells and β-Glucosidase Production*

The culture conditions for *Pseudanabaena* sp. cells were as follows: 250 mL Erlenmeyer flask with a medium containing BG-11, D-cellobiose as carbon source, and a source of nitrogen, at 23 °C ± 1 °C, in the dark, and with continuous shaking at 100 rpm.

More specifically, for the optimization of carbon source concentration, cells were cultured at a range of 0 to 25 g/L cellobiose. After that, six different organic and inorganic nitrogen sources were tested, whereas the concentration of the optimum at the range of 0.5 to 2.5 g/L was also checked. Finally, the effect of temperature on growth and enzyme production was determined by testing 9 different temperatures, ranging from 15 to 35 °C.

For all the experiments, the inoculum constitutes 10% of the culture and was taken from heterotrophic cultures after the determination of their density. Every 24 h, we were taking samples to monitor the growth, biomass, enzyme production, and sugar consumption.

Using the optimum conditions for biomass and enzyme production, a time-course experiment in a 2-L bioreactor was taken place. Prior to heat sterilization (121 °C, 20 min), the pH was adjusted to 6.5 and not regulated during the cultivation. The inoculum constituted of precultures of 48 hold under the optimum culture conditions. Cells were harvested every 24 h.

The determination of cyanobacteria biomass was achieved by collecting samples and measuring the dry biomass. More specifically, a preweighed filter WHATMAN membrane of 0.45 micron diameter was used to filter the samples under vacuum (the volume of the samples was known). The filtrated samples were incubated at 60 °C overnight, and then the dried biomass was weighed. The concentration (mg/L) of the algal in the cultivation was calculated based on the difference in the weight of the membrane before and after the filtration. The growth was determined measuring the absorbance at 600 nm every 24 h. The specific growth rate (µ) was calculated from consecutive OD_{600} measurements at the exponential phase following the formula

$$\mu = \Delta ln \frac{OD_{600}}{\Delta t} \quad (1)$$

where t is time.

The amount of residual sugar during the time course of the experiment was color imetrically determined using a dinitrosalicylic acid (DNS) reagent as described by Miller (1959). Samples of Pamv7 were made up to 0.25 mL with milli-Q water, and 0.5 mL of the DNS reagent was added. The color obtained after boiling the mixture for 5 min and diluting it with 2 mL of milli-Q water was measured at 540 nm. The concentration (mg/mL) of the residual cellobiose was based on the standard equation

$$y = 2.246 * OD_{540} \quad (2)$$

where y is the concentration of cellobiose (g/L).

For β-glucosidase determination, cells were harvested every 24 h via centrifugation at 4000 rpm for 5 min and washed twice with milli-Q water. Then, they were suspended in milli-Q water and disrupted via ultrasonication. The debris and intact cells were removed via centrifugation at 4000 rpm for 5 min to obtain the crude cell extract while the β-glucosidase assay was described.

2.8. β-Glucosidase Assay

β-Glucosidase activity was determined by measuring the hydrolysis of p-nitrophenyl-β-D-glucopyranoside (pNPG). The content of pNPG in the reaction mixture (1 mL) was 2 mM in a 100 mM citrate-phosphate buffer (pH 6.0) with an appropriately diluted enzyme extract. The mixture was incubated for 15 min at 40 °C. The reaction was stopped by adding 0.2 mL of 10% Na_2CO_3, and the released p-nitrophenol was measured at 410 nm. All enzyme assays were performed at least triplicate. One unit (U) of β-glucosidase activity is defined as the amount of enzyme that liberates 1 μmol of p-nitrophenol per minute in the reaction mixture under the above assay conditions.

2.9. FT-IR Spectrum

An FTIR-8400 infrared spectrometer (Shimadzu, Tokyo, Japan) equipped with a deuterated triglycine sulfate (DTGS) detector was used for the Fourier transform infrared spectroscopy (FTIR) analysis. The spectra were recorded in the range of 400 to 4000 cm^{-1} with an average of 32 scans. The samples were in the form of KBr pellets (60 mg) containing 1 wt% of the cyanobacterium cells.

2.10. Protein and Lipid Determination

For protein determination, the bicinchoninic acid assay (BCA) was used as described first by Smith et al. [36].

For lipid determination, a modified version of Folch's method was used [37]. The cell dry biomass was powdered and incubated with a mixture of methanol–chloroform (1:2 v/v) overnight. The next day, the mixture was centrifuged, after the addition of water, creating two phases with the cell pellet in the middle. The lower chloroform phase containing the extracted lipids was transferred to preweighted glass vials, which were subsequently dried. Lipid weight was determined based on the weight difference after drying, and the lipid concentration (g/L) was calculated based on the initial sample.

The lipid content (% w/w) was calculated using the following equation:

$$\text{lipid content} = \frac{\text{lipid concentration}}{\text{biomass concentration}} \times 100$$

2.11. Statistical Analysis

In the current study, all experiments were conducted in triplicate. The data were expressed as mean ± standard deviation and were analyzed using one-way analysis of variance(ANOVA) with Duncan's multiple range test (DMRT), with p values < 0.05 being regarded as significant.

3. Results and Discussion

3.1. Isolation and Phylogenetic Characterization

Several cyanobacterial strains were isolated from water samples of Lake Pamvotis. The results of the phylogenetic analysis revealed that the strain named "Pamv7" is a new cyanobacterial strain. With the sequencing of 16S rDNA and the ITS region of Pamv7 strain, the results of the BLAST analysis revealed sequence identities in comparison with others that are available in GenBank. More specifically, the 16S rDNA sequence displayed 99.3% identity to *Pseudanabaena* sp. 0830-3 in the GenBank database. Furthermore, the ITS sequence displayed 91.9% identity to *Pseudanabaena* sp. RK_4_6 and 88% identity to *Limnothrixredekei* CCAP 1443/1 in the GenBank database as presented in Table 1.

Table 1. Comparison of *Pseudanabaena* sp. 16S rDNA and "Pamv7" ITS sequences to already deposited sequences using BLAST software in NCBI website (https://www.ncbi.nlm.nih.gov (accessed on 15 June 2019).

Pamv7	Species GenBank	% Overlapping	Identity	Accession (15 June 2019)
16S rDNA partial (471 bp)	*Pseudanabaena* sp. 0830-3	100	99.3%	AB936777
ITS full length (703 bp)	*Pseudanabaena* sp. *RK_4_6*	99	91.9%	KY379894
ITS full length (703 bp)	*LimnothrixredekeiCCAP 1443/1*	99	88%	AJ580007

The results of the phylogenetic analysis revealed that the "Pamv7" cyanobacterium is related to *Pseudanabaena* sp. The distance trees, which are presented in Figure 1, are based on the 16S rDNA (Figure 1A) sequence and ITS sequence (Figure 1B) of Pamv7 and several sequences with the highest similarities retrieved from GenBank/EMBL/DDBJ databases. Based on these two constructed phylogenetic trees, the Pamv7 sequence, which is presented with a red symbol, revealed that the filamentous strain isolated from Lake Pamvotis belongs to cyanobacteria and is affiliated to *Pseudanabaena* sp. and *Limnothrix* sp. All in all, the strain of Pamv7 belongs to the Pseudanabaenaceae family, a small family of cyanobacteria, which contains the ecologically important and polyphyletic genera of *Limnothrix* and *Pseudanabaena* [38]. A microscopic picture of the strain is also presented in Figure 1A.

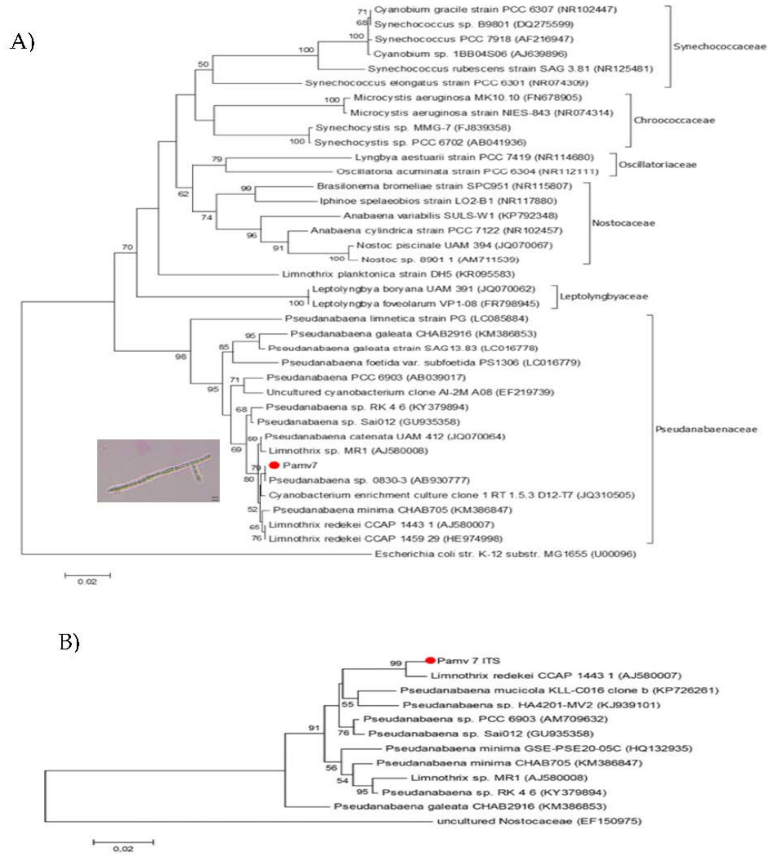

Figure 1. Phylogenetic analysis of *Pseudanabaena* sp. (**A**) Distance tree based on alignment of *Pseudanabaena* sp. 16S rDNA sequence (•), and sequences with highest similarities retrieved from GenBank/EMBL/DDBJ databases (branches with bootstrap values below 50% have been deleted in this presentation). Microscopic picture is also presented. (**B**) Distance tree based on alignment of *Pseudanabaena* sp. ITS sequence (•), and sequences with highest similarities retrieved from GenBank/EMBL/DDBJ databases (branches with bootstrap values below 50% have been deleted in this presentation).

3.2. Heterotrophic Characterization by Biolog-ECO Plate

Biolog-ECO plates, containing 31 carbon sources, can determine the metabolic fingerprint of each microorganism. It is achieved through the microorganism's reaction on the different carbon sources, whichindicates the cell's metabolic situation. The result of the Biolog-ECO test showed that *Pseudanabaena* sp. could accumulate a wide range of organic substrates. Cyanobacterium *Pseudanabaena* sp. could metabolize 29 kinds of organic substrates among all 31 carbon sources in the Biolog-ECO plate, indicating cells' ability to achieve higher biomass under heterotrophic than autotrophic conditions [39]. In this research, according to their biochemical properties, the 31 substrates in the Biolog-ECO plate were classified into six categories: carbohydrates, carboxylic acids, amino acids, amines, phenolic compounds, and polymers, as described in Table 2. The absorbance of each biochemical category at the endpoint (120 h) is presented in Figure 2 as a whisker plot graph. The strain showed diverse performances on different categories of substrates. The assailable substrates of *Pseudanabaena* sp. covered all five categories, which indicates that the strain could utilize a broad range of organic substrates. The metabolic properties of *Pseudanabaena* sp. mainly utilized amino acids, carbohydrates, carboxylic acids, polymers, phenolic compounds, and amines, as presented in Table 2. These results come in accordance with previous studies of heterotrophic cultivations of cyanobacteria, which were using different carbohydrates, i.e., monosaccharides, disaccharides, and polysaccharides, as carbon sources [40,41].

Table 2. Specific growth rates μ (d^{-1}) of *Pseudanabaena* sp. in Biolog-ECO plate. Organic substrates are assigned into diverse biochemical categories, according to description of Keun-Hyung Choi and Fred C. Dobbs [16].

Substrate	Specific Growth Rate	Substrate	Specific Growth Rate
Carbohydrates	**μ (d^{-1})**	**Carboxylicacids**	**μ (d^{-1})**
β-Methyl-D-glucoside	0.17 ± 0.01	*Pyruvicacidmethylester*	0.50 ± 0.01
D-Galactonicacid γ-lactone	0.29 ± 0.01	*D-Galacturonicacid*	0.42 ± 0.01
D-Xylose	0.42 ± 0.01	*γ-Hydroxybutyricacid*	0.15 ± 0.01
i-Erythritol	0.15 ± 0.01	*D-Glucosaminic acid*	0.39 ± 0.01
D-Mannitol	0.52 ± 0.01	*Itaconic acid*	0.26 ± 0.01
N-Acetyl-D-glycosamine	0.40 ± 0.01	*α-KetobutyricAcid*	0.42 ± 0.01
D-Cellobiose	0.60 ± 0.01	*D-Malic acid*	0.28 ± 0.01
Glucose-1-phosphate	0.16 ± 0.01		
α-D-lactose	0.19 ± 0.01	**AminoAcids**	μ (d^{-1})
d.l-A-Glycerol phosphate	0.32 ± 0.01	*L-Arginine*	0.43 ± 0.01
		L-Asparagine	0.70 ± 0.01
Amines	μ (d^{-1})	*L-Phenylalanine*	0.28 ± 0.01
Phenylethylamine	0.08 ± 0.01	*L-Serine*	0.48 ± 0.01
Putrescine	0.18 ± 0.01	*L-Threonine*	0.28 ± 0.01
Polymers	μ (d^{-1})	**Phenoliccompounds**	μ (d^{-1})
Tween 40	0.38 ± 0.01	*2-Hydroxy benzoic acid*	0.00 ± 0.01
Tween 80	0.37 ± 0.01	*4-Hydroxy benzoicacid*	0.37 ± 0.01
α-Cyclodextrin	0.16 ± 0.01		
Glycogen	0.14 ± 0.01		

The metabolic patterns of *Pseudanabaena* sp. for each carbon substrate in Biolog-ECO plates are presented in Table 2, where the specific growth rates of *Pseudanabaena* sp. in the presence of each substrate are presented. More specifically, according to μ (d^{-1}), *Pseudanabaena* sp. metabolizes better the substrates that belong to amino acids and especially L-asparagine, followed by glycyl-L-glutamic acid, L-serine, and L-arginine. Among the carbohydrates, the microorganism prefers D-cellobiose, D-mannitol, D-xylose,

and N-acetyl-D-glycosamine, whereas among the carboxylic acids, pyruvic acid methyl ester acid obviously metabolized better, followed by a-ketobutyric acid, D-galacturonic acid, and D-glucosaminic acid.

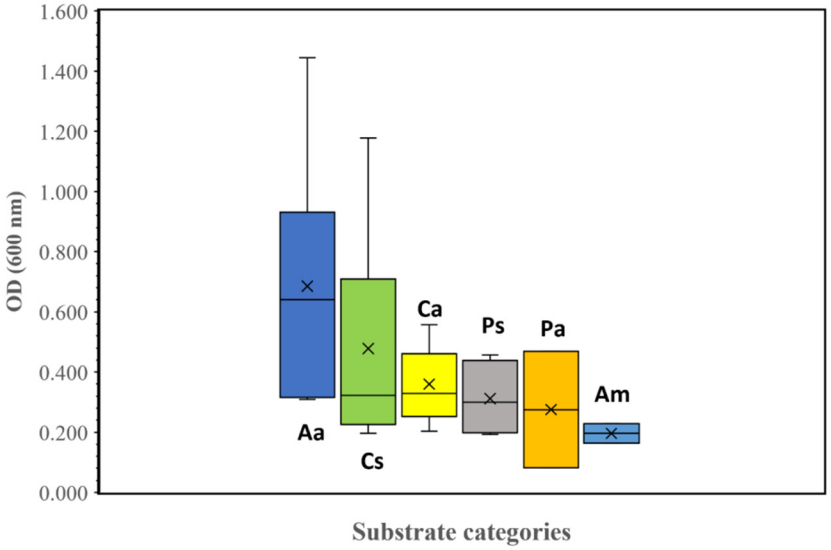

Figure 2. Absorbances of assimilable substrates for cyanobacterial strain *Pseudanabaena* sp. in Biolog-ECO plate. Substrates in Biolog-ECO plates are assigned into six categories according to their biochemical properties, where columns from left to right represent amino acids, carbohydrates, carboxyl acids, polymers, phenolic acids, and amines, respectively.

3.3. Optimization of Cellobiose Concentration on Biomass and β-Glucosidase Production in Pseudanabaena sp. *Cells*

Among the various organic substrates that were tested with Biolog, cellobiose is the preferred carbohydrate source, as it shows a specific growth rate of 0.6 d^{-1}. Moreover, it is observed that *Pseudanabaena* sp. cells can produce β-glucosidase, when heterotrophically cultivated, using cellobiose. We should highlight that, until now, the basic β-glucosidase producers were bacteria and fungi, whereas it is the first time that a cyanobacterial strain produces enzyme. Therefore, it is of crucial importance to find out the optimum conditions for both biomass and enzyme production.

First, we checked the effect of cellobiose concentration, as it constitutes the carbon source, which is necessary for heterotrophic cell growth. Therefore, in this part, we examined the effect of cellobiose concentration on biomass and enzyme production by testing six different concentrations, starting from cellobiose absence until the presence of 25 g/L cellobiose. As shown in Figure 3, the cell dry weight increased when the cellobiose concentration changed from 0 to 20 g/L, whereas over this rate, a decrease in the biomass formation was observed. More specifically, when the carbon source concentration was 20 g/L, biomass had its highest value reaching 2.63 ± 0.03 g/L. This value appears to be the minimum difference from the biomass at the 10 and 15 g/L substrate, which were 2.24 ± 0.02 and 2.59 ± 0.03 g/L, respectively. At the same time, the enzyme activity significantly increased when the cellobiose concentration increased from 5 to 10 g/L, remaining approximately constant on bigger rates. As shown in Figure 3, it increased from 11.73 ± 0.78 (5 g/L) to 26.45 ± 0.78U/L (10 g/L). These results come in accordance with other studies about the heterotrophic growth of microalgae, where low carbon concentrations are not enough for a satisfactory growth of the cells, whereas greater carbon concentrations were causing an inhibitory effect on the biomass or its production [42]. Moreover, the concentration of 10 g/L of carbon source—usually glucose on most of the cases—for the heterotrophic growth of different microalgal strains, seems to be the most appropriate, something that comes in accordance with our study [43,44]. We should mention that cell growth is highly related with both the strain and carbon source. For instance, the cyanobacterium *Phormid-*

ium sp. was able to produce 5.54 g/L biomass when cultivated using fructose as the carbon source compared with1.18 g/L when cultivated using sucrose [22,40]. Cyanobacterium *Nostocflagelliforme* reached a biomass of 0.41 g/L after 7 days of cultivation with fructose, whereas *Anabaena variabilis* reached 10 g/L with the same carbon source [22].

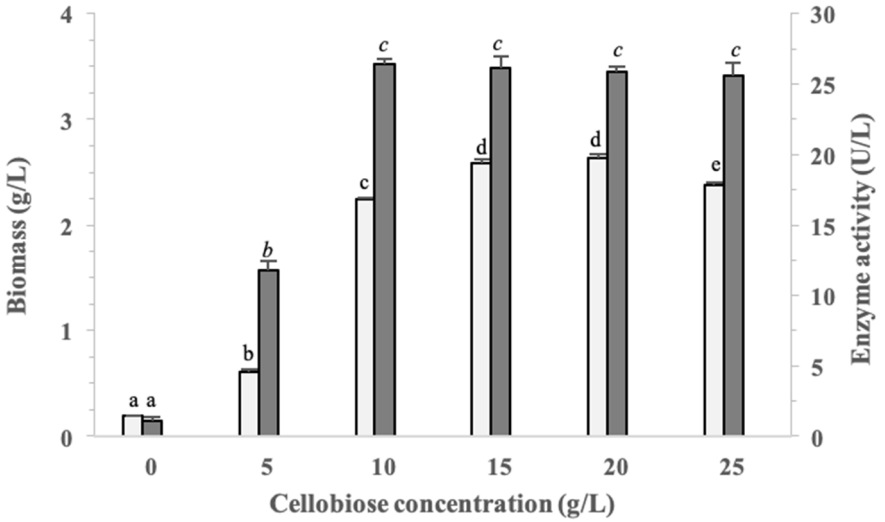

Figure 3. Biomass and β-glucosidase production by *Pseudanabaena* sp. under heterotrophic conditions in different cellobiose concentration; white column represents biomass production, and gray column the enzyme production. All measurements were performed in triplicate, whereas SD is represented. Different letters over bars with same color indicate differences according to Duncan's multiple range test DMRT with $p < 0.05$.

Therefore, regarding the fact that cell growth is highly dependent on the strain, carbon source, and its concentrations, 10 g/L cellobiose constitutes the greater option as *Pseudanabaena* sp. cells can produce a little lower biomass, but with great enzyme activity, and is presented as the most effective carbon concentration.

3.4. Optimization of Nitrogen Source on Biomass and β-Glucosidase Production in Pseudanabaena sp. *Cells*

After the carbon source, which is the cellobiose in the current work, the quality and quantity of the nitrogen source play a crucial role in microalgae growth and production of bioactive molecules [45]. As it is reported that microalgae have the ability to assimilate many nitrogen sources [46], keeping the cellobiose concentration at 10 g/L, we checked the effect of different nitrogen sources, some organic and some inorganic forms.

As shown in Figure 4, *Pseudanabaena* sp. cells did not even manage to grow under the presence of inorganic nitrogen sources. Both ammonium chloride and ammonium sulfate dramatically change the pH of cultures, as they are rapidly assimilated by the cells, having cell death as a result. Additionally, urea is related with urease presence, so the cyanobacterial cells are not able to assimilate the nitrogen source, overcome the adaptive phase, and continue in the exponential phase [47]. On the other hand, both yeast and peptone constitute organic nitrogen sources that are absolutely used by the cells. Therefore, they lead to normal growth and enzyme production. Moreover, complex nitrogen sources contain both growth factors, vitamins and amino acids, which may affect the growth process in a positive way [46]. The use of yeast led to 2.22 ± 0.04 g/L cell dry weight and 26.10 ± 0.75 U/L enzyme activity, whereas the use of peptone to 2.28 ± 0.75 g/L and 24.57 ± 0.64 U/L, respectively. Moreover, the peak of biomass and enzyme production happened earlier in the culture when yeast was the nitrogen source, while they were keeping these values, even when the substrate was exhausted about something that was not presented in the case of peptone. The above results come in accordance with other studies, showing that ammonium sources cause the inhibition of cell growth, whereas yeast

extract shows the greatest growth and protein production [46]. Moreover, *Spirulina platensis* and *Anabaena 7120* cells displayed the same tendency, where ammonium chloride, sulfate, and urea were causing dramatic growth inhibition, with sodium nitrate being the most suitable among them [48,49].

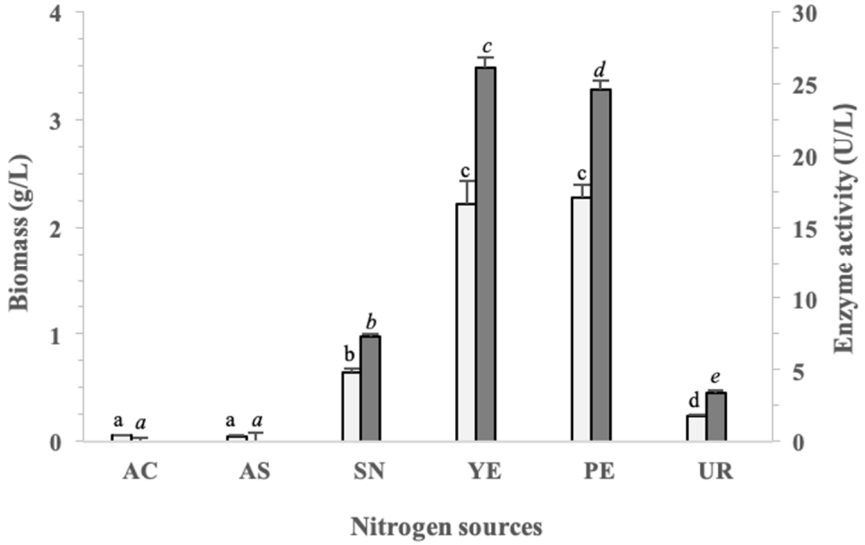

Figure 4. Cell dry production (g/L) and β-glucosidase activity (U/L) of *Pseudanabaena* sp. under heterotrophic conditions in different nitrogen sources (ammonium chloride (AC), ammonium sulfate (AS), sodium nitrate (SN.), yeast extract (YE), peptone (PE), and urea (UR)).In all cases, dark gray column represents biomass production, whereas gray column the enzyme production. Activity (U/L) was measured against 2 mM pNPG in citrate-phosphate buffer, pH 6.0, at 40 °C. In all cases, samples were collected daily, and all reactions and measurements were performed in triplicate, while SD is represented. Different letters over bars with same color indicate differences according to Duncan's multiple range test DMRT with $p < 0.05$.

We should mention that the preferred nitrogen source is also highly dependent on the desirable product. For instance, *Dunaliellasalina* prefers ammonium than nitrate sources for protein production, whereas *Chlorella sorokiniana* prefers urea or nitrate instead of ammonium for lipid production [50,51].

3.5. Optimization of Nitrogen Concentration on Biomass and β-Glucosidase Production in Pseudanabaena sp. *Cells*

It has already been reported that the nutrients of the medium, such as carbon and nitrogen and their concentrations, influence the cell growth [52]. Moreover, nutrient and especially nitrogen limitations can lead to enhancement of production of specific bioactive compounds [42]. The optimal concentrations of carbon and nitrogen sources are both strain- and product-dependent. Therefore, selecting yeast as the most appropriate nitrogen source, we continued with the examination of the effect of yeast concentration on both biomass and enzyme production. For this purpose, we checked four different yeast concentrations, ranging from 0.5 to 2.5 g/L. As presented in Figure 5, the increase in yeast concentration from 0.5 to 1.5resulted in a biomass and enzyme production increase, reaching their peak values of 2.73 ± 0.15 g/L and 42.44 ± 0.09 U/L, respectively. On a yeast concentration of 2 g/L, both biomass and enzyme productions were about the same, whereas on higher yeast concentrations, both biomass and enzyme production were half. Therefore, the optimal yeast concentration and most cost-effective is 1.5 g/L.

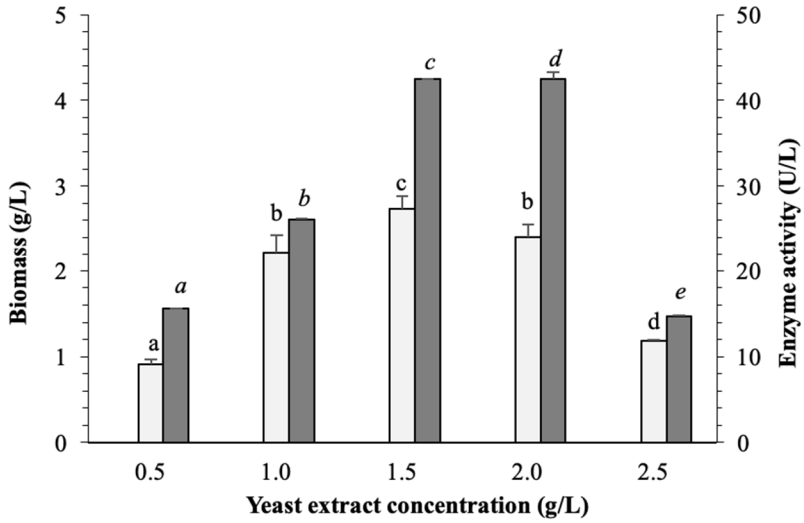

Figure 5. Cell dry production (g/L) and β-glucosidase activity (U/L) of *Pseudanabaena* sp. under heterotrophic conditions in different nitrogen concentrations (0.5, 1.0, 1.5, 2.0, 2.5 g/L—from left to right, respectively). In all cases, dark gray column represents biomass production, whereas gray column the enzyme production. Activity (U/L) was measured against 2 mM pNPG in citrate-phosphate buffer, pH 6.0, at 40 °C. In all cases, samples were collected daily, and all reactions and measurements were performed in triplicate, while the SD is represented. Data associated with the same letter over bars with same color indicates no significant difference at $p < 0.05$.

3.6. Optimization of Temperature on Biomass and β-Glucosidase Production in Pseudanabaena sp. *Cells*

After the purification of nutrient environment with carbon and nitrogen sources, the abiotic factor of temperature plays also a crucial role on growth and enzyme activity. It is reported that most of the microalgal species appear to have an optimum temperature range of 20–30 °C [53]. In most cases, temperatures lower than 16 °C slow the growth, whereas temperatures over 35 °C lead to cell death. For temperatures out of the above ranges, there are specific strains of microalgal species that can survive, grow, and produce biomolecules. For instance, the thermostable *Cyanobacterium aponinum* appears to have its optimal growth at 40 °C [54]. Testing temperatures from 15 to 35 °C, as it is obvious from Table 3, biomass production shows a sharp increase when culture temperature was increased from 15 to 23 °C.

For the temperature range of 23–28 °C, biomass production was about the same, displaying a max of 2.80 ± 0.19 g/L (at 23 °C) and a min of 2.46 ± 0.11 g/L (at 28 °C). At growth temperatures over 30 °C, *Pseudanabaena* sp. cells showeda decrease in their growth, with a harsh reduction at 35 °C, which resulted in less than half biomass production. These results come in accordance with the optimal temperature ranges for most of microalgal species. Moreover, *Pseudanabaena* sp. is isolated from Lake Pamvotis, whose temperature ranges from about 5 to 26 °C depending on the season, with most of the months being over 12 °C [55], something that comes in accordance with the preferred temperatures from the cells.

The highest enzyme activity was 44.69 ± 0.75 at 23 °C, having a slight difference with this at 25 and 28 °C, being 43.05 ± 0.54 U/L and 42.01 ± 0.88 U/L, respectively. Therefore, these temperature values seem to be the optimal values, with temperature values excluded from them resulting in decreasing rates of both the biomass and enzyme.

Table 3. Cell dry production (g/L) and β-glucosidase activity (U/L) of *Pseudanabaena* sp. under heterotrophic conditions in different temperatures (15, 18, 20, 23, 25, 28, 30,32, and 35 °C—from left to right, respectively). In all cases, dark gray column represents biomass production, whereas gray column the enzyme production. Activity (U/L) was measured against 2 mM pNPG in citrate-phosphate buffer, pH 6.0, at 40 °C. In all cases, samples were collected daily, and all reactions and measurements were performed in triplicate, while the SD is represented. Data associated with the same letter at the same column are not significantly different; $p < 0.05$. All means are significantly different from control.

Cultivation Temperature (°C)	Production by *Pseudanabaena* sp.	
	Biomass (g/L)	β-Xylosidase (U/L)
15	1.64 ± 0.12 a	30.27 ± 0.05 a
18	1.63 ± 0.15 a	30.01 ± 0.46 a
20	2.00 ± 0.02 b	37.66 ± 0.41 b
23	2.80 ± 0.19 c	44.69 ± 0.75 c
25	2.76 ± 0.09 c	43.05 ± 0.55 cd
28	2.46 ± 0.11 d	42.01 ± 0.89 d
30	2.10 ± 0.10 b	34.10 ± 0.72 e
32	2.02 ± 0.16 b	30.27 ± 0.34 a
35	1.01 ± 0.06 e	12.64 ± 0.42 f

3.7. Growth of Pseudanabaena sp. *under Optimum Conditions*

Taking all the above into consideration and knowing that the aim of the purification process is the highest enzyme production in parallel with the cost minimizing, for the optimal production, we selected and used the following parameters: 10 g/L cellobiose, 1.5 g/L yeast and 23 ± 1 °C. Figure 6 presents the growth, enzyme production, and cellobiose consumption curves, resulting from cell culture in a bioreactor of 2 L volume, using the optimum conditions referred above. As shown in Figure 6, *Pseudanabaena* sp. cells enter the exponential phase even from the first hours of inoculation, whereas at 96 h, they reach close to the peak. Moreover, as it is observed, enzyme production is drastically increased from the first hours of cultivation, when the cells are at its exponential phase. As it is observed, the enzyme activity is about 18 U/L at the first 48 h of cultivation, whereas it extremely rapidly reaches close to its peak value of 42.2 U/L at 72 h and the peak at 160 h with 44.7 U/L enzyme production; at the same time, cellobiose concentration is less than 1/10 of the initial. Therefore, *Pseudanabaena* sp. cells were producing β-glucosidase during their station phase, while the cellobiose was already consumed. The maximum activity of produced β-glucosidase remained stable until the end of culture.

It is known that heterotrophic culture conditions cause a great enhancement of biomass production, which also happened in our study. We should mention that the production of cell dry biomass is highly related with many conditions such as the carbon source, a possible nutrient starvation, the pH, or the temperature of the culture [18]. Moreover, the above results are in accordance with previous studies indicating the instant insertion of cells in the exponential phase even from the first hours of culture, with enzyme production in parallel [40,56,57].

In addition, FTIR spectra (Figure 7) characterized the specific macromolecules that can be detected and derived from *Pseudanabaena* sp. lyophilized cells, cultivated under the above optimum conditions [58]. Proteins, lipids, and polysaccharides correspond in characteristics bands of FTIR spectra of cells in the range of 4000–400 cm^{-1} [59,60]. O–H and N–H vibration stretching can be observed at 3280 cm^{-1}, resulting in the carbohydrate and protein content. At the wavelength range 3000–2800 cm^{-1}, C–H stretching vibration can be observed that represents the acyl chains. These bands are characteristic of lipid compounds. The CH_3 and CH_2 absorption bands are observed at 2998–2829 cm^{-a} and represent the lipid and carbohydrate content.

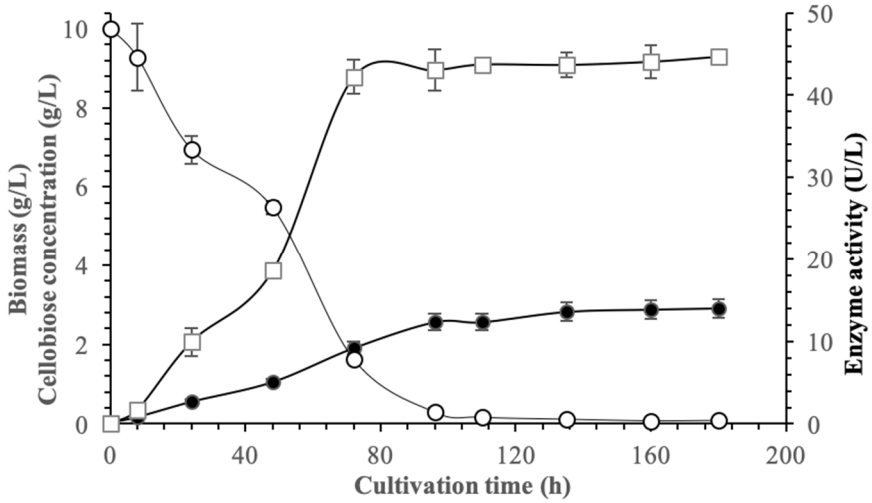

Figure 6. Time course showing cultivation of *Pseudanabaena* sp. under optimum heterotrophic conditions in cellobiose (10 g/L) and yeast (1.5 g/L) and 23 °C. Cell dry weight (g/L) (black circles), β-glucosidase production (U/L) (white squares), and concentration of residual sugars (white circles) during cultivation time are presented with black, dark gray, and light gray lines, respectively. Activity (U/L) was measured against 2 mM pNPG in citrate-phosphate buffer, pH 6.0, at 40 °C. In all cases, samples were collected daily, and all reactions and measurements were performed in triplicate, while the SD is represented.

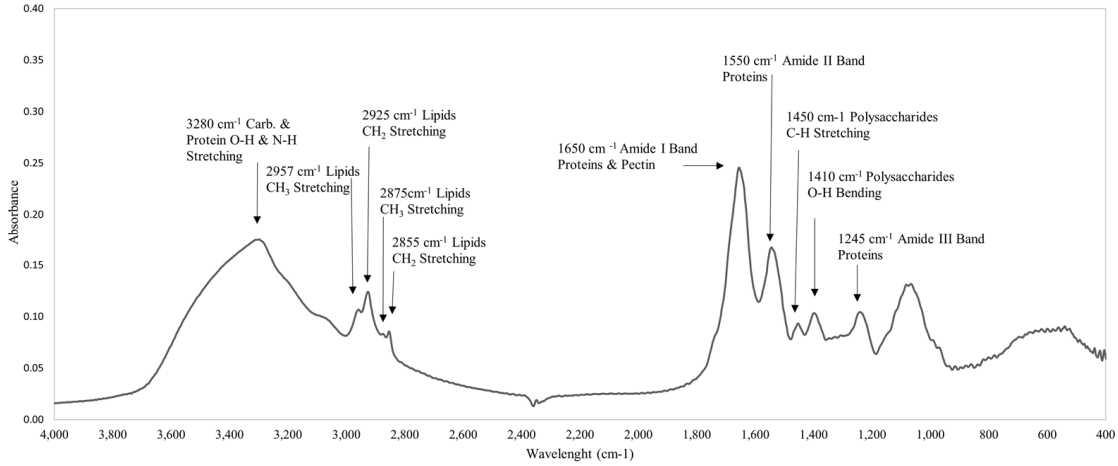

Figure 7. FTIR spectra of *Pseudanabaena* sp. cells. Each peak represents a specific molecular movement. Functional groups are represented.

The band at 1650 cm^{-h} represents the protein C–O stretching vibrations of peptide bonds (amide I). The band at 1550 cm^{-r} represents the N–H bending vibrations and C–N stretching vibrations that are considered for use in the estimation (amide II). At 1186–1350 cm^{-1}, C–H and O–H deformations are present. At the wavelength 1245 cm^{-r}, amide III band is present, resulting from several coordinate displacements. Polysaccharides have characteristic absorption bands around 1200–950 cm^{-o} representing the C–O–C stretching band. The presence of the detected lipids, polysaccharides, amides, proteins, and carbohydrates constitutes characteristic molecules of cyanobacteria species as also previously reported [61,62].

Continuing with a first determination of the protein and lipid content, *Pseudanabaena* sp. cells were capable of producing about 22.5 ± 0.8% *w*/*w* and 43.3 ± 0.9% *w*/*w*, respectively. The above results constitute one more evidence about the importance of the novel cyanobacterial cells, as they constitute the raw material to produce highly valuable products, displaying a plethora of applications. It is known that proteins derived from microalgae are

used in the food industry, nutraceuticals, pharmaceuticals, and cosmetics [63–65]. On the other hand, lipids also constitute a great part of cell content, something that come in accordance with previous studies, an indication that heterotrophic culture conditions enhance lipid production [66]. Depending on the type of produced lipids, the appropriate applications differ. For instance, PUFAs are more appropriate as nutraceuticals, whereas MUFAs and SFAs find application on energy industry and more usual in biodiesel production [67].

4. Conclusions

In this study, an isolated cyanobacterium from the eutrophic lake Pamvotida was identified as *Pseudanabaena* sp. The strain heterotrophically grows with a variety of organic sources. The best assimilable classes of organic molecules are carbohydrates, carboxylic acids, and amino acids, as determined by Biolog microplate experiments. *Pseudanabaena* sp. rapidly heterotrophically grows using cellobiose as a carbon source, reaching high biomass production. At the same time, the strain quantitatively produced an intracellular β-glucosidase reaching high productivity, with characteristics that make it appropriate for many applications. The optimal culture conditions for the greatest and at the same time more affordable enzyme conditions were determined, whereas the enzyme was produced at a bioreactor system of 2 L through a time course experiment, showing that the enzyme was produced early during the culture. This is the first report of β-glucosidase production by a cyanobacterium strain. Work is already in progress toward the purification and characterization of the enzyme.

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