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Pseudomonas sp Isolated from Wastewater and their Interaction with Microalgae

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Abstract

During biodegradation processes in Wastewater Treatment Plants (WWTP), bacteria play an important role, and associations of bacteria with microalgae have the potential to remediate wastewater by reducing pollutants. This work aimed to identify bacteria present in wastewater and to analyze their interaction with *C. vulgaris* to favor their phytoremediation. The physicochemical and microbiological characterization of the wastewater from the Alseseca-Sur WWTP was carried out, identifying the bacterial isolates, a phytoremediation treatment was performed with *C. vulgaris* and wastewater as a culture medium, quantifying the proliferation of *C. vulgaris* in the supplemented culture medium and the wastewater. The physicochemical parameters decreased after phytoremediation, the algae-bacteria system caused a decrease in COD due to its application in symbiosis; *Pseudomonas* sp. prevailed with 75%; *C. vulgaris* showed optimal growth when wastewater was used as culture medium, inoculating *C. vulgaris* in wastewater at 100%, 174 cells/mL were quantified compared to 24 cells/mL of *C. vulgaris* with bold medium plus drinking water. The phytoremediation process in conjunction with the bacterial load effluent from the Alseseca-Sur WWTP showed a decrease in the values of physicochemical parameters; in addition to increasing the number of cells/mL of *C. vulgaris* using water from the effluent of the Alseseca-Sur WWTP as a culture medium.

Keywords: Biodegradation, Environmental, Wastewater, *Pseudomonas*, Microalgae

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Introduction

Biological treatment of wastewater allows for the removal of organic matter and nutrients, enabling its reuse or safe discharge. Bioremediation of wastewater depends in part on the microbial communities present in the bioreactor. Bacteria play an important role in the processes of decomposition and stabilization of organic matter; in wastewater treatment plants the presence of these microorganisms is important due to their participation in the biodegradation process, highlighting their activity in the removal of Biological Oxygen Demand (BOD), coagulation of non-sedimentable colloidal solids and stabilization of organic matter (Barak *et al*., 2020). These activities carried out by bacteria are intended to complete the cycle of degradation and reproduction from the waste material, which is how man uses these microorganisms to purify wastewater. In Mexico, treatment plants concentrate water from domestic, agricultural and industrial sources, the latter containing a large amount of organic and inorganic substances that are toxic for the microorganisms responsible for stabilizing organic matter, making it appropriate to carry out a pre-treatment for the purification of toxic waste, generating after this treatment an association of bacteria with microalgae to establish an adequate control of all the factors involved in the treatment (Yong *et al*., 2021). Studies by Chen *et al*., (2017) have shown that, in natural aquatic environments, microalgae are always associated with bacteria leading to mutual interactions and can be growth promoters, symbionts or simply in coexistence, generating strategies for bioremediation with low action times in the reduction and/or removal of heavy metals present in wastewater used for agricultural irrigation (Matsumoto *et al*., 2015). Microalgae in laboratory culture are often associated with different bacterial strains that have been co-enriched from natural environments together with algal cells, or that come from the site of contamination. *C. vulgaris* has a high mitigation potential as it absorbs certain heavy metals through biosorption; in addition to being in direct contact with the microbiota it works together with the bacteria present in the contaminated environment (Salama *et al*., 2019; Ubando *et al*., 2021) and *C. vulgaris* has also been reported to increase the abundance of bacteria that support oxygen production, so a combined application of microalgae and bacteria-based technologies offers a promising remediation approach (Song *et al*., 2020). Weimer *et al*., (2020) isolated *Pseudomonas putida* from wastewater where they used *C. vulgaris* for tertiary treatment and observed that the bacterial contribution supported the growth and performance of the microalgae in their

work within the tertiary phase of treatment; furthermore Leong and Chang (2020) investigated the use of this microalgae in small communities thanks to its low cost and efficiency, describing that it not only removes heavy metals, but also organic matter and inorganic nutrients by working in conjunction with certain bacteria. In wastewater, the presence of heavy metals and xenobiotic compounds is characteristic, generating problems in high concentrations in the development, growth, and reproduction of organisms (Arora, 2020). The joint use of bacteria with *C. vulgaris* extract or immobilize toxic compounds, leaving the substance unavailable to organisms and preventing them from being affected. It is also important to highlight that bacteria are functionally important in pollutant biotransformation processes and offer resources for their application in bioremediation, as well as achieving various socioeconomic advantages through the use of this process, in particular the environmental advantages of bioenergetics and the economic and social benefits of the economic valorization of wastewater (Ma *et al*., 2020; Rajhi *et al*., 2020). This study aimed to identify bacteria present in wastewater and to analyze their interaction with *C. vulgaris* to promote their phytoremediation.

Materials and Methods

Study Area

The Alseseca-Sur Wastewater Treatment Plant (WWTP) located in the municipality of Puebla, Mexico (18°57'14.9 " N, 98°11'23.5" E), performs advanced primary treatment in the final part of the Alseseca riverbed. The treated water presents a domestic, agricultural and industrial mix, with a maximum treatment in rainy weather of 700 L/s while in the dry season it is 300 L/s.

Sampling

Weekly visits were made to the Alseseca sur-WWTP during November 2017, March, and November 2018, to know the characteristics of the samples, discarding variations of the components in different sampling periods. Each of the samplings was carried out following the NMX-AA-003-1980 standard, where the sample collection was established consisting of 6 L subsamples every 4 h for 24 h to obtain a composite sample transferred to the laboratory to be stored at 4 °C (DOF, 1992).

Physicochemical Characterization

The physicochemical characterization of the wastewater samples was carried out following Mexican Standards and Water Analysis Techniques, which establish the methodological and permissible parameters for the determination of the water quality in the wastewater samples to be used (DOF, 1997).

Microbiological Quantification

Serial dilutions were made of the wastewater samples and the wastewater treated with *C. vulgaris* in nutritive broth, taking dilutions from 10-3 to 10-6, spreading 0.1 mL of each of the dilutions in nutrient agar, incubated at 36 °C for 24 h, then the colony-forming units per milliliter (CFU/mL) were counted, performed in triplicate. Bacterial isolation was performed by striated seeding on nutrient agar, incubating at 36 °C for 24 h, selecting various colonies for their morphological characteristics and by Gram staining, placing them again in nutrient broth to be incubated; these isolates were identified with the Chromogenic Compact Dry "Nissui" EC™ medium. DNA extraction was carried out with the Zymo-Research Quick-DNA Fungal / Bacterial kit and it was amplified by Polymerase Chain Reaction (PCR) with universal oligonucleotides of the 16S RM and RB gene for bacteria (5'- AGA GTT TGA TYM TGG CTC AG-3 ') and (5'- GGA CTA CCA GGG TAT CTA ATC C-3') using an MS mini thermal cycler (BioRad®, USA) and the following amplification program: initial denaturation at 95 °C by 5 min, 35 cycles of 92 °C for 1 min, 57 °C for 30 s, and 72 °C for 1 min (Cabra-Cendales *et al*., 2015). The PCR products were analyzed by 1% agarose gel electrophoresis, stained with Biotium. The sequencing of the PCR products was carried out by the Biomolecular Detection Center of the Benemerita Universidad Autonoma de Puebla, sending vials containing +/- 100 µL of the products of each PCR reaction. The sequences were analyzed for quality using the CLC main Workbench ver. 6.1® 2015 CLC bio, QIAGEN and then annotated using the BLAST program ver. 2.2.27+ comparing them against the NCBI nt database, with a threshold value of 1 × 10-5. These results were filtered with 80% similarity and 80% coverage of the amplified region.

Wastewater Treatment with Chlorella vulgaris

The inoculation of *C. vulgaris* was carried out directly in the effluent water samples from the Alseseca-Sur WWTP, using the wastewater sample as the culture medium of *C. vulgaris* in addition to the Bold medium. 10 mL of *C. vulgaris* were added for each L of residual water, carrying out the inoculation in triplicate. The conditions for the growth of the algae were with continuous aeration at a flow of 19 L/h and exposed to white light at a temperature of 18 °C for 5 days (Magdaleno *et al*., 2012). At the end of the growth time of *C. vulgaris* in the wastewater sample, the concentration of 180 cells / mL of *C. vulgaris* in the wastewater was observed, verifying with this data that the water from the Alseseca-Sur WWTP contains the appropriate characteristics. For the survival and growth of *C. vulgaris* (stock solution), 10 mL of the stock solution were added for each liter of residual water, making concentrations of 100 (total residual water), 75 (750 mL residual water: 250 mL purified water), 50 (500 mL residual water: 500 mL purified water) and 25% (250 mL residual water: 750 mL purified water) respectively plus a 100% purified water control plus Bold medium as culture medium. At the end of the incubation period, the cell count was performed. The organic matter was separated from the water utilizing centrifugation to determine the physicochemical parameters of the water following the Mexican water analysis standards mentioned above.

The cell count was performed on the total of the samples, estimating the growth by the cell count through the microscope, always considering the Neubauer chamber filling technique (Neubauer Improved 0.1 mm), performing the cell concentration calculations (Ramos & Pizarro, 2012).

Statistical Analysis

The comparison of the CFU number of the wastewater with the wastewater treated with *C. vulgaris* was performed with the Mann Whitney U test with a significance level of 0.05 (GraphPad InStat Software v2.04a).

Results and Discussion

The physicochemical characterization of the effluent water from the Alseseca-Sur WWTP was determined during the three days following its collection, except for the BOD which was measured once the sample arrived at the laboratory. The Alseseca-Sur WWTP treated with *C. vulgaris*; the results obtained are shown in **Table 1**, in comparison with the maximum permissible limits of the corresponding regulation.

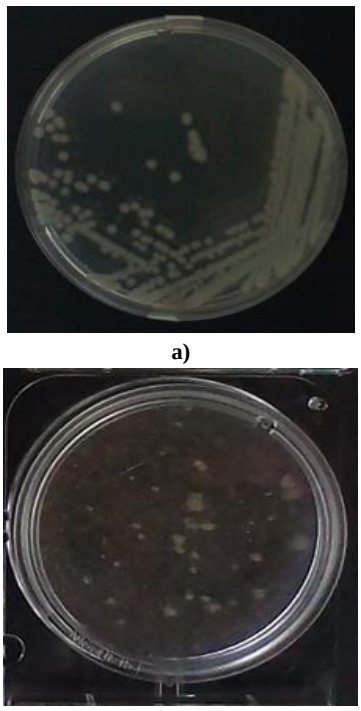
The microbiological quantification showed a decrease in the bacterial load in the phytoremediated sample with *C. vulgaris*, obtaining for the first dilution $108X10^6$ CFU, while in the sample treated with *C. vulgaris* a total of $72X10^6$ CFU was quantified for the dilution equivalent; For the 10-6 dilutions of both samples, 108 and 72 CFU were counted respectively, the statistical analysis did not show a significant difference ($P>0.05$) in the CFU/mL count between the values of the effluent water and the phytoremediation process. The isolates obtained in a nutrient medium presented the colonial characteristics of *Pseudomonas* sp., **(Figure 1a)** when performing the Gram stain, they were negative, bacillary-rectum shape and did not develop spores; characteristics like those of *Pseudomonas* sp. The bacterial cultures seeded in the Chromogenic Compact Dry "Nissui" EC™ medium presented a beige coloration that according to the manufacturer's indications corresponds to *Pseudomonas* sp., **(Figure 1b)** to confirm the identification of the isolates, after their amplification by PCR **(Figure 2)**. **Table 2** shows the BLAST results, obtaining the identification of the microorganisms (coverage and identity greater than 86%) for 75% of the isolates.

The phytoremediation process using wastewater as a culture medium showed favorable results with a higher percentage of wastewater; where, in 100% water, 174 cells/mL of *C. vulgaris*, 75% 120 cells/mL, 50% 90 cells/mL and 25% 35 cells/mL of the algae were quantified; unlike the control where 24 cells/mL were quantified.

In several studies where phytoremediation processes are carried out in wastewater, physicochemical parameters that contribute to water pollution are determined, such as arsenic, organic compounds, phosphorus and nitrogen (Gomez-Guzman *et al*., 2017; Srivastava *et al*., 2018); however, this determination is only focused on specific pollutants, and it is important to carry out a complete characterization once phytoremediation has been carried out. In this study, a physicochemical characterization was carried out following NOM-001-SEMARNAT-199617, considering all the parameters to determine the variations once the phytoremediation process has been implemented. The microbiological study of the treated samples showed that *Pseudomonas* sp., in 75% of the isolates from the wastewater from the Alseseca-Sur WWTP, using the effluent water as a culture medium for the growth of *C. vulgaris* without requiring a simulated medium, thus taking advantage of the characteristics present in the initial sample, in contrast to what has been reported in various studies where phytoremediation processes are carried out with *C. vulgaris* inoculating certain species of *Pseudomonas*, *Bacillus* and bacterial consortia to favor the efficiency of the system in the removal of pollutants present in wastewater (Mu *et al*., 2020); the data on the increase in the concentration of *C. vulgaris* with wastewater as a culture medium are similar to those reported in Shen *et al*., (2017) where they report an increase in the cell density of *C. vulgaris* in symbiosis with *Pseudomonas putida* using municipal wastewater as a culture medium, removing ammonium, phosphates and organic compounds from the wastewater. However, in the present work the decrease was in all the physicochemical parameters established in NOM-001-SEMARNAT-199618, except for hardness. This decrease is due to the use of 100% wastewater inoculated with *C. vulgaris*, increasing the cell density of the algae using the wastewater as a bioreactor and generating adequate phytoremediation, unlike what was reported by Amenorfenyo *et al*., (2019) where the increase in the concentration of algae increased about the amount of *Pseudomonas* inoculated in the photoreactors, generating an increase in treatment costs. The reduction of pollutants in phytoremediation wastewater is directly linked to the use of *C. vulgaris* together with the microbiota present in the wastewater, in particular *Pseudomonas*.

Table 1. Physicochemical Characterization of the Sample from the Alseseca-Sur WWTP

Parameter	NOM-001-SEMARNAT-1996	Initial sample	Sample treated *C. vulgaris*
pH	5-10	8	7
DQO	Not specified	212	140
DBO	200	66.66	50
Nitrogen	60	7.8	0.7
Phosphor	30	1.7	1.2
Arsenic	0.4	0	0
Cadmium	0.4	0.091	0.045
Copper	6	0.09	0.06
Cyanide	3	0.027	0.017
Chrome	1.5	1.37	1.24
Nickel	4	0.62	0.26
Lead	1	0.68	0.52
Zinc	20	1.04	0.97
Hardness ($CaCO_3$)	Not specified	320	335
Magnesium	Not specified	28.5	23
Calcium	Not specified	146	153
Total solids	200	20.69	32.45
Suspended-solids (mL/L)	2	1.68	1.02



a)

b)

Figure 1. Colonial growth of P*seudomonas* sp., in nutrient agar Panel (a), *Pseudomonas* sp., identification in Cromogenic medium Compac Dry Nissui EC™ Panel (b).

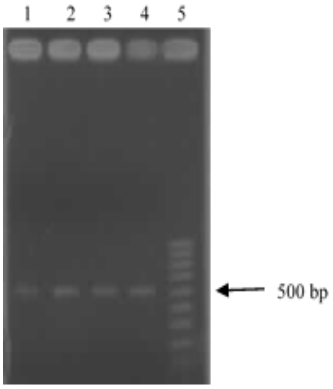


Figure 2. Agarose gel electrophoresis 1%, lanes 1-3 positive samples to 16S gene, lane 4 positive control, lane 5 molecular weight marker (1 kb).

Table 2. Identification of isolates with BLAST using the 16S gene

Code	Description	Coverage (%)	Identity (%)	Accession
AR1	*P. toyotomiensis*	89	95.41	KF993336.1
AR2	*Pseudomonas* sp BAB-3709	93	94.35	KM104685.1
AR3	*Pseudomonas* sp BAB-3709	86	96.26	KM104685.1
AR4	*S.enterica* NCTC10433	80	95.22	LS483428.1

Conclusion

The effluent water from the Alseseca-Sur WWTP can be used as a culture medium for the development of *C. vulgaris*, as it has the necessary characteristics for its proliferation, increasing the number of cells/mL in the wastewater to 100%, thus generating effective phytoremediation of the wastewater, demonstrating the decrease in the values of the physicochemical parameters; without the addition of microorganisms.

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Ethics statement: None

References

Amenorfenyo, D. K., Huang, X., Zhang, Y., Zeng, Q., Zhang, N., Ren, J., & Huang, Q. (2019). Microalgae brewery wastewater treatment: Potentials, benefits, and the challenges. *International Journal of Environmental Research Public Health*, *16*(11), 1910. doi:10.3390/ijerph16111910.

Arora, P. K. (2020). Bacilli-mediated degradation of xenobiotic compounds and heavy metals. *Frontiers in Bioengineering Biotechnology*, *8*, 570307. doi:10.3389/fbioe.2020.570307.

Barak, H., Brenner, A., Sivan, A., & Kushmaro, A. (2020). Temporal distribution of microbial community in an industrial wastewater treatment system following the crash and during recovery periods. *Chemosphere*, *258*, 1-9. doi:10.1016/j.chemosphere.2020.127271.

Cabra-Cendales, T., Meneses-Cabezas, D. C., & Galeano-Venegas, N. F. (2015). Identificación de microorganismos asociados a residuos de higuerilla (*Ricinus communis). Revista Colombiana de Química, 44*(2), 10-15.

Chen, T., Zhao, Q., Wang, L., Xu, Y., & Wei, W. (2017). Comparative metabolomic analysis of the green microalga *Chlorella sorokiniana* cultivated in the single culture and a consortium with bacteria for wastewater remediation. *Applied Biochemical Biotechnology*, *183*(3), 1062-1075. doi: 10.1007/s12010-017-2484-6.

Diario Oficial de la Federación (DOF). Límites Máximos Permisibles de Contaminantes en Las Descargas de Aguas Residuales en Aguas y Bienes Nacionales; NOM-001-SEMARNAT-1996. Ciudad de México, México.1997. Available online: http://www.profepa.gob.mx/innovaportal/file/3290/1/nom-001-semarnat-1996.pdf (accessed on June 23 2019).

Diario Oficial de la Federación (DOF). Norma Mexicana "Aguas

residuales muestreo"; NMX-AA-3-1980. Ciudad de México, México. 1992. Available online: https://www.gob.mx/cms/uploads/attachment/file/166762/ NMX-AA-003-1980.pdf (accessed on June 23 2019).

Gómez-Guzmán, A., Jiménez-Magaña, S., Guerra-Rentería, A. S., Gómez-Hermosillo, C., Parra-Rodríguez, F. J., Velázquez, S., Aguilar-Uscanga, B. R., Solís-Pacheco, J., & González-Reynoso, O. (2017). Evaluation of nutrients removal (NO3-N, NH3-N and PO4-P) with *Chlorella vulgaris, Pseudomonas putida, Bacillus cereus* and a consortium of these microorganisms in the treatment of wastewater effluents. *Water Science Technology, 76*(1-2), 49-56. doi:10.2166/wst.2017.175

Leong, Y. K., & Chang, J. S. (2020). Bioremediation of heavy metals using microalgae: Recent advances and mechanisms. *Bioresource Technology*, *303*, 122886. doi:10.1016/j.biortech.2020.122886.

Ma, Q., Liu, S., Li, S., Hu, J., Tang, M., & Sun, Y. (2020). Removal of malodorant skatole by two enriched microbial consortia: Performance, dynamic, function prediction and bacteria isolation. *Science of the Total Environment, 725*, 1-10. doi:10.1016/j.scitotenv.2020.138416.

Magdaleno, A., Juárez, A. B., Paz, M., Tornello, C., Núñez, L., & Moretton, J. (2012). Evaluación ecotóxica y genotóxica de aguas hospitalarias. *Acta Toxicologica Argentina, 20*, 14-24.

Matsumoto, S., Kasuga, J., Taiki, N., Makino, N., & Arao, T. (2015). Reduction of the risk of arsenic accumulation in rice by the water management and material application in relation to phosphate status. *Journal of Plant Interaction, 10*(1), 65-74. doi:10.1080/17429145.2015.1016129

Mu, R., Jia, Y., Ma, G., Liu, L., Hao, K., Qi, F., & Shao, Y. (2020). Advances in the use of microalgal-bacterial consortia for wastewater treatment: Community structures, interactions, economic resource reclamation, and study techniques. *Water Environmental Research*, 11. doi:10.1002/wer.1496.

Rajhi, H., Bardi, A., Sadok, S., Moussa, M., & Turki, S. (2020). Phytoremediation of samples extracted from wastewater treatment plant and their socioeconomic impact. *Water Science Technology*, *82*(8), 1653-1664. doi:10.2166/wst.2020.429.

Ramos, R., & Pizarro, R. (2012). Growth and bioremediation capacity of Chlorella vulgaris (Trebouxiophycea, Chlorophyta) cultivated in wastewater generated in the fish farming of the yellowtail amberjack Seriola lalandi (Perciformes: Carangidae). *Revista de Biología Marina y Oceanografía*, *53*(1), 75-86. doi:10.4067/S0718-19572018000100075

Salama, E. S., Roh, H. S., Dev, S., Khan, M. A., Abou-Shanab, R.A. I., Chang, S. W., & Jeon, B. H. (2019). Algae as a green technology for heavy metals removal from various wastewater. *World Journal of Microbiology Biotechnology*, *35*(5), 75. doi:10.1007/s11274-019-2648-3.

Shen, Y., Gao, J., & Li, L. (2017). Municipal wastewater treatment vi aco-immobilized microalgal-bacterial symbiosis: Microorganism growth and nutrients removal. *Bioresource Technology, 243*, 905-913. doi:10.1016/j.biortech.2017.07.041

Song, X., Wang, W., Cao, X., Wang, Y., Zou, L., Ge, X., Zhao, Y., Si, Z., & Wang, Y. (2020). *Chlorella vulgaris* on the cathode promoted the performance of sediment microbial fuel cells for electrogenesis and pollutant removal. *Science of the Total Environment, 728*, 1-8. doi:10.1016/j.scitotenv.2020.138011.

Srivastava, S., Srivastava, S., Bist, V., Awasthi, S., Chauhan, R., Chaudhry, V., Shing, C. P., Dwivedi, S., Niranjan, A., Agrawal, L., et al. (2018). *Chlorella vulgaris* and *Pseudomonas putida* interaction modulates phosphate trafficking for reduce arsenic uptake in rice *(Oryza sativa L.). Journal of Hazardous Materials, 5*(351), 177-187. doi:10.1016/j.jhazmat.2018.02.039

Ubando, A. T., Africa, A. D. M., Maniquiz-Redillas, M. C., Culaba, A. B., Chen, W. H., & Chang, J. S. (2021). Microalgal biosorption of heavy metals: A comprehensive bibliometric review. *Journal of Hazardous Materials, 402*, 123431. doi:10.1016/j.jhazmat.2020.123431.

Weimer, A., Kohlstedt, M., Volke, D. C., Nikel, P. I., & Wittmann, C. (2020). Industrial biotechnology of *Pseudomonas putida*: advances and prospects. *Applied Microbiology Biotechnology*, *104*(18), 7745-7766. doi:10.1007/s00253-020-10811-9.

Yong, J. J. J. Y., Chew, K. W., Khoo, K. S., Show, P. L., & Chang, J. S. (2021). Prospects and development of algal-bacterial biotechnology in environmental management and protection. *Biotechnology Advance, 47*, 107684. doi:10.1016/j.biotechadv.2020.107684.