Bioremediation with an Alkali-Tolerant Yeast of Wastewater (Nejayote) Derived from the Nixtamalization of Maize

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Abstract: Nejayote, the wastewater from the nixtamalization of maize, is difficult to biodegrade due to its abundant calcium content; low levels of nitrogen, phosphorus, and easily assimilable sugars; elevated pH; and high chemical oxygen demand (COD). The aim of the present study was to isolate microorganisms capable of utilizing filtered nejayote (NEM) as the only source of carbon for growth and to test the best microorganism for the bioremediation of this wastewater by lowering the level of pH and COD. Of the 15 strains of microorganisms tested, *Rhodotorula mucilaginosa* LCRE was chosen and identified using molecular techniques. Subsequently, its growth kinetics were characterized during cultivation in unenriched NEM (control) and NEM enriched with nitrogen and phosphorus salts. *R. mucilaginosa* LCRE showed a greater growth ($6.9 \leq X \leq 8.9$ g L^{-1}), biomass yield ($0.33 \leq Y_{X/S} \leq 0.39$ g g^{-1}), and specific growth rate ($0.748 \leq \mu \leq 0.80$ day^{-1}) in the enriched versus control NEM ($X = 6.55$ g L^{-1}, $Y_{X/S} = 0.28$ g g^{-1}, and $\mu = 0.59$ day^{-1}). However, a higher total sugar consumption (94.98%), better COD removal efficiency (75.5%), and greater overall COD removal rate (1.73 g L^{-1} h^{-1}) were found in the control NEM. Hence, *R. mucilaginosa* LCRE holds promise for the efficient bioremediation of nejayote without costly pretreatments or nutrient supplementation.

Keywords: bioremediation; nejayote; nixtamalization; *Rhodotorula mucilaginosa*; wastewater treatment; nutrient supplementation

1. Introduction

The process of nixtamalization, which consists of cooking maize in limewater, has been a part of Mexican traditional cooking since the pre-Columbian era. After maize is cooked for several hours, it is washed abundantly with water. The purpose of the process is to remove the hard pericarp layer and the endosperm cells, resulting in a soft kernel and the gelatinization of the starch, respectively. As a consequence, it is easier to grind the maize and form the soft dough used to make tortillas, the basic food of the Mexican culture [1,2]. Nixtamalization improves the nutritional quality of maize by increasing the bioavailability of calcium and vitamin B_3 (niacin) as well as the digestibility of the proteins. Additionally, it decreases the mycotoxins of maize [3].

Although nixtamalization enhances the flavor and nutritional value of maize, it has an ecological cost. The wastewater, known as nejayote, is difficult to biodegrade due to its high calcium content; low concentration of nitrogen, phosphorus, and easily assimilable sugars; elevated pH; and high chemical oxygen demand (COD). The approximately 75 L of water employed for the nixtamalization of 50 kg of maize generates a similar quantity of nejayote [4]. In Mexico alone, around 14.4 million m^3 of highly contaminated wastewater is produced each year from this process [5–7]. The contamination stems mainly from elevated levels of the following: pH (up to 12), COD (2500–40,000 mg O_2 L^{-1}), biochemical oxygen demand (BOD) (190–8100 mg O_2 L^{-1}), and inorganic material. The latter is due to the abundance of calcium (about 1526 mg L^{-1}) derived from the limewater [5,7–10]. Nejayote

far exceeds Mexican norms for the disposal of wastewater, which allows for a maximum COD of 150 mg O_2 L^{-1} and pH of 10 [11,12]. Unfortunately, most of this wastewater is thrown into bodies of water or storm sewers [5–7].

Various alternatives exist for processing nejayote to lower the level of contamination. Firstly, it can be processed to extract certain biomolecules that are valuable for the food, pharmaceutical, cosmetic, and other industries [13]. For example, the ferulic acid in nejayote has antioxidant and anti-inflammatory properties [6,14], and the peptides derived from maize proteins potentially have antihypertensive and chemoprotective effects [15]. Secondly, it might also be utilized as a fertilizer for crops [16] or as an auxiliary substrate for the germination of seeds [17]. Thirdly, microorganisms tolerant to the harsh conditions of nejayote are able to consume organic material in aerobic reactors, mainly for the growth of biomass [7,18,19]. In anaerobic reactors, the strategy of generating clean energy in the form of methane [20] and biohydrogen [21–23] has been explored.

The fourth possibility is to treat nejayote to diminish the levels of pH, COD, and calcium before disposal. Among the technologies proposed are filtration, microfiltration, and ultrafiltration to separate the insoluble solids [8,10,24], as well as coagulation–flocculation with chitosan [4], enzymatic treatments accompanied by adsorption [25], and nanofiltration and flocculation [26]. Many of the aforementioned treatments have been carried out in pilot studies [27]. Even if further research made the implementation of one of these methods feasible on a larger scale, the enzymes required to catalyze the different reaction steps would be costly [28]. Membrane treatment, on the other hand, must address various issues such as the sedimentation caused by a high level of calcium [29].

Some authors have suggested recycling nejayote for subsequent nixtamalization, but this would increase the COD of the wastewater, making its treatment even more challenging [4]. Reducing water consumption in the nixtamalization process has been explored to limit the quantity of effluents and residual calcium [13].

The treatment of nejayote entails biotechnological challenges because the growth of microorganisms is inhibited by its elevated levels of pH, COD, and calcium and the presence of phenolic compounds [4]. The aim of the current contribution was to identify and test a microbial strain capable of decreasing the high level of contamination of nejayote. After the cultivation in filtered nejayote (NEM) of 15 microbial strains from different sources, the one that produced the greatest decrease in the COD was selected. It was subjected to molecular identification and then cultivated in unenriched (control) NEM and NEM enriched with different combinations of nitrogen and phosphorus salts. A comparative analysis was conducted between the control and enriched NEM regarding the growth kinetics of the selected microorganism, total sugar consumption, COD removal, and change in pH.

2. Materials and Methods

2.1. Obtaining and Characterizing Nejayote

After nejayote was obtained from a nixtamal mill located in Villa Nicolás Romero, Mexico State, Mexico (19.590179, −99.294445), it was passed through coarse filter paper to remove the grains of maize and insoluble residues. The filtrate was stored under refrigeration at −20 °C until use. The filtered nejayote (NEM) was characterized by evaluating the level of pH, humidity, proteins, lipids, ashes, carbohydrates, total solids, volatile solids, total hardness, total sugars, reducing sugars, glucose, phosphorus, COD, total organic carbon (TOC), total nitrogen, and assimilable nitrogen.

2.2. Culture Media

After being sterilized in an autoclave (at 121 °C for 15 min), the filtered nejayote (without the addition of salts) served as the culture medium to test the growth of the isolated microorganisms. To form a solid medium, 20 g L^{-1} of bacteriological agar (BD Bioxon, Cuautitlán Izcalli, Mexico State, Mexico) was added to the filtered nejayote, and then the medium was sterilized before pouring it into Petri dishes. This solid culture medium (denominated NEM) was employed to conserve the isolated strains of microorganisms and

to observe their colonial morphology. Liquid YPG medium (10 g L^{-1} yeast extract, 20 g L^{-1} casein peptone, and 20 g L^{-1} dextrose) was utilized for the preparation of the inoculate, while solid YPG served to conserve the isolated strains of the microorganisms.

The microorganisms were tested in NEM to select the one best able to lower the COD. The selected microorganism was then cultivated in unenriched (control) NEM and NEM enriched with nitrogen and phosphorus salts (ammonium sulfate and potassium phosphate, respectively). These salts were added due to the low level of nitrogen and phosphorus in nejayote. Determinations were made of the growth kinetics of the selected strain and the decrease in the COD and pH in all formulations of the medium.

2.3. Isolation of Microorganisms and Pre-Selection of the Most Effective Ones

As a source of microorganisms possibly capable of growing in nejayote, samples were taken of soil from a public park and from sewage water that empties out into an urban river, located in Los Cántaros II, Nicolás Romero, Mexico State, Mexico. A small amount of each sample was placed in Erlenmeyer flasks with NEM. The samples were left to incubate under agitation at 30 °C for 72 h, being periodically moved to fresh NEM medium until the cultures did not present any more changes in their macroscopic characteristics (e.g., color). This process took about one month.

Subsequently, the method of serial decimal dilutions was carried out and a small volume of each microorganism was incubated in Petri dishes with NEM at 30 °C for 72 h. A sample taken from each distinct type of colony was cultivated by cross striation, with each sample in a separate dish. This process continued until each strain of microorganism was completely isolated. The growth of the microorganisms was assessed by placing each one in a separate flask containing liquid NEM.

At the same time that the microbes were being isolated, six amylolytic microorganisms (denominated MR1-MR6) were chosen from the collection of isolates of the Research Lab I (Laboratorio de Investigación I) of the Biochemical Engineering Department of the Escuela Nacional de Ciencias Biológicas (ENCB) of the Instituto Politécnico Nacional (IPN). For one month, they were incubated in a liquid NEM medium, being periodically placed in a fresh medium. The isolated strains (from soil and sewage samples) and the strains from the ENCB collection that showed growth and constant characteristics were pre-selected for the following phase of the experiment.

2.4. Choosing the Most Promising Microorganism

The final selection of the microorganism was based on its capacity to lower the COD of NEM. Firstly, each inoculum was prepared by cultivating one inoculation loop full of each isolated microorganism in liquid YPG, with orbital agitation for 48 h at room temperature. Subsequently, each culture was centrifuged at 3500 rpm for 15 min to obtain a pellet of biomass, which was again suspended in Type II sterile water and centrifuged. The latter procedure was repeated two more times to eliminate the excess YPG, and then the pellet was wet with a small quantity of Type II sterile water and processed to determine the concentration of dry weight of the biomass.

In total, 30 mL of sterile NEM was placed in 125 mL flasks with a previously established level of COD. Then, the necessary amount of suspension of the inoculum to reach an initial biomass concentration of 0.25 g L^{-1} was added to each flask. After 7 days, a sample taken from each isolate was passed through a Whatman Grade GF/F glass microfiber filter (0.7 µm pore size) (Cytiva, St. Louis, MO, USA), and the residual COD was examined in the filtrate. The percentage of COD removal was calculated with Equation (1).

$$\text{COD removal efficiency (\%)} = 100 \frac{\left(COD_0 - COD_f\right)}{COD_0} \tag{1}$$

where COD_0 and COD_f are the initial and final COD (g O$_2$ L^{-1}) values of NEM, respectively. The microorganism most effective at COD removal was chosen for molecular identifica-

tion, and then its growth kinetics in NEM was characterized, first without and then with salts added.

2.5. Kinetic Characterization of the Best Microorganism

The TOC in the NEM was quantified, finding approximately 26.58 g L^{-1}. A part of the NEM was set aside for use as the control, while the other part was enriched with nitrogen and phosphorus salts, given the low concentration of these elements in nejayote. Accordingly, different amounts of analytic-grade ammonium sulfate (1, 3, 5, 7, and 9 g L^{-1}) and monobasic potassium phosphate (0.5, 1, 2, 3, and 4 g L^{-1}) were added to NEM (JT Baker, Xalostoc, Mexico State, Mexico), obtaining five molar ratios of TOC to the total nitrogen added (TOC/N: 146, 49, 29, 21, and 16 mol mol^{-1}) and of TOC to the total phosphorus added (TOC/P: 603, 301, 151, 100, and 75 mol mol^{-1}).

To begin the experiments, an inoculum of the best microorganism was prepared in the same manner as performed during the process of selection. At the same time, 100 mL of the control and all variations of the enriched media (to achieve all combinations of the aforementioned molar ratios of TOC/N and TOC/P) were placed in 500 mL Erlenmeyer flasks. Each culture medium was sterilized in an autoclave at 121 °C for 15 min, followed by a determination of the initial pH, total sugars, and COD of the corresponding sample. Then, 0.25 g L^{-1} of biomass was inoculated into each flask, and the flasks were left under agitation at room temperature for 12 days, a sample being taken every 4 days. The samples were passed through Whatman Grade GF/A glass microfiber filters (1.6 µm pore size) (Cytiva, St. Louis, MO, USA). In each sample, an assessment was made of the biomass produced as well as the level of the pH, total residual sugars, and COD.

Experiments without microbial biomass (biomass-free controls) were also conducted to detect possible abiotic losses of total nitrogen, assimilable nitrogen, COD, total hardness, and calcium hardness brought about by pH, light, temperature, or any other abiotic factor in the absence of living biomass. Biomass-free controls were maintained under the same incubation conditions as the cultures of the best microorganism.

The biomass growth, total sugar consumption, and COD removal were calculated with Equations (2), (3), and (4), respectively.

$$\text{Biomass growth} \left(\text{g L}^{-1}\right) = X_t - X_0 \tag{2}$$

$$\text{Total sugar consumption} \left(\text{g L}^{-1}\right) = S_0 - S_t \tag{3}$$

$$\text{COD removal} \left(\text{g L}^{-1}\right) = COD_0 - COD_f \tag{4}$$

where X_0 and X_t are the concentrations of biomass (g L^{-1}) and S_0 and S_t are the concentrations of total sugars (g L^{-1}) at the initial time (day 0) and time t (in days). The COD (g O$_2$ L^{-1}) was only evaluated at day 0 (COD_0) and at the end of the experiment (COD_f, where "f" is day 12).

The specific growth rate of biomass (μ) (Equation (5)) was found for the time interval of incubation in which the greatest growth rate took place (days 0–4).

$$\mu \left(\text{day}^{-1}\right) = \frac{\ln X_2 - \ln X_1}{t_2 - t_1} \tag{5}$$

where $X_1 = X_0$, $X_2 = X_{4 \text{ days}}$, $t_1 = t_0$, and $t_2 = t_{4 \text{ days}}$.

Also determined was the overall yield of biomass in relation to the total consumption of sugars ($Y_{X/S}$), the overall capacity for COD removal per unit of biomass produced ($Q_{COD/X}$), and the volumetric COD removal rate (R_{COD}) by means of Equations (6), (7), and (8), respectively.

$$Y_{X/S} \left(\text{g g}^{-1}\right) = \frac{X_f - X_0}{S_0 - S_f} \tag{6}$$

$$Q_{COD/X} \,(\text{g g}^{-1}) = \frac{COD_0 - COD_f}{X_f - X_o} \tag{7}$$

$$R_{COD}(\text{g L}^{-1} \, \text{day}^{-1}) = \frac{COD_0 - COD_f}{t_f - t_o} \tag{8}$$

where X_f and S_f correspond to the concentration of biomass and total sugars (g L^{-1}), respectively, and t_f represents the time at the end of incubation (12 days).

2.6. Molecular Identification

The microbial isolate selected was identified using molecular techniques in the National Collection of Microbial Strains and Cell Cultures (la Colección Nacional de Cepas Microbianas y Cultivos Celulares) of the Center for Research and Advanced Studies (Centro de Investigacion y de Estudios Avanzados) of the IPN (CDBB, CINVESTAV-IPN) by amplifying and sequencing fragment 18S-ITS1-5.8S-ITS2-26S and using oligonucleotides ITS5 and ITS4 in accordance with the conditions previously described by White et al. [30].

2.7. Analytical Techniques

Various parameters of NEM were established. The pH was measured with the potentiometric method (Oakton OKT35613-54, Cole-Parmer, Vernon Hills, IL, USA), and the concentrations of total solids and total volatile solids with the method published in the Mexican norm NMX-AA-034-SCFI-2015 [31]. The moisture, crude protein content, ether extract, and ash content were examined using the AOAC methods 934.01, 2001.11, 920.39, and 942.05, respectively [32]. The nitrogen-free extract was calculated by subtracting from 100 the percentages of humidity, crude protein content, ether extract, and ash content.

The total sugars were quantified with sulfuric acid by means of the Rao and Pattarbiraman method [33], utilizing the glucose pattern and reducing sugars found with the 3,5-dinitrosalicylic acid (Merck-Sigma, Toluca, Mexico) method described by Miller [34,35]. The concentration of glucose was ascertained through the enzymatic glucose oxidase–peroxidase technique (Merck-Sigma, Toluca, Mexico) [36]. Total nitrogen was assessed with the Hach Method 10072 (HACH Company, Tlalnepantla, Mexico) [37], and the quantity of assimilable nitrogen with the formaldehyde method [38]. The phosphorus content (quantified as ortho-phosphates only), COD, and TOC were determined with methods 8048 [39], 8000 HR [40], and 10129 of HACH [41], respectively. The Hach method HA-4P GPG (145700) was used to measure total and calcium hardness concentrations. The magnesium hardness concentration was determined by subtracting the calcium hardness concentration from the total hardness concentration [42]. The biomass concentration was determined gravimetrically by measuring dry cell weight. The culture samples were filtered through pre-weighed Whatman GF/A glass microfiber filters (Cytiva, St. Louis, MO, USA) with a pore size of 1.6 µm. The filters were rinsed twice with sterile distilled water and subsequently dried to constant weight at 92 °C. The biomass concentration was calculated based on the weight differential of the microfiber filter before and after filtration and drying [32].

2.8. Statistical Analysis and Software

All evaluations for the characterization of nejayote were performed in triplicate (*n* = 3). The kinetic data on the behavior of the selected microorganism in unenriched and enriched NEM are the average of four assays (*n* = 4). The statistical analysis was conducted on GraphPad Prism Ver 10.1 (GraphPad Software, Boston, MA, USA), considering significance at α = 0.05. The graph of biomass growth and total sugar consumption in the function of the content of TOC/N and TOC/P was created with MATLAB software ver. 9.11 (R2021b) (The MathWorks Inc., Natick, MA, USA).

3. Results and Discussion

3.1. Physicochemical Composition of Nejayote

The data from the physicochemical characterization of NEM are shown in Table 1.

Table 1. Physicochemical characterization of NEM.

	Value	Reported Values	References
pH	10.3 ± 0.1	8–14	[5,7,9,10,23]
Moisture (%)	97.98	96–98	[43,44]
Crude protein (%) *	6.93	2.9–8	[44–47]
Ether extract (%) *	5.44	0.12–6.47	[45–48]
Ash (%) *	16.34 ± 0.1	0.767–31.2	[8,45,47]
Nitrogen-free extract (%) *	71.29	24.4–76	[14,44,47]
Total solids (mg L^{-1})	$26{,}128 \pm 24$	11,680–46,523	[7,8,10,20,43,49]
Total volatile solids (mg L^{-1})	$19{,}349.5 \pm 135.5$	19,293.3	[21]
Total sugars (mg L^{-1})	$24{,}950 \pm 125$	23,570–63,410	[50]
Reducing sugars (mg L^{-1})	147.5 ± 0.62	149–818	[21,50,51]
Glucose (mg L^{-1})	81.0 ± 5.0		
Phosphorus (mg PO_4^{3-} L^{-1})	0.739 ± 0.001	7.6–190	[7,9,18,20]
Total nitrogen (mg L^{-1})	591.48 ± 5.68	120–440	[7,9,18,20,21,26,49]
Assimilable nitrogen (mg L^{-1})	112.0 ± 1.0		
Calcium hardness (mg L^{-1})	2622 ± 152		
Magnesium hardness (mg L^{-1})	570 ± 152		
Total hardness (mg $CaCO_3$ L^{-1})	3192.0 ± 152	1340–4000	[5,7–9]
Chemical oxygen demand (COD) (mg L^{-1})	$27{,}400 \pm 125$	2500–68,000	[5,8,9]
Total organic carbon (TOC) (mg L^{-1})	$26{,}584.05 \pm 105.79$	2984–9836	[10,49]

* Based on dry weight.

The preparation of nixtamal (the dough used for making tortillas) is not a standardized process in Mexico, given that variation exists in several parameters, including the quantity of lime added, the quality and type of maize kernels used, the cooking time, and the agitation rate. Consequently, there is variability in the physicochemical properties of nejayote [43], as evidenced by the wide range of values for the properties evaluated herein (Table 1). However, nejayote has typical characteristics that were found in the current study, such as an elevated alkaline pH (10.3) together with a high level of the content of solids (total solids = 26,128 mg L^{-1}; total volatile solids = 19,349.5 mg L^{-1}), solid hardness (3192 mg $CaCO_3$ L^{-1}), and COD (27,400 mg L^{-1}). These values closely resemble those described for nejayote by Trejo-González et al. [52], García-Depraect et al. [21], and Valderrama-Bravo et al. [8]. The current COD value is approximately 183 times greater than the maximum established by Mexican norms for the disposal of wastewater in storm drains or the sewer system (maximum 150 mg O_2 L^{-1}) [12], where unfortunately the majority of wastewater from nixtamalization is discharged without treatment.

For assessing the quality of water, the TOC is considered a valuable parameter that is complementary to the COD. The value of the TOC has not always been included in reports on nejayote in the literature. The value detected presently (26,584.05 mg L^{-1}) is far above any previously published value (<10,000 mg L^{-1}) [10,49] and almost 700-fold greater than the maximum established by the Mexican norm for the disposal of residual water in rivers (38 mg L^{-1}) [11].

The present humidity value (97.98%) closely aligns with that documented by Díaz-Montes et al. [43]. The maize kernel has a low content of proteins (8–11%) and lipids (4–7%) and a high content of carbohydrates (70–77%) [53], which is reflected in the composition of these biomolecules in the nejayote in the current work (6.93%, 5.44%, and 71.29%, respectively). Although the percentage of ash in maize is about 1.3% [53], nejayote has a percentage far superior (16.34% found in this study) due to the lime employed for nixtamalization. The value for the total hardness of nejayote is approximately 3192.0 mg L^{-1}, which is due to the values for calcium (2622 mg L^{-1}) and magnesium (570 mg L^{-1}). Regarding the principal carbohydrates of maize, almost 70% are of starch and a lesser proportion of cellulose, hemicellulose, and glucose [29]. Nejayote contains a similar proportion of the same compounds. The elevated percentage of total carbohydrates observed presently (71.29%) concurs with the 72.5% identified by Villela-Castrejón et al. [14].

The concentration of total sugars, determined utilizing a glucose pattern (24,950 mg L^{-1}), is similar to the value reported by Ramírez-Romero et al. [50] (23,570 mg L^{-1}). Of the total sugars, only 0.59% were reducing sugars (147.5 mg L^{-1}), a very low quantity compared to the amount obtained by Ramírez-Romero et al. [50] (818 mg L^{-1}) but very close to the finding of García-Depraect et al. [21] (148.6 mg L^{-1}) and superior to that documented by Pedroza-Islas et al. [19] (~108 mg L^{-1}). Among the reducing sugars in nejayote are glucose and fructose. There were only 81 mg L^{-1} of glucose, constituting 55% of the reducing sugars and 0.32% of total sugars.

Raw maize kernels have low concentrations of glucose (6.71%) and fructose (2.84%), and nixtamal exhibits a 94.3% and 91.2% decrease, respectively, in these quantities [54]. Hence, these reducing compounds are apparently leached into nejayote. According to Renk et al. [55], glucose and fructose reach very low levels or are undetectable in nejayote. They seem to be transformed into other compounds, including other carbohydrates, such as their isomers, saccharic acids, and derivatives of the Maillard reaction, or they might be reconverted into sucrose.

Nitrogen is a vital nutrient for the growth of the maize plant and the development of kernels on the ears of maize (constituting the seeds of the plant). Most of the nitrogen in the kernels is in prolamins (zein), glutelins, and globulins located mainly in the endosperm. The limited solubility of these proteins diminished further during nixtamalization [54]. A portion of these proteins leach into the cooking water together with some amino acids [15], the latter of which form a portion of the total nitrogen.

This nitrogen cannot be well utilized by all microorganisms for their growth. Prolamins have a lot of proline, and glutamines and are considered to be the storage proteins in the grains of cereals, serving as a source of nitrogen during the sprouting and early development of the seedling [56]. However, proline is an amino acid that certain microorganisms (like yeasts under conditions of low oxygenation) cannot transaminate into alpha-keto acids. Therefore, the cells of such microorganisms are not capable of assimilating proline to form other amino acids for growth and development [57]. Moreover, some amino acids released during nixtamalization can produce lysinoalanines, compounds known not to have good bioavailability and to cause toxicity in animals. They even appear to have an antimicrobial effect [58,59].

Since the nitrogen contained in proline and lysinoalanines may not be assimilable by microorganisms, both the total nitrogen and assimilable nitrogen were quantified in NEM, finding a value of 112.0 mg L^{-1} for the latter, representing about one-fifth of the total nitrogen (591.48 mg L^{-1}). As with other parameters currently evaluated in NEM, the total nitrogen is well above (~23.6 times) the Mexican norm for the disposal of residual water in bodies of water (25 mg L^{-1}) [11].

The amount of phosphorus found (0.739 mg PO_4^{-3} L^{-1}) is around one-tenth of the value reported by other researchers [20], probably because in the current contribution a determination was made only of ortho-phosphates (being the easiest phosphorus compounds to be assimilated by organisms) and not of the total phosphorus in NEM.

3.2. Isolation and Selection of the Microorganism

The wastewater from nixtamalization is an extremely harsh environment for the growth of microorganisms. Apart from the elevated pH (>10), nejayote has low levels of nitrogen and phosphorus and a high concentration of calcium. Thus, it was necessary to find an alkaliphile or alkali-tolerant microorganism with a minimal requirement of essential nutrients (e.g., nitrogen and phosphorus).

In total, 5 microorganisms were isolated from the samples of sewage water and 4 from the samples of soil. Of these 9 microorganisms, there were 6 bacteria, 2 filamentous fungi, and 1 yeast. Of the 6 strains taken from the IPN collection (5 bacteria and 1 yeast), 2 bacteria were not able to grow in nejayote (MR3 and MR5). A description of the colonial morphology of the 15 strains is shown in Table 2.

Table 2. For the microorganisms cultivated in NEM, the growth and colonial morphology are characterized. Additionally, the percentage of COD removal is specified.

Identification Key	Source	Microorganism	Growth	COD Removal Efficiency (%)	Colonial Morphology
OM-97	Sewage	Bacterium	+	35.24	Yellow colony; greasy aspect, irregular edges.
LCRE	Sewage	Yeast	+	75.11	Pink colony; bright, wavy, and punctiform surface.
LC-98B	Sewage	Bacterium	+	34.31	Yellow colony; bright, punctiform surface.
B-16B	Sewage	Bacterium	+	42.65	White colony; bright, punctiform surface.
A-201	Sewage	Bacterium	+	48.22	White colony; bright, coarse, flat surface.
E-31H	Contaminated soil	Fungus	+	48.22	Tall white filamentous fungus; green center.
GJ-25H	Contaminated soil	Fungus	+	51.93	Black fungus; cottony appearance.
E-31B	Contaminated soil	Bacterium	+	32.08	Yellow colony; punctiform, matte surface.
GJ-25B	Contaminated soil	Bacterium	+	39.57	Pink colony; punctiform, flat surface.
MR1	Culture collection	Yeast	+	32.17	Pink colony; bright, circular, convex surface.
MR2	Culture collection	Bacterium	+	45.21	White colony; elevated around the entire edge.
MR3	Culture collection	Bacterium	−	–	White colony; elevated around the entire edge.
MR4	Culture collection	Bacterium	+	32.69	Beige colony; elevated around the entire edge, convex surface.
MR5	Culture collection	Bacterium	−	–	White colony; smooth surface around the edge.
MR6	Culture collection	Bacterium	+	41.03	White colony; wavy around the edge.

Symbols: no growth (−); growth (+).

Bacteria, which constituted the majority of the microorganisms (either isolated or from the collection) capable of growing in NEM, are generally known to grow in an optimum pH near neutral (6–7.5). However, some bacteria can tolerate or even require alkaline conditions (pH > 9) due to their evolutionary adaptation to extreme environmental conditions, allowing them to colonize various habitats [60,61]. On the other hand, the growth of two fungi and two yeasts in NEM is noteworthy, considering that these microorganisms typically prefer acidic environments (pH < 6). The survival and growth of these strains indicate their potential adaptability to diverse conditions. The thirteen surviving strains were preselected, and their COD removal efficiency in NEM was determined. The best result, being a 75.11% COD removal efficiency, was shown by LCRE (Table 2).

In the limited number of reports on the isolation of microorganisms and the testing of their growth in nejayote without pH adjustment, bacteria, especially those belonging to genera such as *Bacillus*, *Clostridium*, *Burkholderia*, and *Pseudomonas*, have shown promising results [22,62]. Therefore, the growth of LCRE in this harsh medium is particularly surprising. Apart from the elevated pH and COD levels, nejayote contains low nutrient availability and a high calcium concentration. Additionally, it contains phenolic compounds like ferulic acid, which can inhibit the growth of certain yeasts [63].

3.3. Identification of the LCRE Strain

The DNA from the LCRE strain was amplified to obtain the DNA from the ribosomal internal transcribed spacer (ITS) regions. The product of PCR was sequenced and edited to furnish a consensus sequence of the fragment 18S-ITS1-5.8S-ITS2-26S with a total of 642 nucleotides (Figure S1, Supplementary Materials). Based on the BLAST search of the National Center for Biotechnology Information (NCBI), the most similar sequence (with a sequence coverage of 100% and an identity of 100%) was the species *Rhodotorula mucilaginosa* of the PMM08-3684L strain (access number KP132584.1). A dendrogram was created with the different sequences of the distinct species related to the genus *Rhodotorula* in order to explore the phylogenetic relationships (Figure 1).

R. mucilaginosa, a yeast-like fungus, has been isolated from diverse sources. It generates mucoid colonies in various tones of pinkish orange, a color given by its production of carotenoids such as β-carotene, torulene, and torularhodin [64–66]. It has the capacity to assimilate different sources of carbon, including glucose, sucrose, and fructose, and to grow in complex substrates (e.g., molasses, whey, ketchup, and raw glycerol) [64,66–68].
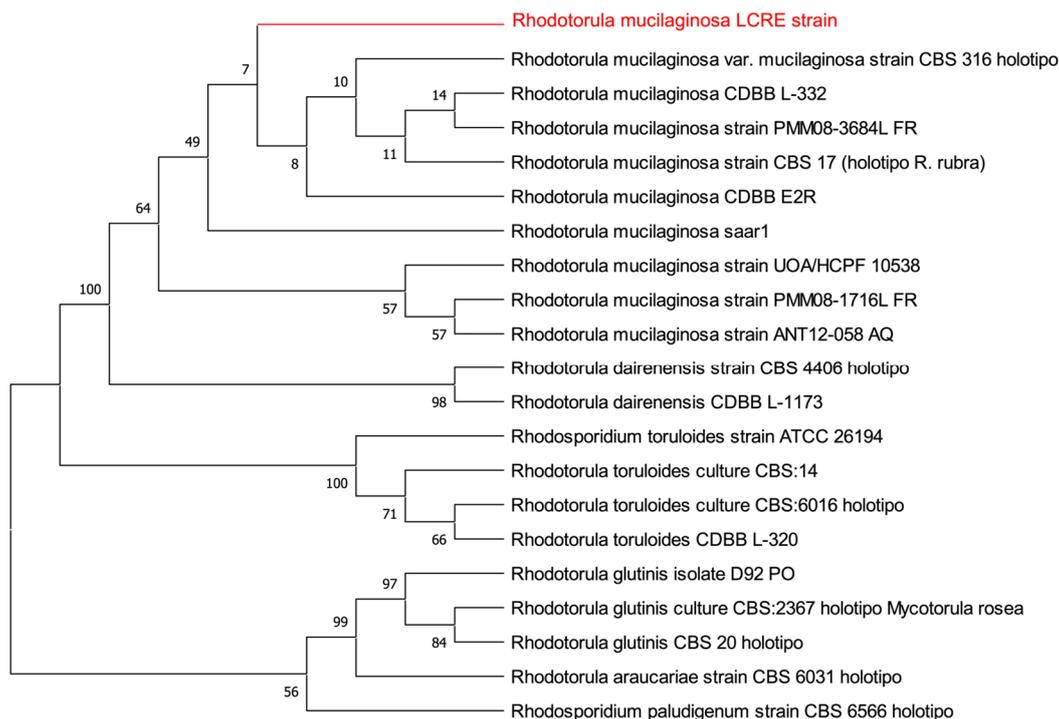
Figure 1. Dendrogram of the similarity of the consensus sequences of the genus *Rhodotorula*, considering the fragment 18S-ITS1-5.8S-ITS2-26S of the LCRE strain (depicted in red). This fragment had a total of 642 nucleotide positions, found by the Neighbor-Joining (3-Tamura) algorithm. The bootstrap values are based on 1000 repetitions.

Apart from being an important source of carotenoids, *R. mucilaginosa* is able to produce lipids and enzymes, and it has potential as a microbial fertilizer and antifungal agent [65]. It has also proven useful in the treatment of olive mill wastewater [69], and in the removal of colorants [70] and heavy metals, such as Hg^{2+}, Pb^{2+}, Cu^{2+}, Cr^{6+}, and Ni^{2+}, in polluted water [70–73]. The utility of *R. mucilaginosa* in the field of industry, biotechnology, and bioremediation is widely recognized due to the benefits it offers, even though it is known to be an emerging opportunistic pathogen that can affect immunodepressed and immunocompromised individuals [74].

The culture of *R. mucilaginosa* isolated in the current contribution is illustrated in a Petri dish (Figure 2a) and under an optical microscope (Figure 2b).

(**a**)   (**b**)
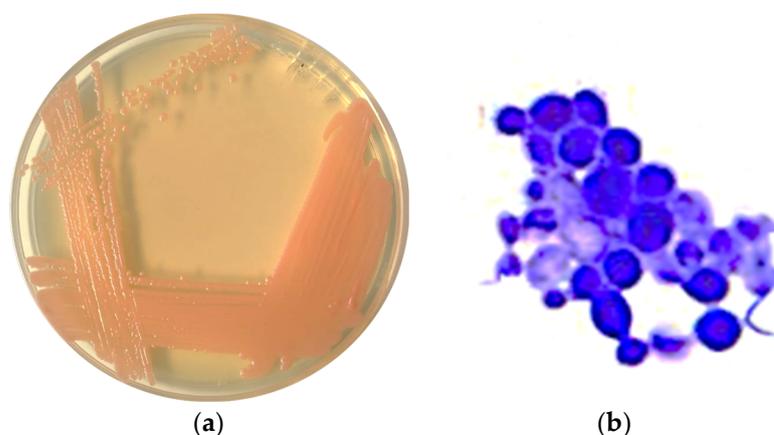
Figure 2. *Rhodotorula mucilaginosa* LCRE visualized (**a**) in a Petri dish in Sabouraud agar medium (incubated at 30 °C for 48 h) and (**b**) under an optical microscope (5000×) after being stained with crystal violet dye.

3.4. Kinetic Characterization of Rhodotorula mucilaginosa LCRE in NEM

Once identified, the isolated yeast was subjected to a kinetic study to characterize its growth. Accordingly, it was cultivated in unenriched (control) NEM and in NEM enriched with nitrogen and phosphorous salts added in different TOC/N and TOC/P ratios. Additionally, control cultures free of biomass were incubated for 12 days. No statistically significant differences were found ($p > 0.05$ by the Bonferroni multiple comparison test, $n = 4$, $\alpha = 0.05$) in the pH or the concentrations of total nitrogen, assimilable nitrogen, COD, total hardness, and calcium hardness between day 0 and day 12 of the abiotic cultures. These findings indicate that the results reported herein are solely due to the biological activity of *R. mucilaginosa*.

The initial pH apparently depended on the TOC/P rather than the TOC/N ratio (Figure 3). When a small amount of phosphorus (TOC/P = 603 mol mol^{-1}) was added to the control medium, the initial pH was close to 10. With a greater quantity of phosphorus (TOC/P = 151 mol mol^{-1}) and the highest concentrations tested (TOC/P = 100 and 75 mol mol^{-1}), the initial pH of the medium was close to 7.0 and 6, respectively (Figure 3a–e).
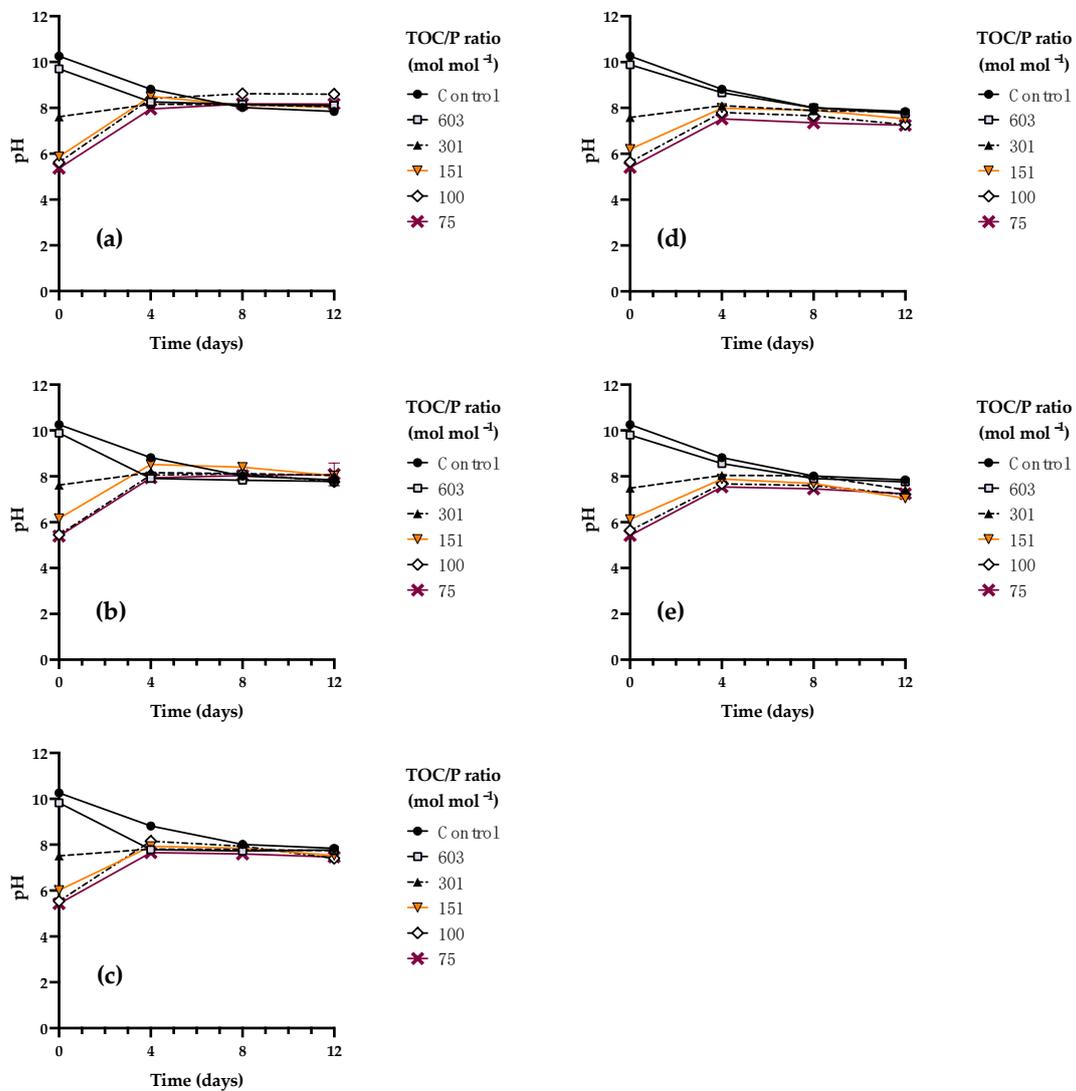

Figure 3. During the growth of *Rhodotorula mucilaginosa* in NEM, the change in pH is portrayed for the unenriched (control) NEM and NEM enriched with salts of nitrogen ((NH$_4$)$_2$SO$_4$) and phosphorus (KH$_2$PO$_4$) to achieve distinct TOC/P ratios and the following ratios of TOC/N: (**a**) 146 mol mol^{-1}, (**b**) 49 mol mol^{-1}, (**c**) 29 mol mol^{-1}, (**d**) 21 mol mol^{-1}, and (**e**) 16 mol mol^{-1} ($n = 4$). When the error bars are not shown, the standard deviation is smaller than the symbol size.

Independently of the initial ratio of TOC/N or TOC/P, as of day 4, the pH of all media had changed to about 8.0. From day 4 to day 12, the pH remained at an almost constant value, despite the ongoing growth of the yeast and consumption of the substrate (explained later). *R. mucilaginosa* has been reported to grow adequately at pH 4–7, with its generation of biomass becoming slow at pH 8 [66,67]. However, it has been able to increase the pH from approximately 5.5 to almost 9.0 when used in the treatment of olive mill and potato wastewater and in the manufacture of cheddar cheese, a phenomenon attributed to the deamination of amino acids and the production of ammonia [69,75,76].

According to the literature, the growth of *R. mucilaginosa* decreases drastically at values of pH above 9 [66,67]. Nevertheless, the yeast isolated presently could grow adequately at the initial pH of NEM (10.3) (Figure 4), registering maximum biomass at day 12 of incubation in both unenriched NEM and NEM enriched with salts in different proportions.
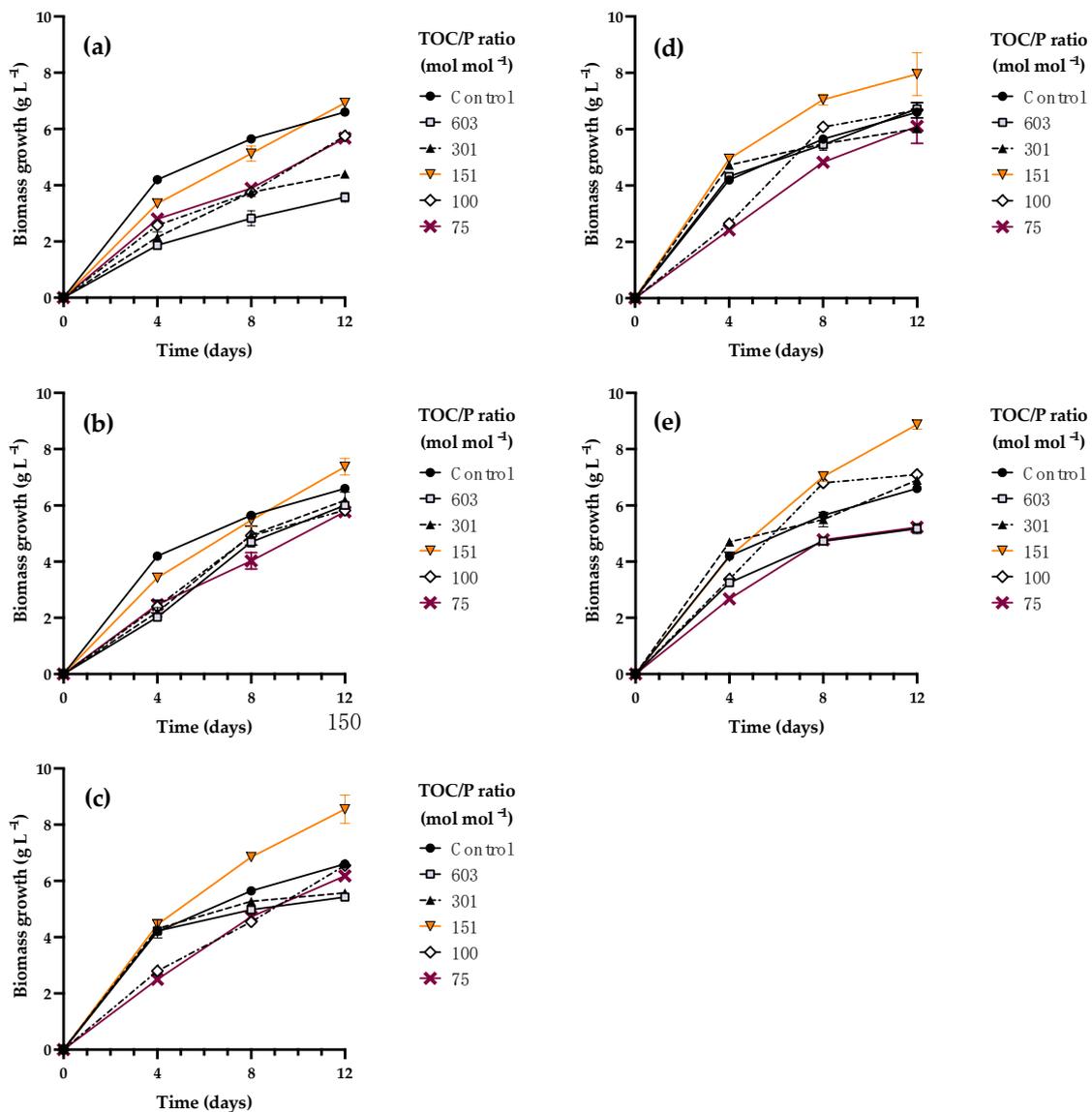
Figure 4. Growth kinetics of *Rhodotorula mucilaginosa* in unenriched (control) NEM and in NEM enriched with salts of nitrogen ($(NH_4)_2SO_4$) and phosphorus (KH_2PO_4) to achieve distinct TOC/P ratios and the following ratios of TOC/N: (**a**) 146 mol mol^{-1}, (**b**) 49 mol mol^{-1}, (**c**) 29 mol mol^{-1}, (**d**) 21 mol mol^{-1}, and (**e**) 16 mol mol^{-1} ($n = 4$). When the error bars are not shown, the standard deviation is smaller than the symbol size.

At the point of maximum growth in the control NEM, the biomass of *R. mucilaginosa* LCRE reached 6.55 g L^{-1}, a concentration higher than that reported by Moran-Mejía et al. [77] for the growth of *Clavispora lusitaniae* Hi2 in nejayote as the sole substrate (1.93 g L^{-1}). The enriched medium with a TOC/P of 151 mol mol^{-1} and a TOC/N of 146 mol mol^{-1} afforded a greater biomass than the control medium (6.9 vs. 6.55 g L^{-1}; Figure 4a), representing a significant difference (p = 0.0195). All the other media with a TOC/N of 146 mol mol^{-1} (involving a range of values for TOC/P) resulted in a significantly lower value for the biomass. As the ratio of TOC/N decreased to 49, 29, and 21 mol mol^{-1}, maintaining a TOC/P ratio of 151 mol mol^{-1} (Figure 4b, Figure 4c, and Figure 4d, respectively), the biomass of *R. mucilaginosa* LCRE increased between day 8 and day 12. Once again, the TOC/P ratio of 151 mol mol^{-1} was the only one capable of promoting greater biomass production than the control NEM. Thus, with the TOC/P ratio of 151 mol mol^{-1} (requiring the addition of 2 g L^{-1} monobasic potassium phosphate) and the lowest TOC/N ratio of 16 mol mol^{-1} (with 9 g L^{-1} of ammonium sulfate, the greatest quantity added), the biomass had a maximum level of 8.9 g L^{-1} (Figure 4e), which was a 35.9% higher level than the value of the control NEM.

Interestingly there was a decline in the biomass of *R. mucilaginosa* when Aksu and Eren [67] used a concentration of ammonium sulfate superior to 2 g L^{-1} in a substrate of molasses. Hence, the effect caused by a given concentration of ammonium sulfate on the growth of *R. mucilaginosa* depends on the substrate. In the current study, the enriched NEM with the greatest amount of nitrogen combined with the greatest amount of phosphorus (TOC/N = 16 mol mol^{-1}; TOC/P = 75 mol mol^{-1}; Figure 4e) generated a biomass 20.2% inferior to that of the control. For the growth of *R. mucilaginosa* LCRE in NEM, the most effective medium had a high concentration of nitrogen combined with a medium level of phosphorus.

According to the analysis of the data used to create Figure 4, in all cases, the best specific growth rate (μ day^{-1}) occurred from day 0 to day 4 of incubation. Figure 5 shows the μ values of the yeast cultivated in the control NEM and in the NEM enriched with distinct ratios of TOC/N and TOC/P. Overall, the value of μ found in the control NEM (0.59 day^{-1}) was increased via supplementation with nitrogen and phosphorus salts. The highest values of μ (0.748 day^{-1} < μ < 0.80 day^{-1}) were obtained with TOC/P ratios of 603, 301, and 151 mol mol^{-1} and TOC/N ratios of 29, 21, and 16 mol mol^{-1}.

To our knowledge, there are no reports to date on the growth of *R. mucilaginosa* in nejayote, making a direct comparison of μ impossible. The specific growth rate for *R. mucilaginosa* obtained in the present study from days 0–4 (0.62 day^{-1} < μ < 0.79 day^{-1}) is lower than that reported for the same yeast cultivated in sugar molasses (0.25 h^{-1} = 6.0 day^{-1}) [67] or glucose (0.21 h^{-1} = 5.04 day^{-1}) as the substrate [78]. Conversely, μ values were much lower when *R. mucilaginosa* was tasked with treating residual waters, a substrate not easily assimilable. For instance, the use of *R. mucilaginosa* to treat residual waters from the olive mill industry and potato starch production resulted in μ values ranging from 0.06 to 0.2 day^{-1} [69] and 0.0318 h^{-1} (0.763 day^{-1}) [76], respectively. The latter value of μ is close to the values found in this study.

Compared to substrates with simple sugars, the lower values of μ for NEM could be due to its lack of easily assimilable simple sugars (Table 1). In nejayote, *R. mucilaginosa* LCRE faced a greater complexity for the degradation of the substrate, translating into a lower growth rate. The challenge presented by *R. mucilaginosa* LCRE in consuming the substrate is further evidenced by the relatively slow consumption of total sugars throughout the 12 days of incubation. Indeed, the total sugars were not completely consumed by day 12 of incubation ($S_0 \approx 24.95$ g L^{-1}; Figure 6). Although *R. mucilaginosa* consumed about 94.98% of the initial amount of total sugars in the control NEM by day 12, it needs only a few hours to almost completely consume the total sugars in glucose and molasses (depending on the conditions of incubation and initial concentrations) [78,79].
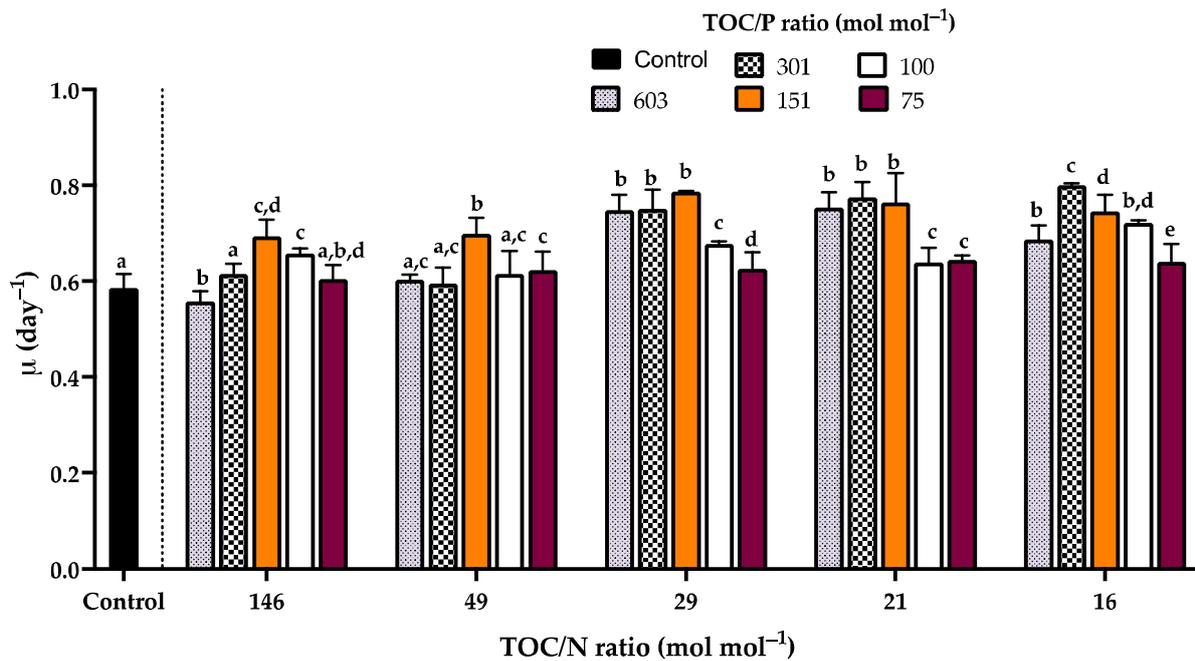
Figure 5. The specific growth rate (μ) of *Rhodotorula mucilaginosa* LCRE obtained from days 0–4 of incubation. The statistical analysis was made for each TOC/N and TOC/P ratio in relation to the control NEM (two-way ANOVA for multiple comparisons with Tukey's test to create confidence intervals; $n = 4$; significance was considered at $\alpha = 0.05$). The same letters indicate the lack of a significant difference between the corresponding groups. When the error bars are not shown, the standard deviation is smaller than the thickness of the line of the bars.

Compared to the control NEM, the NEM enriched with the least quantity of nitrogen herein tested (TOC/N = 146) and phosphorus at any TOC/P ratio caused a significant (68–81%) decrease in the consumption of the substrate (Figure 6a). The yeast took longer to begin to utilize the substrate with a TOC/P ratio of 151, 100, or 75 mol mol^{-1}, or a TOC/N ratio of 49 or 29 mol mol^{-1} (Figures 6b and 6c, respectively). Despite the delay in the consumption of the substrate in these cases, the final consumption at day 12 was close to that obtained in the control medium. The consumption of the substrate increased with ammonium sulfate at 7 and 9 g L^{-1} (TOC/N = 21 and 16 mol mol^{-1}, respectively) and monobasic potassium phosphate at 2 g L^{-1} (TOC/P = 151 mol mol^{-1}) (about 90% at day 12; Figure 6d,e). When comparing the latter ratios of TOC/N and TOC/P in enriched NEM with the control NEM, there was no significant difference in the consumption of total sugars (S_0 = ~23 vs. ~24.95 g L^{-1}, respectively). Contrarily, the highest concentrations of salts of nitrogen and phosphorus added to the medium (TOC/N = 16 mol mol^{-1} and TOC/P = 75 mol mol^{-1}) (Figure 6e) provided the lowest consumption of total sugars (18.4 g L^{-1}, 74%). Hence, the consumption of total sugars followed the same general pattern as that observed for the production of biomass. In all TOC/N ratios evaluated, the highest consumption of total sugars occurred with the TOC/P ratio of 151 mol mol^{-1}. As the concentration of ammonium sulfate in the medium increased (with the TOC/N ratio from 146 to 16 mol mol^{-1}), the consumption of total sugars went from 87 to 93%. Thus, the greatest quantity of ammonium sulfate tested (TOC/N = 16 mol mol^{-1}) combined with an intermediate amount of monobasic potassium phosphate (TOC/P de 151 mol mol^{-1}) led to a good level of consumption of the substrate that was not significantly different from the level found in the control NEM (93% vs. 94.98%; $p = 0.258$).

Ramírez-Romero et al. [50] cultivated *Lactobacillus rhamnosus* GG in nejayote, finding a 29.85% consumption of the initial total sugar concentration (S_0) of 44.89 g L^{-1}. This result was inferior to the percentage and quantity of total sugars consumed by *R. mucilaginosa* LCRE.
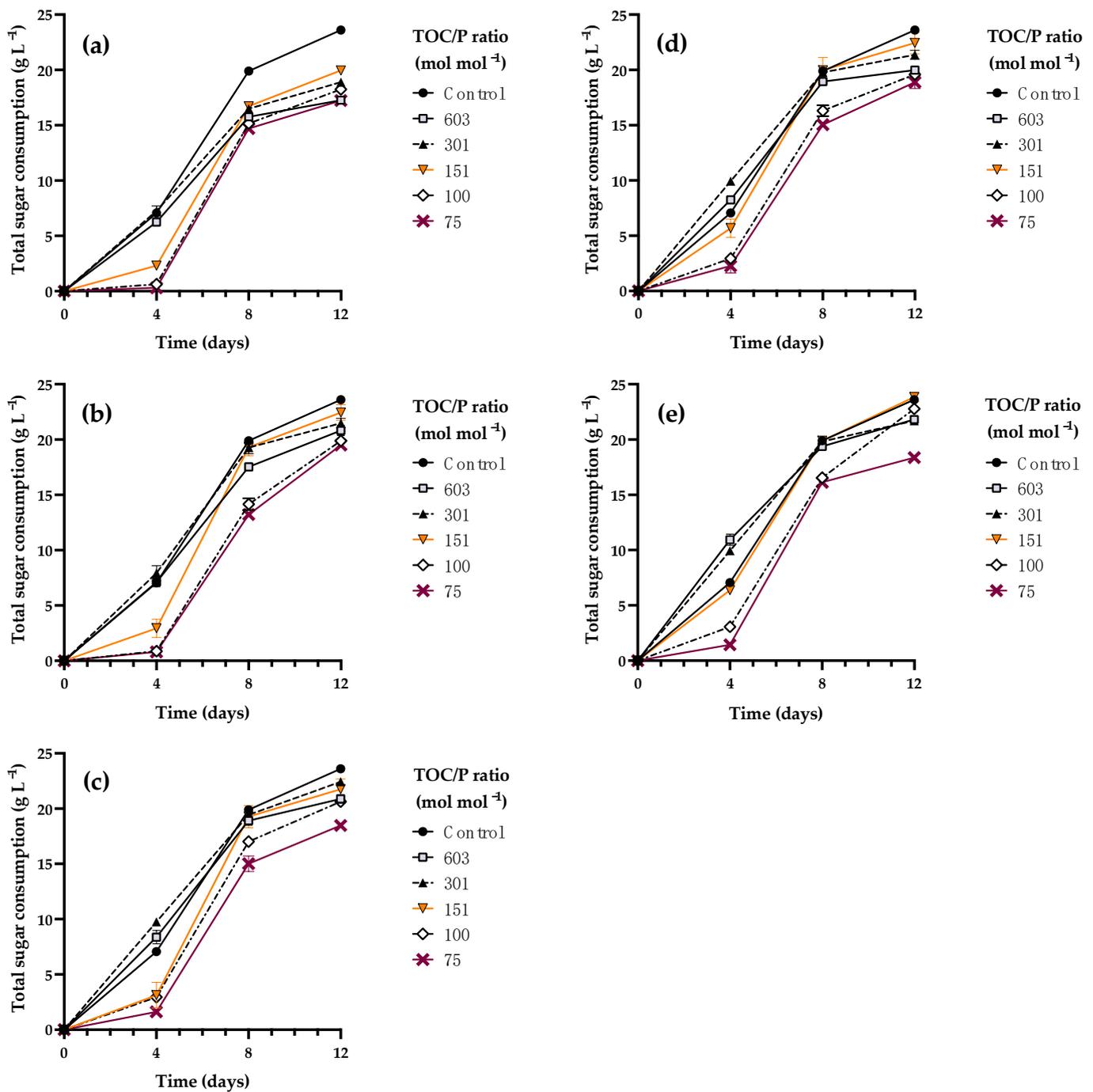
Figure 6. Kinetics of the consumption of total sugars by *Rhodotorula mucilaginosa* in the control NEM and in NEM enriched with salts of nitrogen ($(NH_4)_2SO_4$) and phosphorus (KH_2PO_4) to achieve distinct TOC/P ratios and the following ratios of TOC/N: (**a**) 146 mol mol^{-1}, (**b**) 49 mol mol^{-1}, (**c**) 29 mol mol^{-1}, (**d**) 21 mol mol^{-1}, and (**e**) 16 mol mol^{-1} ($n = 4$). When the error bars are not shown, the standard deviation is smaller than the symbol size.

Another parameter herein considered was the overall yield of biomass in relation to the consumption of total sugars ($Y_{X/S}$) (Figure 7).
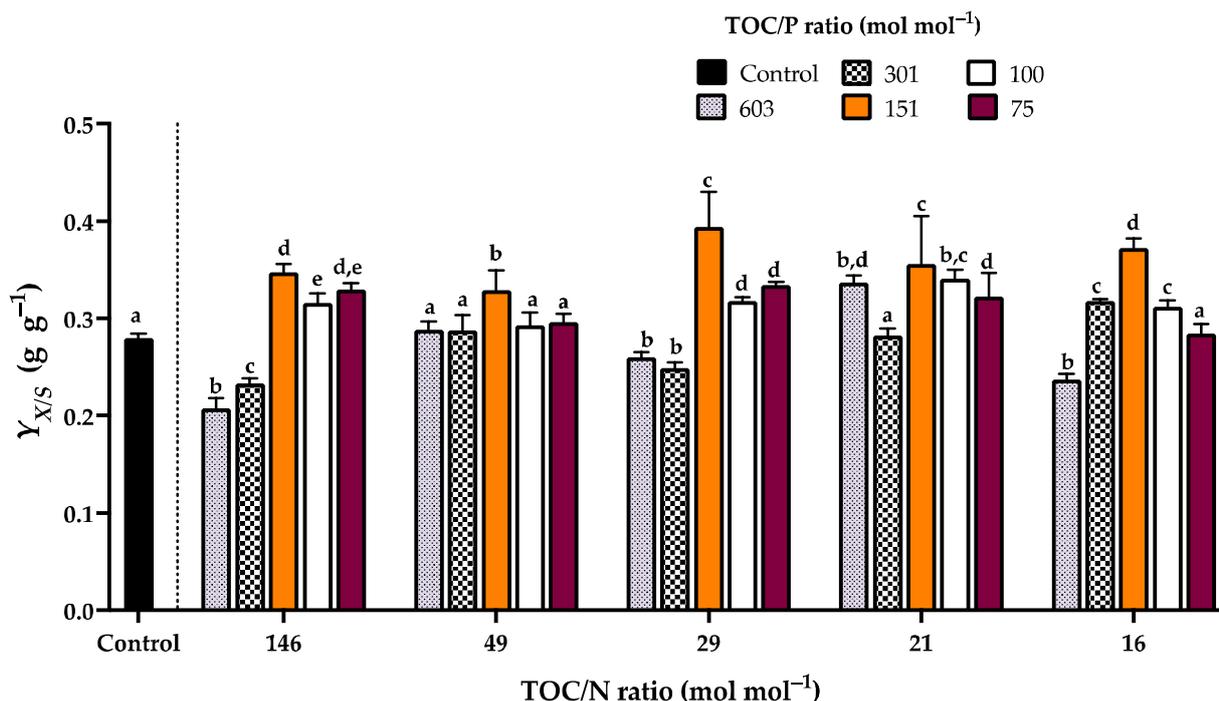
Figure 7. The overall yield of the biomass of *Rhodotorula mucilaginosa* LCRE in relation to its consumption of total sugars ($Y_{X/S}$) in control NEM and NEM enriched with salts of nitrogen (($NH_4)_2SO_4$) and phosphorus (KH_2PO_4) to achieve distinct TOC/P ratios and the following ratios of TOC/N: 146 mol mol^{-1}, 49 mol mol^{-1}, 29 mol mol^{-1}, 21 mol mol^{-1}, and 16 mol mol^{-1}. The combination of each TOC/P ratio with each TOC/N ratio was compared to the control (two-way ANOVA for multiple comparisons with Tukey's test to create confidence intervals; $n = 4$; significance was considered at $\alpha = 0.05$). The same letters indicate the lack of a significant difference between the corresponding groups. When the error bars are not shown, the standard deviation is smaller than the thickness of the line of the bars.

The NEM enriched at any given TOC/N ratio generated the greatest overall yield of biomass ($0.33 < Y_{X/S} < 0.39$ g g^{-1}) when combined with a TOC/P ratio of 151 mol mol^{-1}. These values were all higher than the 0.28 g g^{-1} found for $Y_{X/S}$ in the control NEM. The values of $Y_{X/S}$ reported for *R. mucilaginosa* incubated in glycerol, glucose, sucrose, and molasses as substrates were 0.4, 0.5, 0.6, and 2.35 g g^{-1}, respectively [67,78–80].

Figure 8 was constructed to clearly illustrate the effect of the TOC/P and TOC/N ratios on the growth of *R. mucilaginosa* LCRE (Figure 8a) and the consumption of total sugars (Figure 8b). As can be appreciated by the reddest zones, the TOC/P ratio of 151 mol mol^{-1} and the TOC/N ratio of 16 mol mol^{-1} coincided with the greatest production of biomass (>8.5 g L^{-1}) and the highest consumption of total sugars (>23 g L^{-1}). Conversely, the bluest zones show the lowest growth of the yeast (<4 g L^{-1}) and consumption of total sugars (<18 g L^{-1}) in the NEM resulting from the addition of the smallest amount of ammonium sulfate (TOC/P = 603 mol mol^{-1}) and monobasic potassium phosphate (TOC/N = 146 mol mol^{-1}).

The main objective of the current contribution was to evaluate the capacity of the selected organism (*R. mucilaginosa* LCRE) to diminish the high level of contamination of nejayote by lowering the levels of pH and COD. Although the level of pH reached the range acceptable for disposal into the environment, this was not the case for COD. The overall decrease in COD was analyzed by determining this parameter at time 0 and at 12 days of incubation (Figure 9). The best COD removal efficiency was the 75.7% decrease found in the control medium (from 27.4 to 6.65 g O_2 L^{-1}). All combinations of the TOC/N and TOC/P ratios tested afforded a lesser COD removal efficiency (<65%).
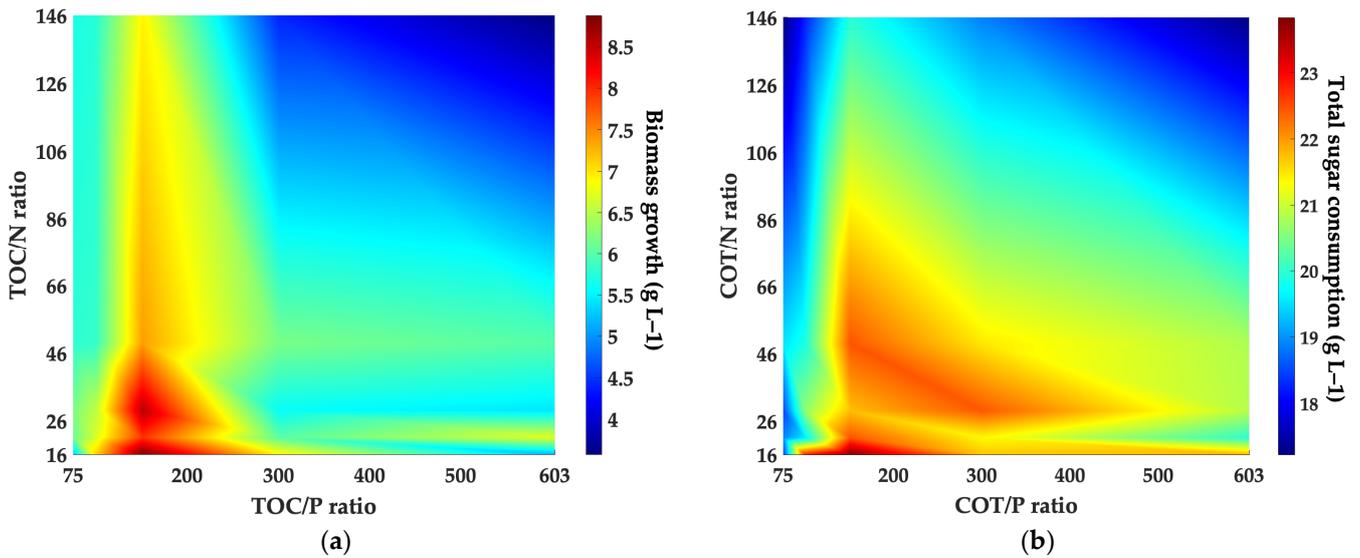
Figure 8. At different TOC/P and TOC/N ratios in enriched NEM (at 12 days of incubation), the following are illustrated with red (a high level) and blue (a low level): (**a**) the biomass growth of *Rhodotorula mucilaginosa* LCRE and (**b**) the consumption of total sugars by the yeast.
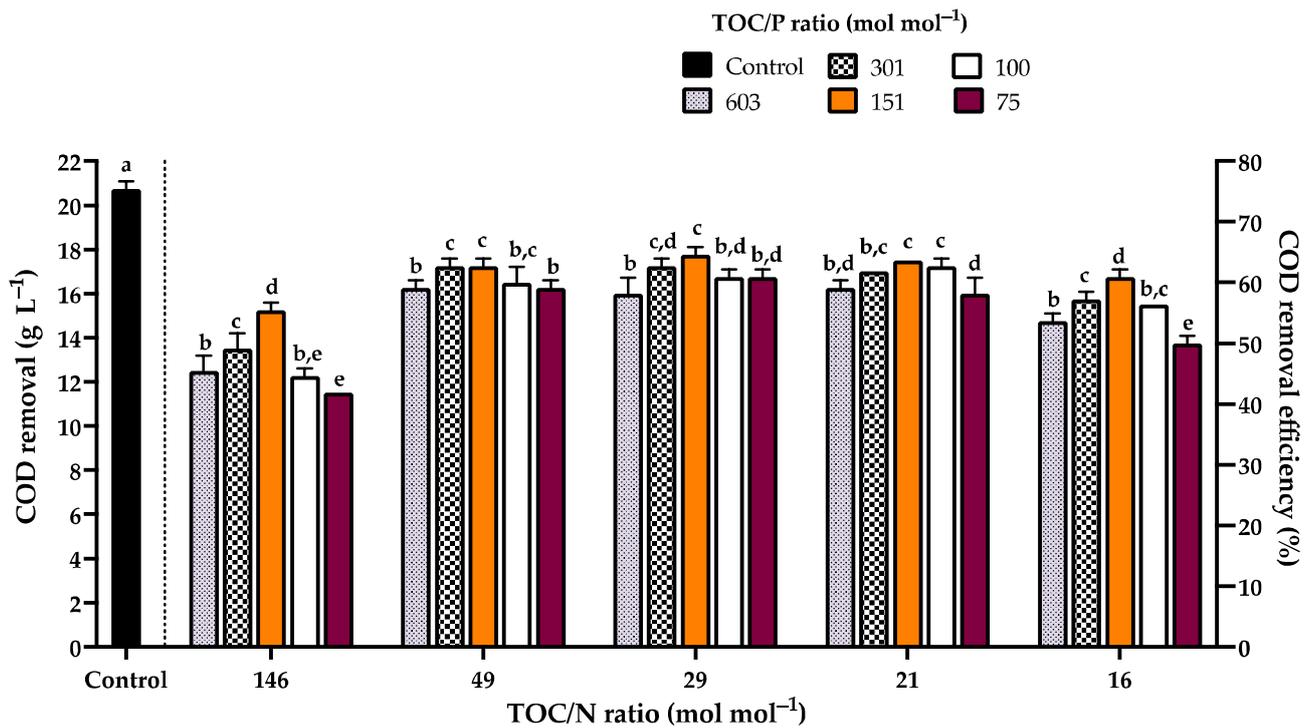

Figure 9. The COD removal (left vertical axis) and the COD removal efficiency (right vertical axis) achieved with *Rhodotorula mucilaginosa* LCRE at day 12 of incubation. The statistical analysis was made for each TOC/N ratio (*x* axis) combined with each TOC/P ratio (bars) in relation to the control (two-way ANOVA for multiple comparisons with Tukey's test to create confidence intervals, with significance considered at $\alpha = 0.05$; $n = 4$). The same letters indicate the lack of a significant difference between the corresponding groups. When the error bars are not shown, the standard deviation is smaller than the thickness of the line of the bars.

Also assessed was the capacity of COD removal in relation to the biomass generated ($Q_{COD/X}$) (Figure 10a). The $Q_{COD/X}$ value of the control NEM (3.14 g g^{-1}) was similar to or higher than that of any NEM enriched with salts, except for the molar ratio of 146 for

TOC/N and 603 for TOC/P. The combination of the latter ratios furnished the maximum $Q_{COD/X}$ of 3.497 g g^{-1}. Since the value of $Q_{COD/X}$ is a function of the biomass, and the TOC/P of 151 mol mol^{-1} provided the greatest biomass, it gave the lowest values of $Q_{COD/X}$ (1.9 g g^{-1} < $Q_{COD/X}$ < 2.3 g g^{-1}). The values obtained presently are superior to those reported by Jarboui et al. [69] (0.715 g g^{-1}) and Díaz-Vázquez et al. [81] (0.73 g g^{-1}) when utilizing *R. mucilaginosa* to diminish the COD of residual water from an olive mill and the vinasse of the tequila industry, respectively.
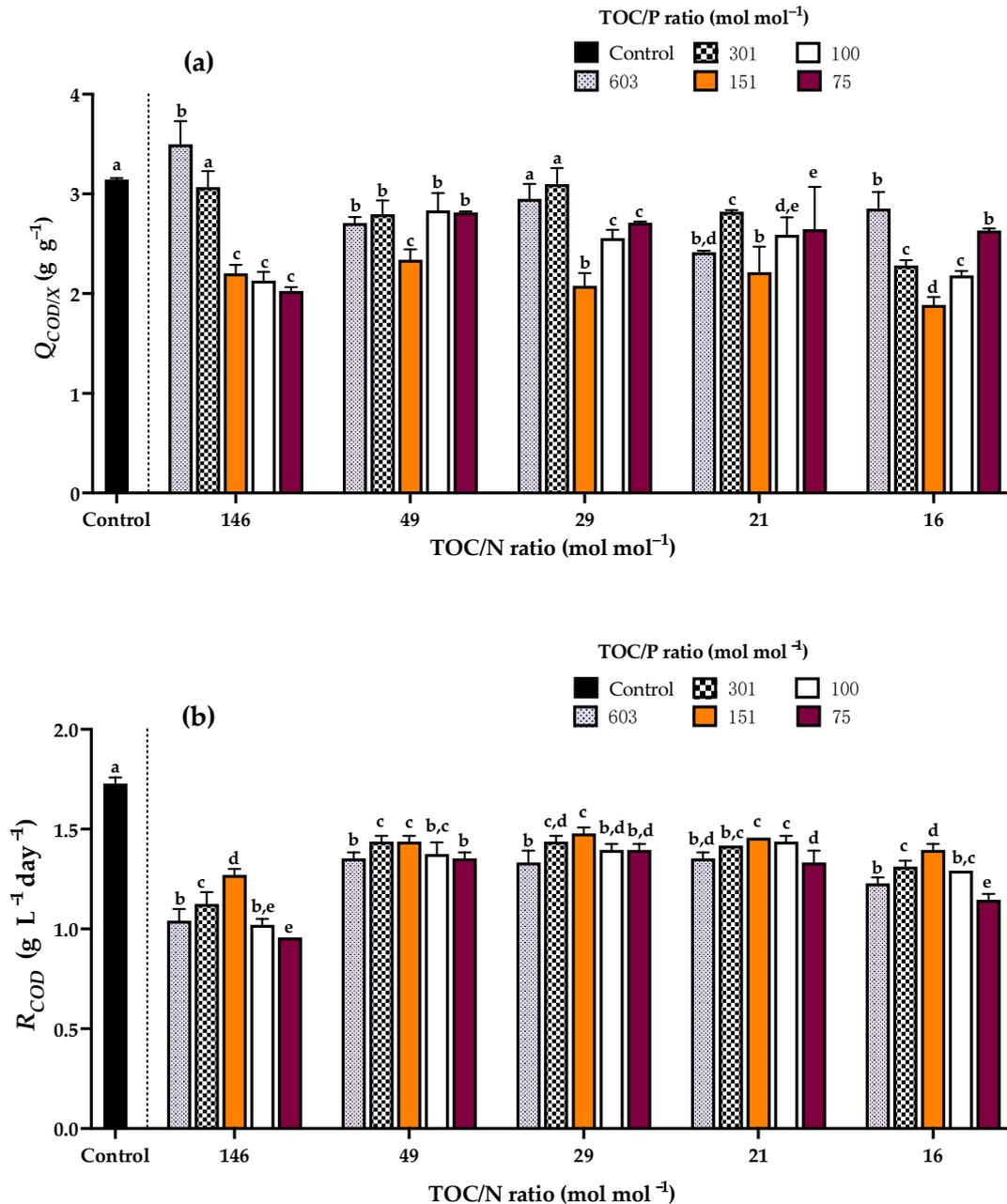
Figure 10. *Rhodotorula mucilaginosa* LCRE was incubated in NEM for 12 days, resulting in the graphed values for (**a**) Q_{COD} and (**b**) R_{COD}. The statistical analysis was made for each TOC/N ratio (*x* axis) combined with each TOC/P ratio (bars) in relation to the control (two-way ANOVA for multiple comparisons with Tukey's test to create confidence intervals, with significance considered at $\alpha = 0.05$; $n = 4$). The same letters indicate the lack of a significant difference between the corresponding groups. When the error bars are not shown, the standard deviation is smaller than the thickness of the line of the bars.

The overall COD removal rate (R_{COD}) was examined as well (Figure 10b). A better value was provided by the control NEM (R_{COD} = 1.73 g L^{-1} h^{-1}) than the NEM enriched with salts. Hence, *R. mucilaginosa* LCRE more efficiently diminished the COD in NEM when no salts were added.

Table 3 lists some reports on the use of *R. mucilaginosa* for the treatment of diverse types of wastewater with complex substrates and elevated values of COD. As can be appreciated, this yeast has a great capacity for lowering the COD of wastewater. To our knowledge, research has not been reported to date on *R. mucilaginosa* as a treatment for nejayote, despite the prevalence of this wastewater in Latin America.

Table 3. Studies on COD removal in wastewater and other media utilizing *Rhodotorula mucilaginosa*.

Strain	Substrate	Initial pH	Final pH	COD$_0$ (g L^{-1})	COD Removal Efficiency (%)	Time (Days)	Final Product	Reference
R. mucilaginosa	Olive oil mill wastewater	5.52	7.82	26.7	38.8	6	NS	[69]
R. mucilaginosa F-1	Waste ketchup	3.32	ND	20.0	84	9	Carotenoids	[66]
R. mucilaginosa ATCC 66034	Potato wastewater	12	9.5	30.82	49	5	Lipids and carotenoids	[76]
R. mucilaginosa LP-2	Crude glycerol	6	6.19	1533	54.6	9	Lipids	[80]
R. mucilaginosa LP-2	Sugarcane molasses	5.8	7	1230	52.3	9	Lipids	[80]
R. mucilaginosa ATCC 9450	Tequila vinasse waste	3.75	ND	43.93	20	2	Animal feed supplement	[81]
R. mucilaginosa URM7509	Sugarcane molasses + cassava wastewater	5.8	7.0	35.33	82.9	7	Lipids and carotenoids	[82]
R. mucilaginosa SML	Food flavoring wastewater + glucose	6.6	6.8	2.69	99.8	2.83	Biodiesel	[83]
R. mucilaginosa LCRE	Nejayote	10.3	7.84	27.4	75.5	12	NS	This work

ND, no data; NS, not specified.

On the other hand, Table 4 lists reports on the microorganisms found with good COD removal efficiency when nejayote was used as a substrate or co-substrate, mentioning key conditions of the process. The times of treatment were long. In some cases, nejayote was pretreated to lower the initial pH. For the aerobic and anaerobic processes conducted, most of the microorganisms involved are bacteria, with a few microalgae. To our knowledge, the current contribution is the first to test yeast for its COD removal efficiency in nejayote. One advantage of the current procedure is the lack of pretreatment of nejayote to change the initial pH prior to cultivation with the yeast.

R. mucilaginosa LCRE was herein found to have an enormous capacity for the bioremediation of nejayote. In relation to the Mexican norm for the disposal of this wastewater in bodies of water, the current process left the pH at an acceptable level (<10) but did not decrease the COD to a sufficiently low level (from 27,400 to 6650 mg L^{-1} versus the maximum permissible level of 150 mg L^{-1}). The lowest level of COD found presently is still 44 times greater than the maximum permissible level stipulated in the norm [11,12]. Hence, further research is needed to improve the COD removal efficiency of *R. mucilaginosa* LCRE. Additionally, it would be advantageous to seek a beneficial industrial use of the biomass of this yeast in order to make the treatment of nejayote economically attractive.

Table 4. Biological treatments of nejayote.

Condition	Reactor/ Mode of Operation	Initial pH	Final pH	COD_0 (g L^{-1})	COD Removal Efficiency (%)	Time (Days)	Supplementation	Microorganism	Product	Reference
Aerobic	Rotating biological reactor/continuous	5.8	7.0	6.4	84.6	20 (HRT = 2.5 days)	None	Amylolytic, proteolytic, and lipolytic bacteria (genera were not specified)	Biomass for livestock feed	[19]
Aerobic	Cascade bubble reactor/batch	6	7.9	26.5–28.0	86.4	36	Supplemented with KH_2PO_4 and $(NH_4)_2SO_4$	*Paenibacillus amylolyticus*, *Pseudomonas putida*, and *Acinetobacter* sp.	Biomass	[7]
Aerobic	Flask/batch	8–10	NR	6.44	75	15	90% nejayote 10% swine pork wastewater	*Chlorella vulgaris*	Biomass	[18]
Aerobic	Flask/batch	8–10	NR	6.44	76	15	90% nejayote 10% swine pork wastewater	*Arthrospira maxima*	Biomass	[18]
Aerobic	Flask/batch	10.3	7.84	27.4	75.5	12	None	*Rhodotorula mucilaginosa*	Biomass	This work
Anaerobic	APCR-UASB/CR	9	7.45	22.85	96.5	30 (28.9 m^3 m^{-2} d^{-1})	Nejayote	Native-mixed microbial consortium from deep soil, cattle manure, and pig manure (genera were not specified).	Methane	[20]
Anaerobic	MSB/batch	5.5	NR	52.8	27.5	5–5.8	80% tequila vinasses 20% nejayote	A mixed culture in which the main bacteria were *Acetobacter orientalis*, *Lactobacillus casei*, *Clostridium beijerinckii*, *Acetobacter lovaniensis*, and *Sporolactobacillus terrae*	Biohydrogen	[21]
Anaerobic	Serum bottles/batch	10.4	4.9	28,950	18.7	20 approx.	60% Nejayote 40% Brewery wastewater	The predominant communities of bacteria were *Clostridium* and *Burkholderia*	Biohydrogen	[22]
Anaerobic	MSB/batch	5.5	5.5	NR	47	0.5	Nejayote Abattoir Wastewater, C/N ratio = 30	Mixed culture, mainly consisting of *Clostridium butyricum*	Biohydrogen	[23]

NR, not reported; MSB, mechanically stirred bioreactor; HRT, hydraulic retention time; APCR-UASB, anaerobic packed bed column reactor and up-flow anaerobic sludge blanket reactor; CR, continuous reactor.

4. Conclusions

Of the 15 microorganisms presently tested for the bioremediation of the wastewater (nejayote) from the nixtamalization of maize, a yeast derived from sewage water yielded the best results. The alkali-tolerant yeast, identified as *R. mucilaginosa* LCRE, exhibited good growth under the very complex physical and chemical conditions of filtered nejayote (NEM), including an elevated pH (>10); a high level of COD; an abundant level of calcium; low concentrations of nitrogen, phosphorus, and easily assimilable sugars; and the presence of other biomolecules (e.g., phenolic compounds), that naturally inhibit the growth of many microorganisms. Although the supplementation of NEM with nitrogen and phosphorus salts benefited the growth of *R. mucilaginosa* LCRE and the consumption of sugars, similar results were found in the unenriched (control) NEM. Both the enriched and unenriched medium brought the pH of NEM within acceptable levels. However, the best COD removal efficiency was found in the unenriched (control) versus enriched NEM. Further research is needed to diminish the COD even further in order to reach a level acceptable for the disposal of wastewater. According to the current findings, *R. mucilaginosa* LCRE holds promise for the bioremediation of nejayote in the absence of costly pretreatments.

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