

Accepted Manuscript

Integral use of sugarcane vinasse for biomass production of Actinobacteria:
Potential application in soil remediation

Juan D. Aparicio, Claudia S. Benimeli, César A. Almeida, Marta A. Polti, Verónica L. Colin



PII: S0045-6535(17)30643-4

DOI: 10.1016/j.chemosphere.2017.04.107

Reference: CHEM 19169

To appear in: *Chemosphere*

Received Date: 06 February 2017

Revised Date: 07 April 2017

Accepted Date: 23 April 2017

Please cite this article as: Juan D. Aparicio, Claudia S. Benimeli, César A. Almeida, Marta A. Polti, Verónica L. Colin, Integral use of sugarcane vinasse for biomass production of Actinobacteria: Potential application in soil remediation, *Chemosphere* (2017), doi: 10.1016/j.chemosphere.2017.04.107

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

HIGHLIGHTS

- Actinobacteria biomass was produced in vinasse-based culture media.
- Recovered supernatants were used until three times for additional biomass production.
- Over 70% of the BOD in vinasse was removed by actinobacteria mixed cultures.
- High biomass performance for removal of lindane and chromium from soil was detected.

**Integral use of sugarcane vinasse for biomass production of
Actinobacteria: Potential application in soil remediation**

Juan D. Aparicio ^{a, b}, Claudia S. Benimeli ^{a, c}, César A. Almeida ^d, Marta A. Polti ^{a, e},
Verónica L. Colin ^{a,*}

^a Planta Piloto de Procesos Industriales Microbiológicos (PROIMI), CONICET. Av.
Belgrano y Pasaje Caseros. 4000 Tucumán, Argentina

^b Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán. 4000.
Tucumán, Argentina

^c Universidad Santo Tomás de Aquino. 4000. Tucumán, Argentina

^d Instituto de Química de San Luis, INQUISAL (UNSL-CONICET), Universidad Nacional
de San Luis. 5700. San Luis, Argentina

^e Facultad de Ciencias Naturales e Instituto Miguel Lillo, Universidad Nacional de
Tucumán. 4000. Tucumán, Argentina

***Corresponding author:** Verónica Leticia Colin, PROIMI-CONICET, Av. Belgrano y
Pasaje Caseros. 4000. Tucumán, Argentina. Tel: 54-381-4344888. Fax: 54-381-4344887 e-
mail: veronicacollin@yahoo.com.ar

ABSTRACT

The use of living actinobacteria biomass to clean up contaminated soils is an attractive biotechnology approach. However, biomass generation from cheap feedstock is the first step to ensure process sustainability. The present work reports the ability of four actinobacteria, *Streptomyces* sp. M7, MC1, A5, and *Amycolatopsis tucumanensis*, to generate biomass from sugarcane vinasse. Optimal vinasse concentration to obtain the required biomass (more than 0.4 g L⁻¹) was 20% for all strains, either grown individually or as mixed cultures. However, the biomass fraction recovered from first vinasse was discarded as it retained trace metals present in the effluent. Fractions recovered from three consecutive cycles of vinasse re-use obtained by mixing equal amounts of biomass from single cultures or produced as a mixed culture were evaluated to clean up contaminated soil with lindane and chromium. In all cases, the decrease in pesticide was about 50% after 14 d of incubation. However, chromium removal was statistically different depending on the preparation methodology of the inoculum. While the combined actinobacteria biomass recovered from their respective single cultures removed about 85% of the chromium, the mixed culture biomass removed more than 95%. At the end of the reused vinasse cycle, the mixed culture removed more than 70% of the biological oxygen demand suggesting a proportional reduction in the effluent toxicity. These results represent the first integral approach to address a problematic of multiple contaminations, concerning pesticides, heavy metals and a regionally important effluent like vinasse.

Keywords: Actinobacteria; Biomass; Vinasse; Lindane; Chromium

1. Introduction

The Northwest of Argentina, more precisely the Tucuman province, has an important concentration of sugar industries combined with autonomous ethyl alcohol distilleries. One of the main problems of the sugar-alcohol industries is the generation of large volumes of vinasse, a final residue in liquid state obtained after alcoholic distillation. Vinasse has a pH that usually ranges from 3.5 to 5.0, and a production volume of 9–14 L per liter of alcohol obtained (España-Gamboa et al., 2012). The raw effluent consists of a complex mixture of water and organic and inorganic compounds, and its release into the environment causes an undesirable impact, mainly as a consequence of the high content of organic matter. The chemical oxygen demand (COD) is usually estimated between 50 and 150 g L⁻¹, while the biological oxygen demand (BOD) is about 30–70% of the COD (Pant and Adholeya, 2007). Application of vinasse in landfill produces unpleasant odor emissions as a result of the putrefaction of organic matter (Belhadj et al., 2013). The high content of total solids and trace metals is another typical characteristic of this raw effluent (España-Gamboa et al., 2012; Soler da Silva et al., 2013). Consequently, vinasse represents a polluting potential for soil and water bodies when inadequately managed and discharged into rivers and lakes without previous treatments.

A variety of technologies, many of them based on revaluation of vinasse as a byproduct with potential applications, are being evaluated in order to mitigate the environmental impact of vinasse. Use of vinasse as feedstock to obtain microbial products of biotechnological interest is a promising practice which would reduce production costs and simultaneously decrease the effluent volume. Colin et al. (2016) recently reported the first advances in bioemulsifier production by the actinobacterium *Streptomyces* sp. MC1 in

a vinasse-based culture medium. Other microbial products such as biopolymers, biofuel and enzymes have also been successfully produced from raw vinasse (Bhattacharyya et al., 2012; Lazaro et al., 2014; Tapia-Tussell et al., 2015). Under certain circumstances, microbial biomass itself could be the desirable product of the fermentation (Durão et al., 2013). In fact, the use of biomass to clean up contaminated soils is an effective biotechnological approach because of the critical role the microorganisms play in biodegradation of organic pollutants and removal/stabilization of heavy metals (Mrozik and Pjotrowska-Seget, 2010; Polti et al., 2014).

Among environmental microorganisms, actinobacteria have been widely reported as potential bioremediation agents (Alvarez et al., 2017). They may be well suited for inoculation in soil as a consequence of their mycelial growth, their relatively rapid growth rates and ability to colonize substrates (Ravel et al., 1998). In fact, Polti et al. (2014) previously demonstrated the potential of an actinobacteria mixed culture whose biomass was produced in a commercial synthetic medium, to clean up contaminated soils with hexavalent chromium and lindane. From this study, it was inferred that the amount of biomass necessary for in situ treatment of soils at field scale would be equal to 40 kg ha⁻¹. Taking into account the importance of the cost/benefit ratio to ensure sustainability of any biotechnological process, the first issue that should be considered is the production of microbial biomass from cheap feedstock. Based on this background, the aim of the present work was to produce actinobacteria biomass using raw vinasse as low-cost feedstock. Subsequently, the performance of the produced biomass to clean up soil contaminated with lindane and chromium was assayed.

2. Materials and methods

2.1. Microorganisms and maintenance

Based on their ability to remove lindane and chromium from soil samples, four native actinobacteria were selected for this study: *Streptomyces* sp. M7, isolated by Benimeli et al. (2003), *Amycolatopsis tucumanensis* DSM 45259T (strain AB0), isolated by Albarracín et al. (2005), *Streptomyces* sp. MC1, isolated by Polti et al. (2007), and *Streptomyces* sp. A5, isolated by Fuentes et al. (2010). The strains were maintained on Starch-Casein agar plates (SC agar) containing (g L⁻¹): starch, 10.0; casein, 1.0; K₂HPO₄, 0.5; agar, 12.0. The pH was adjusted to 7.0 prior to sterilization.

2.2. Biomass production

2.2.1. Sampling and physicochemical characterization of vinasse

Vinasse was obtained from a distillery in the province of Tucumán, Argentina, which uses molasses as raw material for ethyl alcohol production. Samples were taken from a container that received hot vinasse (70 °C), and then bottled, cooled and subsequently stored at 4 °C. Physicochemical characterization of the vinasse was performed according to the Standard Methods for the Examination of Water and Wastewater (A.P.H.A. et al., 2012): the pH was measured electrometrically, the conductivity through the electrical conductivity method, determination of total solids by drying at 105 °C, total fixed solids by incineration at 550 °C, total volatile solids by the difference between total solids and total fixed solids, and the BOD using the 5-d BOD test. Presence of trace elements such as Cr,

Mn, Cu, Zn, As, Cd, Hg and Pb was determined using inductively coupled plasma mass spectrometry (ICP-MS). Effluent color was determined by spectrophotometric measurement at 455 nm (de Souza et al., 2013).

2.2.2. Formulation of vinasse-based biomass production medium

Spores of the four strains harvested from SC agar were inoculated in flasks containing different vinasse concentrations in distilled water (1% to 50%, v/v) at a final spore concentration of 1×10^9 CFU mL⁻¹, either individually (single cultures) or all together (mixed culture) mixing equal amounts of spore of each strain to obtain the final concentration. Media were labeled from V₁ to V₅₀ according to the vinasse concentration; pH was adjusted to 7.0 using 1N NaOH prior sterilization. Biomass production in commercial Tryptic Soy Broth (TSB) medium was used as a reference. All cultures were incubated at 30 °C on an orbital shaker (200 rpm) for 72 h. After incubation, the microbial biomass was harvested by centrifugation at 8,385 g for 15 min at 4 °C. Cells were washed twice with sterile distilled water and dry weight was determined at 105 °C until constant weight. In each case, the highest vinasse concentration that allowed a biomass of 0.4 g L⁻¹ or more was selected (Polti et al., 2014). After separation of the biomass produced, the recovered supernatants were re-inoculated several times (re-inoculation cycles), individually and all strains together, in order to assess their potential (re)-use as production medium.

2.2.3. Determination of trace metals

In order to determine the concentrations of trace elements retained by the microbial biomass obtained from vinasse, a microwave digestion using a mixture of acids (65% HNO₃ and 40% HF) was carried out according to the method described by Liu et al. (2013). Concentrations of trace elements such as Cr, Mg, Cu, Zn, As, Cd, Hg, and Pb were quantified using ICP-MS.

2.3. Bioremediation assays

2.3.1. Preparation and inoculation of soil

Soil samples were taken at a depth of 5–15 cm from a non-polluted site near the city of Tucumán, Argentina, and subsequently stored in the dark at 10–15 °C until use. The main physicochemical characteristics of the soil are shown in Table 1. Glass pots were filled with 200 g of soil and water holding capacity was maintained at 30% using distilled water. The soil samples were artificially contaminated with 40 µg kg⁻¹ of lindane and 80 mg kg⁻¹ of Cr(VI). Samples were inoculated with actinobacteria biomass at a final concentration of 2 g kg⁻¹ of soil, using two different inoculation strategies: a) mixing an equal amount of biomass of the four actinobacteria harvested from single cultures, and b) direct inoculation of the biomass harvested from the actinobacteria mixed culture. Soil, inoculum, lindane and chromium were mixed thoroughly to ensure uniform distribution. Flasks were then incubated at 30 °C for a total time of 14 d. Soil water holding capacity was regularly monitored twice a week. Soil samples contaminated with both toxic substances but without inoculum were used as controls. At the end of the assay period, samples were examined for residual lindane and bioavailable chromium content.

2.3.2. Determination of residual lindane

Extraction of lindane residues from soil was performed as follows: 1) Aliquots of 10 g of dry homogenized soil were transferred to centrifuge tubes and mixed with 8, 2, and 10 mL of water, methanol, and hexane, respectively, 2) tubes were hermetically sealed, shaken vigorously for 15 min on a vortex to allow extraction of lindane from the soil to the organic phase and subsequently centrifuged (8,000 g, 20 min, 4 °C) to separate the organic and aqueous phases, and 3) organic phases were evaporated to dryness under reduced pressure and afterwards residues were re-suspended in hexane. Then, extracts were quantified on a Gas Chromatograph (Agilent 7890A) equipped with a HP5 capillary column (30 m × 0.53 mm × 0.35 µm) and ⁶³Ni µECD detector, a split/splitless Agilent 7693B injector and Agilent Chem-Station software. Quantitative sample analysis was performed using appropriate calibration standards (AccuStandard) (Fuentes et al., 2010). The results of the soil assay were expressed as the percentage of lindane removal.

2.3.3. Determination of bioavailable chromium

Environmental bioavailable chromium (Cr_(EB)) was measured in the soil using a physical method: 100 g of soil were centrifuged at 5,050 g for 60 min (soil water potential: 1,500 kPa, wilting point) (Csillag et al., 1999). This fraction corresponds to the dissolved metal specie in the water which can be taken up by plant roots or other soil organisms (Kim et al., 2015). After centrifugation, supernatants were filtered (0.45 mm pore size) and analyzed by atomic absorption spectrometry using a Perkin Elmer Analyst 400 (AAS) to determine total Cr_(EB) content (A.P.H.A. et al., 2012). The results of the soil assay were expressed as the percentage of chromium removal with Cr_(EB) fraction representing 100%.

2.4. Statistical analysis

Statistical analysis was performed using Infostat (version 2004) and Minitab (version 16) software for Windows. Results are presented as means \pm standard deviation (SD), and all assays were carried out in triplicate. Statistically significant values for the means were assayed using one-way analysis of variance. Subsequent comparisons were performed using Tukey's post-hoc test. Differences were accepted as significant when $p < 0.05$.

3. Results

3.1. Actinobacteria biomass production using vinasse as feedstock

The experimental procedure used to formulate the biomass production medium is summarized in Fig. 1. To select the maximum vinasse concentration that yielded the required amount of biomass, six effluent concentrations were assayed as production medium (V_1 to V_{50}) (Fig. 1A). Only the biomass recovered from V_{20} medium, labeled as B_1 , corresponds to a biomass production (more than 0.4 g L^{-1} in all cases) similar to that in TSB (Fig. 2). For single cultures, the biomass concentration ranged from 0.65 to 0.70 g L^{-1} , while growth for the mixed culture was around 0.82 g L^{-1} .

Since the supernatants recovered from V_{20} cultures were still rich in nutrients, their reuse for additional biomass production was assayed during four consecutive cycles. These supernatants were denoted $V_{20(1)}$ to $V_{20(4)}$ according to the cycle number, while the biomasses of the batches were labeled B_2 to B_5 (Fig. 1B). As shown in Fig. 3, no significant differences were detected in growth of V_{20} , $V_{20(1)}$ and $V_{20(2)}$, and biomass production was

higher than 0.6 g L^{-1} for both single and mixed cultures. During the 3rd reuse [$V_{20(3)}$], biomass production decreased in all cases, but it was still more than 0.4 g L^{-1} . Because the biomass recovered from $V_{20(4)}$ (4th supernatant reuse cycle) was below the previously established critical level, B_5 was discarded for future bioremediation assays (Fig. 1B).

3.2. Effectiveness of actinobacteria to degrade vinasse

To estimate the effectiveness of the actinobacteria to degrade vinasse, BOD levels were determined before (V_{20}) and after of the successive re-inoculation steps of the supernatants used as additional production media [$V_{20(1)}$ to $V_{20(4)}$] (Fig. 4). As expected, the initial value ($17,600 \text{ mg L}^{-1}$) decreased linearly with increasing re-inoculation cycles; final BOD values in $V_{20(4)}$ supernatants ranged from 8,200 (a reduction of 53.4%) to 6,050 mg L^{-1} (a reduction of 65.6%) for single cultures. These results are consistent with a reduction in organic matter, which fluctuated between 53% and 61%. The performance of the mixed culture to degrade vinasse was even higher than that of single cultures, with a reduction in BOD of 72% at the end of the re-used vinasse cycles ($V_{20(4)}$: $4,966 \text{ mg L}^{-1}$).

Variations in other physicochemical parameters were also detected when the initial production medium (V_{20}) was compared with the corresponding $V_{20(4)}$ supernatant (Table 2). Under the current assay conditions, no significant differences were detected in the pH during the re-inoculation cycles. However, conductivity decreased from 15.2 to $10.5 \pm 0.3 \text{ mS cm}^{-1}$, which is a reduction of 30%. Concerning total solids (fixed and volatile), a reduction of about 23% and 15%, respectively, was observed in single cultures. The mixed culture showed a slightly higher decrease with a reduction of 31% and 23% in fixed and volatile solids, respectively. Finally, the effluent color was quantified

spectrophotometrically (*item 2.2.1*), and the optical density of V_{20} was defined as 100%. As shown in Table 2, the highest color removal at the end of re-used vinasse cycles was obtained with the mixed culture, but *S. M7* and *S. A5* grown as single cultures showed a similar performance.

3.3. Performance of actinobacteria biomass regarding lindane and chromium removal

Presence of trace metals such as Mn, Zn, As, Hg, and Pb was detected in the initial V_{20} production medium, but not in $V_{20(1)}$ (Table 3). Based upon the presumption that the B_1 biomass could retain trace metals present in V_{20} , a microwave digestion followed by ICP-MS quantification was performed. As was expected, the pattern and the amount of trace metals in B_1 were similar to those present in V_{20} (Table 3). Therefore, B_1 was discarded for future soil experiments (Fig. 1B).

Lindane and chromium removal from artificially contaminated soil was evaluated using B_2 , B_3 and B_4 fractions, which were obtained by mixing equal amounts of biomass recovered from single cultures or directly produced by a mixed culture (Fig. 5). Lindane removal did not show significant differences regarding the methodology used for inoculum preparation ($p > 0.05$) (Fig. 5A). B_2 , B_3 and B_4 did not demonstrate any differential performance regarding pesticide removal either. In all cases, the decrease in lindane was about 50% after 14 d of incubation.

Although the soil samples were initially contaminated with 80 mg kg^{-1} of chromium, $\text{Cr}_{(\text{EB})}$ fraction was 45 mg kg^{-1} . Thereby the removal assay was conducted on this fraction. Under such conditions, percentage of chromium removal was substantially higher than elimination of lindane with values that were statistically different depending on the preparation

methodology of the inoculum: 85% of the chromium was removed with the combined actinobacteria biomass recovered from their respective single cultures, while a mixed culture biomass removed more than 95% (Fig. 5B). Similar to lindane, no significant differences were detected in chromium removal using B₂, B₃ and B₄ biomasses. At the end of the assays period, no variations on lindane and chromium concentrations were observed in control flasks.

4. Discussion

Inadequate management and disposal of vinasse into the environment is receiving increased attention, because of the ecological problems associated with this practice (Christofolletti et al., 2013). Argentine law on waste disposal prohibits dumping of untreated vinasse in water bodies because of its physical and chemical characteristics, including a high content of minerals, organic matter, and heavy metals, a low pH, and a significant degree of corrosiveness (Federal Hazardous Waste Law N° 24051). However, the same characteristics make vinasse a frequent object of scientific and technological inquiry (Sanchez Moore et al., 2016).

The concept of circular economy considers the re-use and recycling of any type of waste, contributing to the development of a bio-based economy (Pleissner et al., 2016). Thereby, efforts are not only made to reduce pollution generated by these residues, but also to use waste products in general as substrates. As vinasse contains substantial quantities of sugars, amino acids, phosphate and other minerals, it could be used as a nutrient for the development of microorganisms in biotechnological processes. Actually, a variety of projects that use raw vinasse as cheap feedstock to obtain value added products have been

conducted in the last years (Bhattacharyya et al., 2012; Lazaro et al., 2014; Tapia-Tussell et al., 2015; Colin et al., 2016). In the bioremediation field, obtaining microbial biomass as main product of the fermentation is one of the most promising approaches to clean up large areas of contaminated soil. In fact, aerobic processing of wastewater could generate a biomass overproduction because of the high content of organic matter (Jimenez et al., 2003).

Considering that the feasibility of technologies to solve environmental problems is directly related to the profitability of the process, the current study assessed the performance of the biomass produced from sugarcane vinasse to remove chromium and lindane from artificially contaminated soil. From the results of this study it could be inferred that an approximate volume of 10,000 to 12,000 L of vinasse would produce the required biomass for treatment of one hectare of contaminated soil. This volume would be the equivalent of the amount of vinasse generated each 1,000 L of ethylic alcohol. Hence, the use of vinasse to generate biomass could be a promising biotechnological approach to improve management of waste from the sugar-alcohol industry.

The actinobacteria assayed as single cultures or as a mixed culture of the 4 strains showed generally high vinasse tolerance, even at concentrations that usually inhibit growth of other microorganisms. Production of useful biomass without significant vinasse dilutions is essential, since this allows saving water resources and thus reducing the handled volume. Contrary to our results, certain nitrogen fixing bacteria *Azospirillum brasiliense*, *Bacillus polymyxa*, and *Azotobacter chroococcum* and fungi like *Fusarium oxysporum*, *Sclerotinia clerotiorum*, *Pythium aphanidermatum*, and *Phytophthora parasitica* only grew at vinasse concentrations below 15% (Omar et al., 2002; Santos et al., 2008). Pramanik et al. (2012) reported inhibitory of growth of *Haloarcula marismortui* at vinasse concentrations above

10%, whereas Barrocal et al. (2010) only observed tolerance of the cyanobacterium *Spirulina maxima* in media containing vinasse from beet molasses at a concentration below 0.5% (w/v).

In addition to a reduction in the production cost for microbial biomass, the use of vinasse as alternative substrate could represent an adequate strategy to improve the effluent quality. In fact, technologies based on recovery of industrial effluents via microbial pathways are continually evaluated. Recently, Colin et al. (2016) reported on the ability of *S. MC1* to remove over 50% of the biodegradable organic matter from a vinasse sample in a short period of time. This implied a reduction of about 50% of the polluting power, which was verified using *Lactuca sativa* as a bioremediation indicator. The current study showed an even higher BOD removal from vinasse with a mixed culture of actinobacteria. Fuentes et al. (2016) emphasized multiple advantages of mixed cultures over individual strains, remarking their lower nutritional requirements and higher activities. Actually, a traditional process known as anaerobic digestion used a mix of methanogenic Archaea bacteria removing about 90% of the BOD of vinasse (Baez-Smith, 2006).

In the present study, color reduction by the mixed culture compared with that by individual cultures did not substantially improve. In fact, conventional anaerobic-aerobic treatments have proven to be effective to reduce the organic load of wastewater to a significant extent although they usually do not successfully remove the color (Ferreira et al., 2010). Because of their synthetic origin and complex molecular structures, coloring compounds are less amenable to biodegradation, and consequently physicochemical methods are commonly applied to remove the effluent color (de Souza et al., 2013). However, some biological treatments such as the use of white-rot fungi have been proposed

as an alternative to the decolorization of effluents since their extracellular enzymatic system is able to break down a large number of chemical bonds (Ferreira et al., 2010).

Heavy metals are also present in various types of wastewater including vinasses. Although relatively low metal concentrations are vital to microorganism growth, introduction of too many and at fairly large concentrations into the soil causes considerable modification of the microbial community (Dixit et al., 2015). In fact, certain metals such as lead can alter the biological processes even at low concentrations (Saidi, 2010). The alternative use of vinasse requires thereby a preconditioning treatment in order to eliminate heavy metals and other toxins. Many of the metal recovery techniques are based on microbial pathways (Siddiquee et al., 2015). E.g., Colin et al. (2016) demonstrated the ability of *S. MC1* to remove all metals (Mn, Zn, As and Pb) from sugarcane vinasse samples after 4 d of treatment. The current study is the first to use actinobacteria to remove heavy metals from a vinasse-based medium (V_{20}). The metal-free biomass recovered from consecutive re-inoculations of V_{20} was subsequently used to clean up contaminated soil with lindane and chromium. As metals are involved in a series of complex chemical interactions with the components of the soil (e.g., clay mineral and organic matter), in the present study only slightly more than 50% of chromium added to soil was available to be taken by actinobacteria. Besides, the presence of heavy metals can affect organic biodegradation either inhibiting enzymatic pathways and/or by impacting on the ecology of degrading microorganisms (Amor et al., 2001; Sandrin and Maier, 2003; Olaniran et al., 2009).

Treatment of co-contaminated soils is complex; thereby, use of mixed cultures instead of single cultures could be more suitable for an effective bioremediation since the biodiversity could increase the catabolic pathways available for multiple contaminant biodegradation. In fact, the present study demonstrates an effective removal of both lindane and chromium by

using mixed culture biomass resulting from three re-inoculation vinasse cycles. These findings demonstrate the feasibility to improve the bioremediation process and to produce efficient biomass by revaluation of effluents like vinasse.

5. Conclusions

The current study presents an integral approach to multiple contamination problems considering toxins such as pesticides and heavy metals as well as regionally important effluents like vinasse. We have demonstrated that the use of vinasse in microbial fermentations is a highly-valued biotechnological strategy to simultaneously reduce the microbial biomass production cost and environmental impact of raw effluents. Our approach allows establishing the basis for future production of biomass at a large scale, and it is an adequate alternative to improve effluent management. However, additional studies are required in order to evaluate the feasibility of actinobacteria biomass production using vinasse from different distilleries and with varying composition.

Acknowledgements

This work was supported by Secretaría de Ciencia, Arte e Innovación Tecnológica (SCAIT) (PIUNT D504), Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) (PICT 2013 0141), Programa CAPES-CONICET-MINCYT 2014 (PCB II), and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) (PIP 112-201101-00085). The authors gratefully acknowledge the technical assistance of Mr. Guillermo Borchia.

References

- A.P.H.A., A.W.W.A., W.E.F, 2012. Standard Methods for Examination of Water and Wastewater, 22nd edition, Washington: American Public Health Association, ISBN 978-087553-013-0.
- Albarracín, V.H., Amoroso, M.J., Abate, C.M., 2005. Isolation and characterization of indigenous copper resistant actinomycete strains. *Chem. Erde – Geochem.* 65, 145–156.
- Alvarez, A., Saez, J.M., Dávila Costa, J.S., Colin, V.L., Fuentes, M.S., Cuozzo, S.A., Benimeli, C.S., Polti, M.A., Amoroso, M.J., 2017. Actinobacteria: current research and perspectives for bioremediation of pesticides and heavy metals. *Chemosphere* 166, 41–62.
- Amor, L., Kennes, C., Veiga, M.C., 2001. Kinetics of inhibition in the biodegradation of monoaromatic hydrocarbons in presence of heavy metals. *Bioresour Technol.* 78 (2), 181–185.
- Baez-Smith, C., 2006. Anaerobic digestion of vinasse for the production of methane in the sugar cane distillery, SPRI Conference on Sugar Processing, pp. 268–287.
- Barrocal, V.M., García-Cubero, M.T., Gonzáles-Benito, G., Coca, M., 2010. Production of biomass by *Spirulina maxima* using sugar beet vinasse in growth media. *New Biotechnol.* 27 (6), 852–856.
- Belhadj, S., Karouach, F., El Bari, H., Joute, Y., 2013. The biogas production from mesophilic anaerobic digestion of vinasse, *IOSR J. Environ. Sci. Toxicol. And Food Technol.* 5 (6), 72–77.

- 405 Benimeli, C.S., Amoroso, M.J., Chaile, A.P., Castro, G.R., 2003. Isolation of four aquatic
 406 streptomycetes strains capable of growth on organochlorine pesticides. *Bioresource*
 407 *Technol.* 89, 133–138.
- 408 Bhattacharyya, A., Pramanik, A., Kumar Maji, S., Haldar, S., Mukhopadhyay, U.K.,
 409 Mukherjee, J., 2012. Utilization of vinasse for production of poly-3-
 410 (hydroxybutyrate-co-hydroxyvalerate) by *Haloferax mediterranei*, *AMB Express* 2,
 411 34.
- 412 Christofolletti, C.A., Escher, J.P., Correia, J.E., Marinho, J.F., Fontanetti, C.S., 2013.
 413 Sugarcane vinasse: environmental implications of its use. *Waste manage.* 33 (12),
 414 2752–2761.
- 415 Colin, V.L., Juárez Cortes, A.A., Aparicio, J.D., Amoroso, M.J., 2016. Potential application
 416 of a bioemulsifier-producing actinobacterium for treatment of vinasse. *Chemosphere*
 417 144, 842–847.
- 418 Csillag, J., Partay, G., Lukacs, A., Bujtas, K., Nemeth, T., 1999. Extraction of soil solution
 419 for environmental analysis. *Int. J. Environ. Anal. Chem.* 74, 305–324.
- 420 de Souza, R.P., Girardi, F., Santana, V.S., Camargo-Fernandes Machado, N.R., Gimenes,
 421 M.L., 2013. Vinasse treatment using a vegetable-tanning coagulant and
 422 photocatalysis. *Acta Sci.* 35, 89–95.
- 423 Dixit, R., Wasiullah, D., Malaviya, K., Pandiyan, U.B., Singh, A., Sahu, R., Shukla, B.P.,
 424 Singh, J.P., Rai, P.K., Sharma, H., Lade, D.P., 2015. Bioremediation of heavy metals
 425 from soil and aquatic environment: An overview of principles and criteria of
 426 fundamental processes. *Sustainability* 7, 2189–2212.

- 427 Durão Vieira, E., Andrietta, M.G.S., Andrietta, S.R., 2013. Yeast biomass production: a
428 new approach in glucose-limited feeding strategy. *Braz. J. Microbiol.* 44 (2), 551–
429 558.
- 430 España-Gamboa, E. Mijangos-Cortés, J.O., Hernández-Zárate, G., Domínguez Maldonado,
431 J.A., Alzate-Gaviria, L.M., 2012. Methane production by treating vinasses from
432 hydrous ethanol using a modified UASB reactor. *Biotechnol. Biofuels* 5, 82.
- 433 Ferreira, L.F., Aguiar, M., Pompeu, G., Guido Messias, T., Rosim Monteiro, R., 2010.
434 Selection of vinasse degrading microorganisms. *World J. Microbiol. Biotechnol.* 26,
435 1613–1621.
- 436 Fuentes, M.S., Benimeli, C.S., Cuzzo, S.A., Amoroso, M.J., 2010. Isolation of pesticide-
437 degrading actinomycetes from a contaminated site: Bacterial growth, removal and
438 dechlorination of organochlorine pesticides. *Int. Biodeter. Biodegr.* 64, 434–441.
- 439 Fuentes, M.S., Colin, V.L., Amoroso, M.J., Benimeli, C.S., 2016. Selection of an
440 actinobacteria mixed culture for chlordane remediation: Pesticide effects on microbial
441 morphology and bioemulsifier production. *J. Basic Microbil.* 56, 127–137.
- 442 Jimenez, A.M., Borja, R., Martin, A., 2003. Aerobic-anaerobic biodegradation of beet
443 molasses alcoholic fermentation wastewater. *Process Biochem.* 38, 1275–1284.
- 444 Kim, R.Y., Joon, J.K., Kim, T.S., Yang, J.E., Owens, G., Kim, K.R., 2015. Bioavailability
445 of heavy metals in soils: definitions and practical implementation-a critical review.
446 *Environ. Geochem. Health.* 37 (6), 1041–1061.
- 447 Lazaro, C.Z., Perna, V., Etchebehere, C., Varesche, M.B., 2014. Sugarcane vinasse as
448 substrate for fermentative hydrogen production: The effects of temperature and
449 substrate concentration. *Int. J. Hydrogen Energy* 30 (12), 6407–6418.

- 450 Liu, N., Jorgensen, U., Laerke, P.E., 2013. Quality determination of biomass for
451 combustion: A new high throughput microwave digestion method prior to elemental
452 analysis by inductively coupled plasma-optical emission spectroscopy. *Energy Fuels*
453 27, 7485–7488.
- 454 Mrozik, A., Pjotrowska-Seget, Z., 2010. Bioaugmentation as a strategy for cleaning up of
455 soils contaminated with aromatic compounds. *Microbiol. Res.* 165 (5), 363–375.
- 456 Olaniran, A.O., Balgobind, A., Pillay, B., 2009. Impacts of heavy metals on 1,2-
457 dichloroethane biodegradation in co-contaminated soil. *J. Environ. Sci.* 21, 661–666.
- 458 Omar, M.N.A., Mostafa, A.T., Ahmed, A.S., 2002. Concentrated vinasse as anovel
459 diazotrophs growth medium (biovinasse inoculant) and soil conditioner to improve
460 faba bean yield under dripping irrigation system. Symposium No. 3, Paper No. 137,
461 17th WCSS, Thailand, pp. 14–21.
- 462 Pant, D., Adholeya, A., 2007. Biological approaches for treatment of distillery wastewater:
463 a review. *Bioresource Technol.* 98, 2321–2334.
- 464 Pleissner, D., Qi, Q., Gao, C., Perez Rivero, C., Webb, C., Ki Lin, C.S., Venus, J., 2016.
465 Valorization of organic residues for the production of added value chemicals: A
466 contribution to the bio-based economy. *Biochem. Eng. J.* 116, 3–16.
- 467 Polti, M.A., Amoroso, M.J., Abate, C.M., 2007. Chromium (VI) resistance and removal by
468 actinomycete strains isolated from sediments. *Chemosphere* 67, 660–667.
- 469 Polti, M.A., Aparicio, J.D., Benimeli, C.S., Amoroso, M.J., 2014. Simultaneous
470 bioremediation of Cr(VI) and lindane in soil by actinobacteria. *Int. Biodeter. Biodegr.*
471 88, 48–55.
- 472 Pramanik, A., Mitra, A., Arumugam, M., Bhattacharyya, A., Sadhukhan, S., Ray, A.,
473 Halder, S., Mukhopadhyay, U.K., Mukherjee, J., 2012. Utilization of vinasse for the

production of polyhydroxybutyrate by *Haloarcula marismortui*. Folia Microbiol. 57 (1), 71–79.

Ravel, J., Amoroso, M.J., Colwell, R.R., Hill, R.T., 1998. Mercury resistant actinomycetes from Chesapeake Bay. FEMS Microbiol. Lett. 162, 177–184.

Saidi, M., 2010. Experimental studies on effect of heavy metals presence in industrial wastewater on biological treatment. Int. J. Environ. Sci. 1 (4), 666–676.

Sanchez Moore, C.C., Rodrigues Nogueira, A., Kulay, L., 2016. Environmental and energy assessment of the substitution of chemical fertilizers for industrial wastes of ethanol production in sugarcane cultivation in Brazil. Int. J. Life Cycle Assess. DOI: 10.1007/s11367-016-1074-0.

Sandrin, T.R., Maier, R.M., 2003. Impact of metals on the biodegradation of organic pollutants. Environ. Health Perspect. 111, 1093–1101.

Santos, M., Diáñez, F., de Cara, M., Tello, J.C., 2008. Possibilities of the use of vinasses in the control of fungi phytopathogens. Bioresour. Technol. 99, 9040–9043.

Siddiquee, S., Rovina, K., Al Azad, S., Naher, L., Suryanil, S., Chaikaew, P., 2015. Heavy metal contaminants removal from wastewater using the potential filamentous fungi biomass: A Review. J. Microb. Biochem. Technol. 7, 6.

Soler da Silva, M.A., Kliemann, H.J., Borges De-Campos, A., Madari, B.E., Borge, J.D., Gonçalves, J.M., 2013. Effects of vinasse irrigation on effluent ionic concentration in Brazilian Oxisols. Afr. J. Agric. Res. 8 (45), 5664–5677.

Tapia-Tussell, R., Pérez-Brito, D., Torres-Calzada, C., Cortés-Velázquez, A., Alzate-Gaviria, L., Chablé-Villacís, R., Solís-Pereira, S., 2015. Laccase gene expression and vinasse biodegradation by *Trametes hirsuta* Strain Bm-2. Molecules 20, 15147–15157.

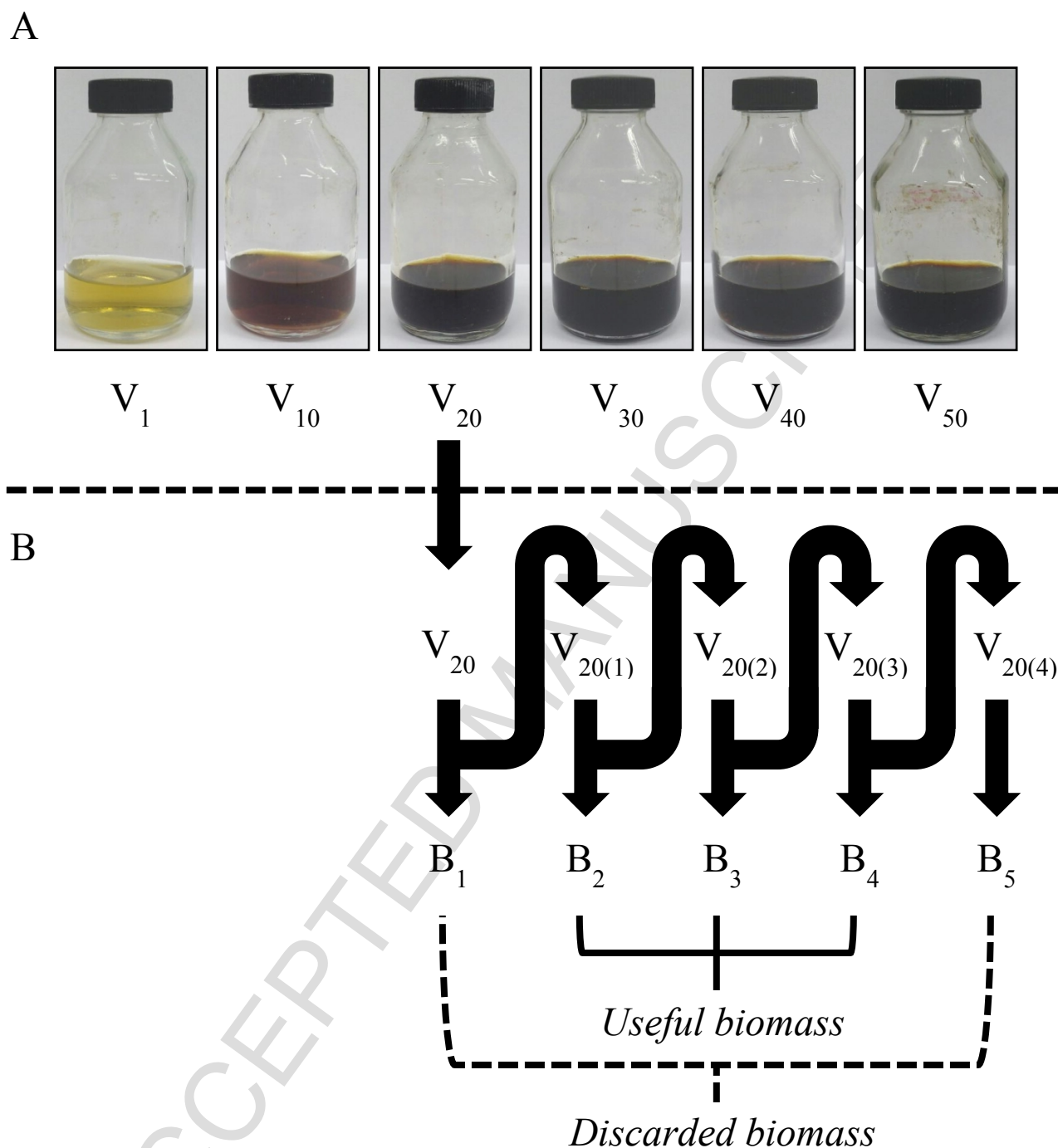


Fig. 1. Flow chart summarizing the experimental procedure used to formulate the vinasse-based production medium: (A) Selection of vinasse concentration (V_1 to V_{50}), and (B) assessment of the vinasse-reuse [$V_{20(1)}$ to $V_{20(4)}$]. B_1 to B_5 represent biomass production.

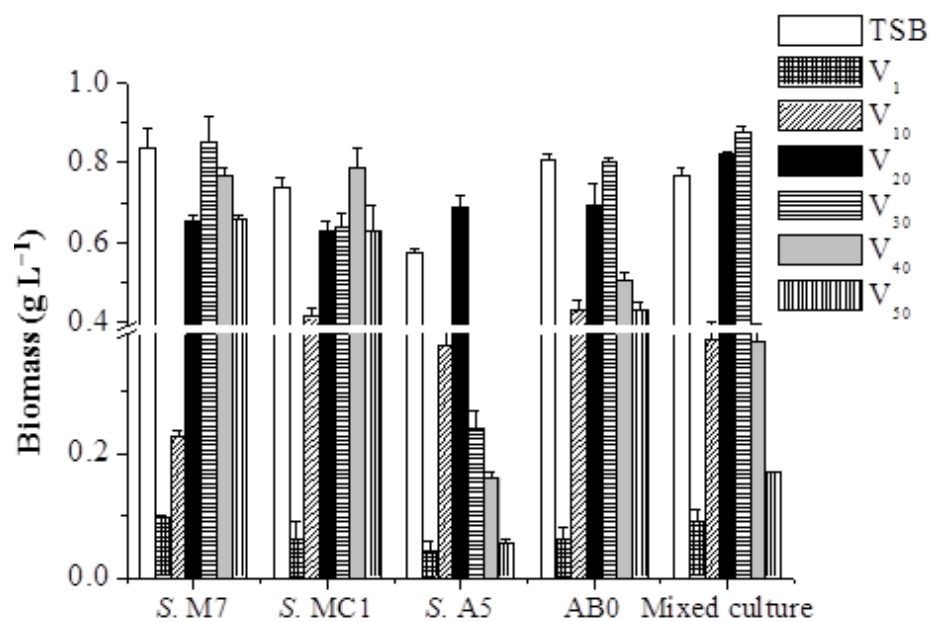


Fig. 2. Effect of the vinasse concentration (V_1 to V_{50}) on the actinobacteria biomass production grown individually or all mixed together. TSB: Tryptic Soy Broth.

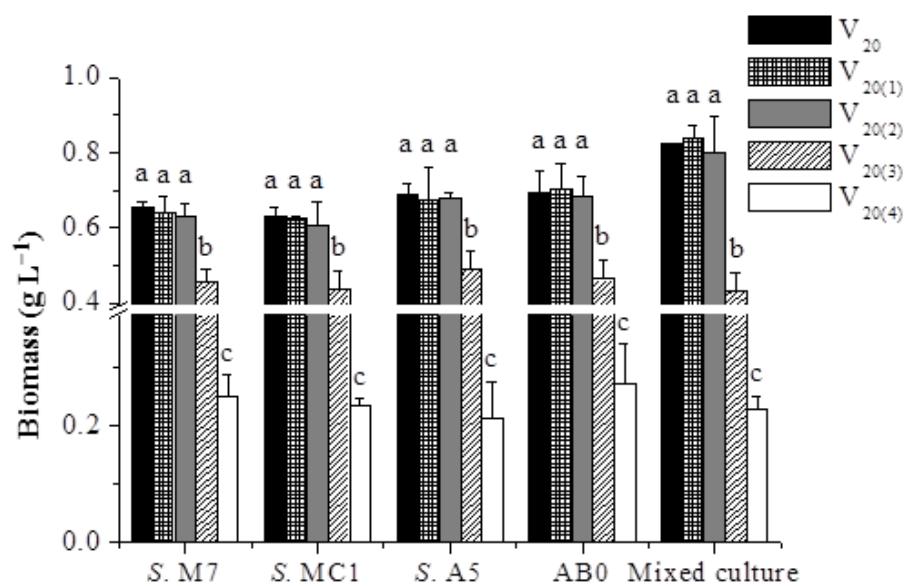


Fig. 3. Biomass production in vinasse-based culture media of the actinobacteria strains grown individually or as a mixed culture of all 4 strains. Bars with different letters (a–c) are significantly different ($p < 0.05$).

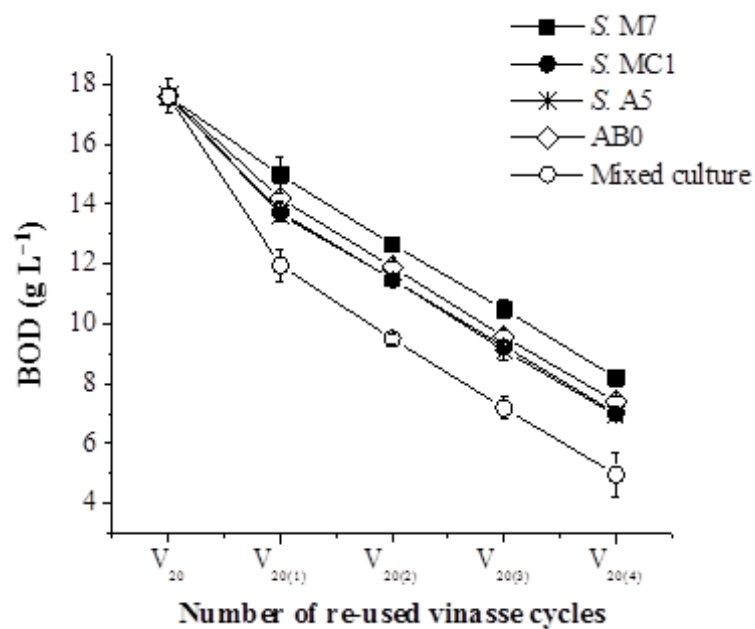


Fig. 4. BOD levels measured in the initial production medium (V₂₀) and during the consecutive cycles of re-used vinasse [V₂₀₍₁₎ to V₂₀₍₄₎].

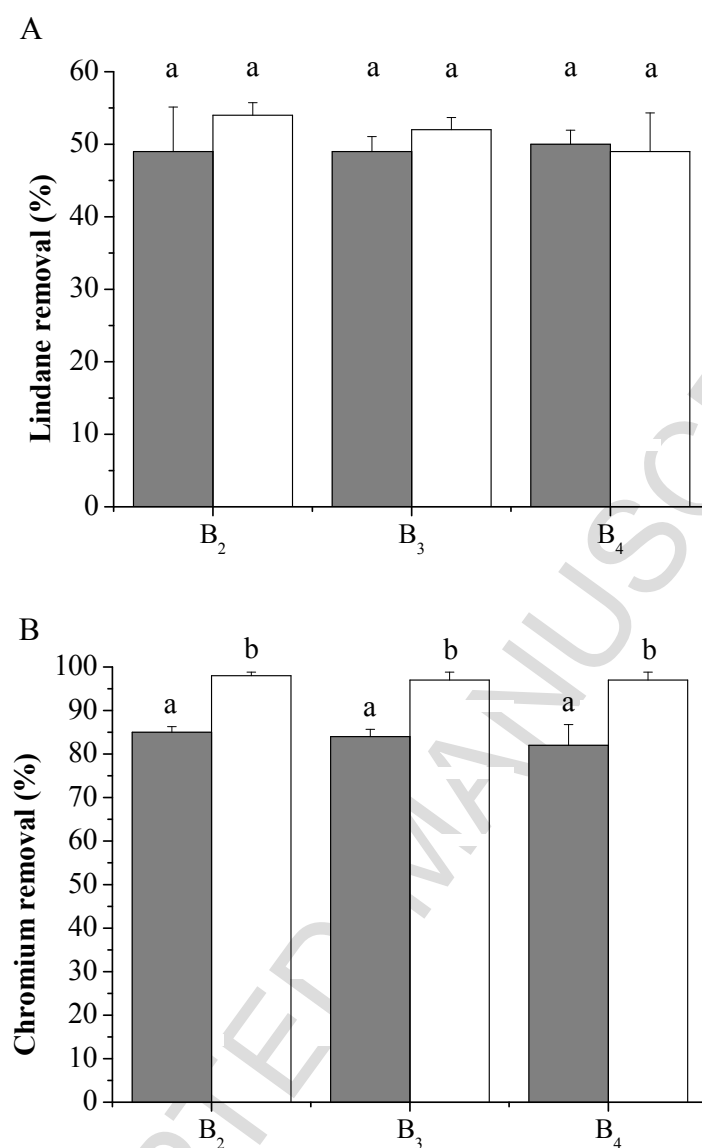


Fig. 5. Removal of lindane (A) and chromium (B) using B₂, B₃ and B₄, obtained either by mixing the biomass harvested from the single cultures (closed bars) or as a mixed culture (open bars).

Table 1

Physicochemical soil characteristics.

Parameters	
pH ^a	6.9
EC, dS m ^{-1b}	0.8
Calcium carbonate, % ^c	1.3
Soil Texture ^d	Loam
Organic carbon, % ^e	2.7
Phosphorus, ppm ^f	15.8
Sodium Exchange capacity, cmol kg ^{-1g}	0.5
Potassium Exchange capacity, cmol kg ^{-1g}	0.1
Calcium Exchange capacity, cmol kg ^{-1g}	0.2
CEC, cmol kg ^{-1g}	17.9

^a Soil/distilled water ratio: 1:2.5.^b Electrical conductivity method.^c Gasometric method.^d Capillary method.^e Walkley and Black method.^f Bray and Kurtz I.^g Cation exchange capacity determined by ammonium-sodium acetate method.

Table 2

Physicochemical parameters measured in the initial production medium (V_{20}) and at the end of re-used vinasse cycles [$V_{20(4)}$].

Parameters	V_{20}	$V_{20(4)}$				
		<i>S. M7</i>	<i>S. MC1</i>	<i>S. A5</i>	AB0	Mixed culture
pH	7.0 ± 0.0^a	7.0 ± 0.1^a	7.1 ± 0.2^a	7.2 ± 0.2^a	7.1 ± 0.1^a	7.2 ± 0.2^a
EC, mS cm ⁻¹	15.2 ± 0.0^a	10.9 ± 0.7^b	10.7 ± 0.3^b	10.5 ± 0.1^b	10.5 ± 0.1^b	10.1 ± 0.4^b
Total Solids (g L ⁻¹)	28.5 ± 0.3^a	24.0 ± 0.7^b	23.2 ± 0.1^b	22.4 ± 1.4^{bc}	23.2 ± 0.3^b	20.9 ± 0.6^c
Fixed Solids (g L ⁻¹)	12.7 ± 0.1^a	10.1 ± 0.3^b	9.7 ± 0.4^b	8.9 ± 0.1^c	10.3 ± 0.6^b	8.7 ± 0.3^c
Volatile Solids (g L ⁻¹)	15.8 ± 0.1^a	13.9 ± 0.1^b	13.5 ± 0.7^{bc}	13.5 ± 0.1^{bc}	12.9 ± 0.1^{cd}	12.2 ± 0.6^d
Color removal (%)	–	18 ± 2.0^a	11 ± 0.0^{bc}	15 ± 3.0^{ab}	9 ± 2.0^c	20 ± 2.0^a

Values with different letters (a–c) are significantly different ($p < 0.05$).

V_{20} : initial production medium. $V_{20(4)}$: supernatant recovered at the end of re-used vinasse cycles.

Table 3

Metal concentrations in the production media and the produced biomass before and after the first inoculation.

Metal Concentration							
Metal (LOD, $\mu\text{g L}^{-1}$)	Production medium ($\mu\text{g L}^{-1}$)		B_1 biomass ($\mu\text{g g}^{-1}$)				
	V_{20}	$V_{20(1)}$	<i>S. M7</i>	<i>S. MC1</i>	<i>S. A5</i>	AB0	Mixed culture
Cr (0.7)	ND	ND	ND	ND	ND	ND	ND
Mn (0.2)	494.8 ± 3.7	ND	23.2 ± 2.9	21.9 ± 3.1	21.5 ± 3.8	21.6 ± 4.1	17.2 ± 8.1
Cu (0.3)	ND	ND	ND	ND	ND	ND	ND
Zn (3.4)	10509.2 ± 59.7	ND	458.7 ± 5.3	500.0 ± 7.6	456.5 ± 12.9	454.3 ± 9.8	383.9 ± 3.2
As (0.1)	1653.7 ± 12.7	ND	73.3 ± 6.5	79.6 ± 7.1	76.3 ± 9.8	71.2 ± 8.7	73.6 ± 7.4
Cd (0.1)	ND	ND	ND	ND	ND	ND	ND
Hg (0.1)	6.1 ± 2.7	ND	0.5 ± 0.2	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
Pb (1.6)	58.2 ± 6.8	ND	2.4 ± 0.3	2.8 ± 0.4	2.6 ± 0.2	2.4 ± 0.3	2.1 ± 0.3

V_{20} and $V_{20(1)}$: Production media before and after the first inoculation, respectively. B_1 : Biomass production during first inoculation.

LOD: Limit of detection. ND: not detected.