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Evaluation of Mezcal Distillery Vinasse at Various Concentrations as a Culturing Medium for *Stigeoclonium nanum*

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Vinasse is an effluent generated during the distillation process of mezcal and other alcoholic beverages. This substance typically shows an acidic pH and a high concentration of organic matter, causing a potentially polluting byproduct if not subjected to appropriate treatment and disposal methods. However, used efficiently, the high organic load within this effluent can be a rich source of soluble carbon and other organic nutrients, such as nitrogen and phosphorus, for microalgae growth. This study evaluated the tolerance of *Stigeoclonium nanum* to mezcal vinasse, using dilutions of 10, 20, 30, and 40 %, using complementary inoculum fractions in BG11 mineral medium over an eleven-day kinetic period. Daily determinations of photosynthetic pigments, nitrate nitrogen, and total phosphorus were made, along with the determination of Chemical Oxygen Demand (COD) at the beginning and end of the kinetic. The results revealed optimal growth with a higher production of photosynthetic pigments (compared to the control kinetic in mineral medium) at the 20 % dilution. The results obtained for this dilution were 18 mg L^{-1} of chlorophyll total production at the end of the kinetic period; additionally, a reduction of nitrate nitrogen on the effluent of 97.23 %, equivalent to 22.77 mg L^{-1}, and a decrease of total phosphorus by 88.33 %, corresponding to 1.74 mg L^{-1}. There was also a decrease of COD by 757 mg L^{-1}, constituting 56.36 % of the total content in the effluent.
When contaminants persist in the water with values higher than the allowed limits, it may cause some harmful effects on the environment. Microalgae exhibit remarkable adaptability and have the potential of consuming high amounts of nitrogen and phosphorus from wastewater treating pollutants from it to release them into the environment in compounds with less or no toxicity; thus, avoiding the risk of eutrophication of the water bodies and reducing the toxicity to human health. Utilizing effluents with high organic loads, such as vinasse, presents challenges. Therefore, this study provides a foundation for developing a culturing medium that ensures microalgae biomass growth and offers a phytoremediation alternative for treating industrial effluents.

1. Introduction

In a context where the concerns about the environmental impact of human activities are on the rise, finding sustainable and efficient solutions for treating industrial effluents has become a highly relevant matter. In addition, one of the problems of the high production cost of microalgal biomass production is the use of expensive mineral mediums (Martínez-Roldán and Ibarra-Berumen, 2019).

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The increasing trend in the consumption and production of alcoholic beverages, especially in the mezcal industry, has brought the challenge to manage the effluent produced, known as vinasse, which has a high organic content that can be used as a complement in culturing medium as a source of organic nutrients.
In recent decades, there has been a growing interest in the innovation and use of microalgae in biological methods for waste water treatment (Martínez-Roldán, 2024).
Most conventional water treatment methods present limitations such as incomplete treatment, high energy requirements, and high operating and maintenance costs. Microalgae is a promising alternative for effluent treatment while simultaneously producing biomass, which is an attractive option for biofuel production, food supplements, and other value-added products. Furthermore, the rapid growth ability of microalgae and their ease of adaptation to adverse conditions make them favourable candidates for applications in bioremediation (Ravikumar et al., 2021).
In waste water treatment, bacteria and fungi are effective for the removal of organic matter, but for the removal of nitrogen and phosphorus not that much. Microalgae can be a better fit for the reduction of those elements and even under the right conditions, can completely eliminate them from the effluent (Martínez-Roldán, 2024).
This study evaluates the tolerance of *Stigeoclonium nanum* to the effluent from the mezcal industry in Durango, Mexico, using inoculum fractions of 10, 20, 30, and 40 %. The primary purpose of this study is to assess the tolerance of this microalgae strain and evaluate the possibility of its use in treating effluents from mezcal distillation, thereby increasing efficiency in nutrient removal and reducing the associated organic load in these effluents. Simultaneously, the tolerance study will show biomass production during the process.
The relevance of this research resides in the lack of information about the tolerance of microalgae to mezcal vinasse. The majority of, existing studies are focused on tequila vinasse and its characteristics are different from mezcal production residues. *Stigeoclounium nanum* was selected because it is a native strain, there is limited research dedicated to it and its capability to growth in diverse industrial effluents was demonstrated.

2. Growth of *Stigeoclonium nanum* in mineral medium

It was necessary to evaluate a control kinetic to assess the tolerance of the *Stigeoglonium nanum* strain, where the culture medium consisted only of BG11 mineral medium. This kinetic serves as a baseline against which the microalgae behavior at various effluent dilutions will be compared. Figure 1 illustrates the production of photosynthetic pigments in mineral medium.

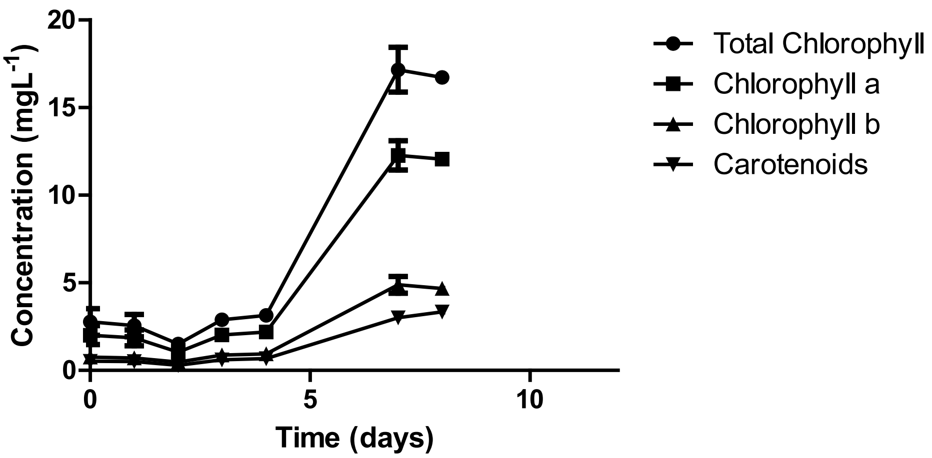

Figure 1: Photosynthetic pigments production in mineral medium.

Along with the BG11 medium as a control, in laboratory conditions. The experimental units consisted of 1 L Kimax® kimble glass bottles constantly bubbled with air at a approximately rate of 4.5 $Lmin^{-1}$, illuminated using cool white light at ≈250 $\mu Em^{-2}s^{-1}$ and a temperature of ≈25°C. The reported results are the mean of three independents experiments.
The quantification of chlorophylls was monitored by using the spectrophotometric method proposed by Wellburn (1994). This technique was used as a tool for monitoring cell growth. It can be noticed that the initial concentration of total chlorophylls was 2.77 mg L^{-1}. At the end of the eight days, the kinetic was 16.73 mg L^{-1},

having a total production of 13.96 mgL^{-1} for total chlorophylls, 10.04 mg L^{-1} for chlorophyll a, 3.91 mg L^{-1} for chlorophyll b, and 2.77 mg L^{-1} for carotenoids.

An exponential adjustment determined the specific growth rate of 0.2868 d^{-1}, considering that no limiting nutrient exists. Therefore, the maximum rate is taken as the specific growth rate.

During the kinetic in mineral medium, the determination of nutrient consumption of phosphorus and nitrogen as nitrate (NO_3, one of the most common forms available in aquatic environments) was carried out using the method proposed by Gales et al. (1996) and the method proposed by Zhang and Fischer (2006), respectively. Figure 2 illustrates the behavior of these nutrients.

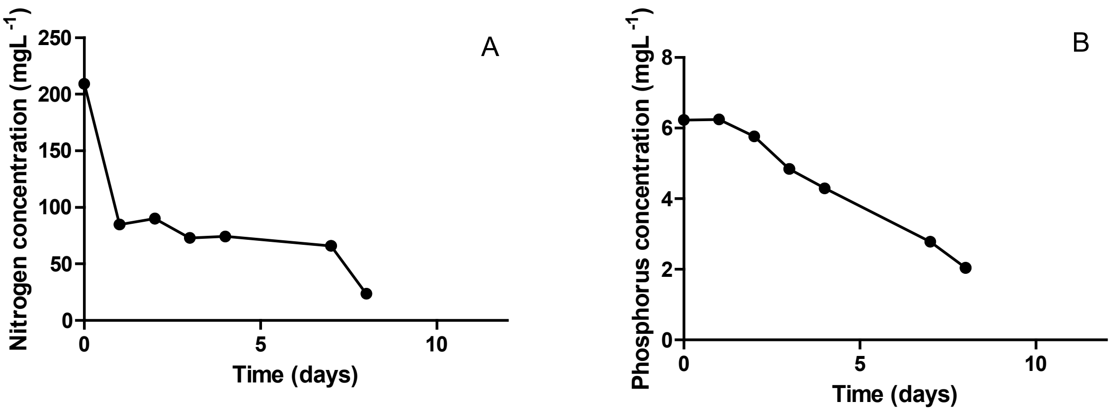

Figure 2: Nutrients consumption by Stigeoclonium nanum in mineral medium. 2A: Nitrogen. 2B: Phosphorus.

For Nitrogen, a removal of 88.67 % was achieved, equivalent to a consumption of 185.61 mg L^{-1}. For Phosphorus, 4.19 mg L^{-1} was removed, equivalent to 67.25 %. Considering the total consumption of nitrogen and phosphorus and the culture duration, the volumetric consumption rate was calculated. The consumption rates were 23.20 mg L^{-1} d^{-1} for Nitrogen and 0.52 mg L^{-1} d^{-1} for Phosphorus, these values are similar to the reported for different microalgal strains.

Microorganisms such as microalgae require micronutrients for their growth. Microalgae can use nitrogen from many sources with different oxidation states, such as nitrate, ammonia, nitrite or organic compounds whereas phosphorus is used mainly in the form of phosphate (Martínez-Roldán, 2024).

3. Growth of *Stigeoclonium nanum* at different vinasse concentrations

A sequence of experiments was conducted with 4 replicates for each dilution, and each determination was carried out in triplicate. The effluent was pretreated filtering out coarse solids and suspended particles and adjusting the pH to 9. This is because the optimal pH for green microalgae typically falls within this range (Hoff & Snell, 2001). The neutralization of the mezcal effluent was carried out using sodium hydroxide (NaOH). Figure 3 shows the dilutions of vinasse at 10, 20, 30, and 40 %, respectively.

Figure 3. Microalgae using dilution of vinasse as a culturing medium.

Figure 4 shows the daily determination of photosynthetic pigments along the growth kinetics, where it can be observed that every dilution started at an average of 6.56 mg L^{-1} of total chlorophylls. At the end of the 11-day kinetic, the 20 % dilution exhibited the highest total production of photosynthetic pigments. Compared with the control, the 20 % dilution demonstrated a 29.23 % increase in biomass production, equivalent to 18.04 mg L^{-1} of total chlorophylls. Other pigments, such as chlorophyll a, chlorophyll b, and carotenoids, were evaluated during the kinetics. Chlorophylls a and b kept the same behavior as the total chlorophylls. However, the carotenoids show an exciting behavior since no dilution overcomes the control kinetic in the mineral medium. This may indicate that adding the effluent leads to a decrease in irradiation due to the turbidity of the vinasse. Carotenoids are typically produced in higher quantities when a protective function is required; this normally happens when light stress exists in the environment. As the content of the distillery effluent increases in the culturing medium, the concentration of carotenoids decreases, suggesting that the used vinasse may contain phenolic compounds such as phenolic acids and flavonoids, which possess antioxidant properties and consequently cause lower carotenoids production.

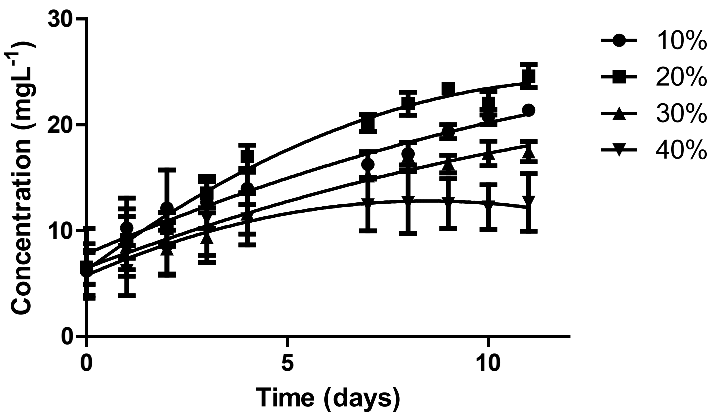

Figure 4: Total chlorophylls production evaluated at different concentrations.

The nitrogen consumption curve illustrated in Figure 5 shows how microalgae utilize and deplete this nutrient over time. This understanding is crucial for optimizing biomass production concerning the concentration of this nutrient. It can be observed that the initial concentration is inversely proportional to dilution. In other words, the higher concentration of this nutrient can be attributed to the mineral medium's contribution and it is also found in the most easily assimilable form for microalgae consumption.

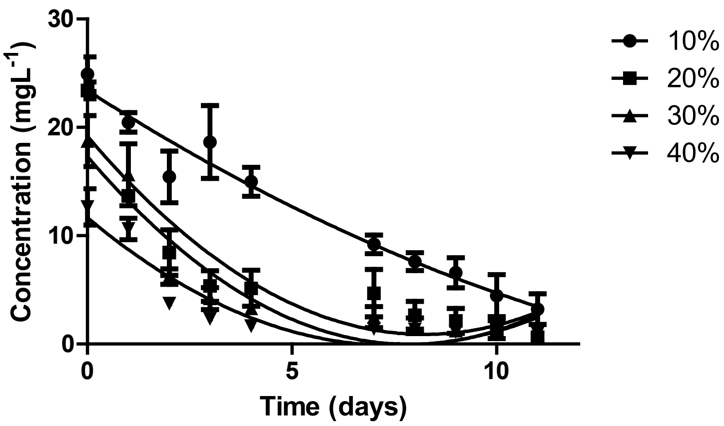

Figure 5: Nitrogen consumption (growth in different concentrations of vinasse).

Dilutions of 20, 30, and 40 % surpass the removal percentages provided by the control kinetics in the mineral medium. However, the most effective removal was achieved with the 20 % dilution, exhibiting a total consumption of 22.77 mg L^{-1} at the end of the 11-day kinetic, representing a 97.23 % total removal of NO_3.
The highest volumetric consumption rate was also found in the 20 % dilution kinetic which was 2.07 mg$L^{-1}d^{-1}$. This is consistent with the behavior observed in figure 5 that shows that by the third day, 77.16% of the available nitrogen is consumed. This behavior can be explained by the growth and adaptation phase in which the culture was in at the time. After this stage, a slower nutrient consumption occurs because the culture enters the stationary phase, where the microalgae nutritional requirements are much lower.
The behavior of the consumption of phosphorus showed an evident almost total absorption of the nutrient in less than three days. Significant removal percentages were evident in all four dilutions up to 80 %, equivalent to an average of 2.17 mg L^{-1}. The addition of effluent favors nutrient consumption. This phenomenon may be attributed to the fact that, at higher concentrations, the nutrient ceases to be limiting, thereby increasing microalgal absorption.
The determination of COD provides a quantitative estimation of the concentration of organic matter present in vinasse, and it was estimated using the method proposed by Rice et al (2012). The contribution of COD to the microalgae culture medium is only attributed to the addition of the effluent. Therefore, it is a determination that was not quantified in the control kinetics in the mineral medium.
All four dilutions exhibit a removal of COD up to 40 %. The dilution that had the highest removal, and consequently, a better degradation of the organic load present in the medium, was a 20 % dilution, achieving a total removal of 757 mg L^{-1} at the end of the kinetics, which represents a 56.16 % removal. The highest volumetric consumption rate was also found in the 20 % dilution kinetic which was 68.81 mg$L^{-1}d^{-1}$.
A higher biomass concentration corresponds to a more significant degradation of organic matter. As a confirmation of this, the optimal production of microalgal biomass was also observed at this particular vinasse concentration.
The determination of the specific growth rates shows that the rate corresponding to the 20 % dilution was 0.1749 d^{-1}. However, it does not surpass the growth rate of the control; the kinetics with the addition of the effluent extends for an additional three days compared to the mineral medium control. This extension represents a higher biomass production.
Figure 6 shows the evolution in the coloration of the culture containing 20 % dilution throughout the entire 11-day duration of the kinetics.

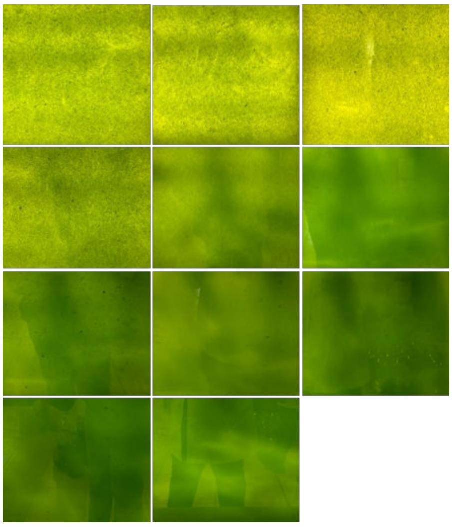

Figure 6: Evolution of coloration in the 20 % dilution kinetic.

4. Conclusions

Adding the effluent stimulated the kinetics duration, extending it to 11 days and increasing biomass production by 29.23 % (In the 20 % dilution). Additionally, the highest concentration of photosynthetic pigments is chlorophyll a, constituting 73.06 % of total chlorophyll. The nitrogen consumption reached removal values of up to 97.23 % and a 56.16 % reduction of COD for the best operational conditions using a 20 % dilution as a culturing medium, this removal could be optimized by implementing a semi-continuous operation.

The use of microalgae could be a potential alternative for nutrient degradation of industrial effluents, thereby reducing their environmental impact. The tolerance tests conducted in this study show that a dilution percentage greater than 30 % inhibits the growth of *Stigeoclonium nanum*.
The evaluation of the growth behaviours of the culture allows us to conclude that depending on the objective of the kinetics, the duration and the most suitable dilution can be defined. If the goal is biomass production, it is recommended to extend the kinetics for 11 days using a 20% dilution. However, if the purpose is nutrient reduction and the decrease of organic load, it is possible to terminate on day eight and use higher dilutions, still achieving substantial removals.
The results can be approached from two different perspectives: optimizing the culture medium using effluents for biomass production, or contaminant removal through the growth of green microalgae strains. *Stigeoclonium nanum* can auto-flocculate, which represents a significant advantage in biomass recovery as it does not require additional energy consumption or the addition of inorganic coagulants.
The results obtained in this investigation demonstrate the potential of this strain for the removal of contaminants such as nitrogen, phosphorus and COD from the mezcal distillery effluent. Some of these contaminants can reach values within limits stipulated in the Mexican Regulation.
Microalgae offer numerous potential applications, but developing these processes sustainably is crucial**.** This is why it is important to promote the use of industrial waste water as a nutrient source for the development of microalgae cultures, which allow, the reduction of contaminants and at the same time, increases the production of biomass which is a source of a large number of different molecules, several of them have high market value, generating an economical benefit (Borowitzka, 2013).
These results expect to contribute to scientific knowledge regarding the use of microalgae in treating industrial effluents. Additionally, the study aims to identify the optimal conditions to maximize organic load removal and biomass production using mezcal vinasse, building the bases for future applications on a larger scale system or under different operational conditions in a sequential batch regime.

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