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Potential application of a bioemulsifier-producing actinobacterium for treatment of vinasse

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HIGHLIGHTS

- First advances on use of *Streptomyces* sp. MC1 for vinasse treatment are presented.
- Over 50% of the biodegradable organic matter in vinasse was removed by this strain.
- High bacterial effectiveness for removing metals trace from vinasse was detected.
- Application of treated vinasse increased the vigour index of *Lactuca sativa.*
- Use of vinasse for bioemulsifier production can reduce effluent volume.

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ABSTRACT

Vinasse is a complex effluent created during production of ethyl alcohol, which can present serious pollution hazard in areas where it is discharged. A variety of technologies, many based upon recovery of the effluent via microbial pathways, are continually being evaluated in order to mitigate the pollution potential of vinasse. The present work reports on initial advances related to the effectiveness of the actinobacterium *Streptomyces* sp. MC1 for vinasse treatment. Alternative use of raw vinasse as a substrate for producing metabolites of biotechnological interest such as bioemulsifiers, was also evaluated. The strain was able to grow at very high vinasse concentrations (until 50% v/v) and remove over 50% of the biodegradable organic matter in a time period as short as 4 d. Potentially toxic metals such as Mn, Fe, Zn, As, and Pb were also effectively removed during bacterial growth. Decrease in the pollution potential of treated vinasse compared to raw effluent, was reflected in a significant increase in the vigour index of *Lactuca sativa* (letucce) used as bioremediation indicator. Finally, significant bioemulsifier production was detected when this strain was incubated in a vinasse-based culture medium. These results represent the first advances on the recovery and re-valuation of an actual effluent, by using an actinobacterium from our collection of cultures.

1. Introduction

The growing use of bioethanol is a concrete fact. However, a major limiting factor for production of bioethanol is the large volume of vinasse generated during the process (9–14 L of vinasse per L of alcohol produced) (España-Gamboa et al., 2012). Vinasse is an acidic effluent of variable composition that poses an environmental pollution hazard, mainly due to its high concentration of recalcitrant organic matter. Many studies have indicated that disposal of vinasse in landfills can lead to groundwater contamination, and it can result in emission of disagreeable odours caused by the putrefaction of organic matter (Belhadj et al., 2013). The chemical oxygen demand (COD) of vinasse has been estimated as between 50 and 150 g L^{-1}, while its biological oxygen demand (BOD) is about 30–70% of the COD (Pant and Adholeya, 2007).

A high concentration of potassium is another typical characteristic of vinasse, which makes it particularly attractive as a soil amendment or fertilizer (de Mello Prado et al., 2013). However,

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there are practical and legal restrictions on potassium content and other elements contained by the irrigation medium for agricultural soils, and vinasse often exceeds such standards (Soler da Silva et al., 2013). The presence of a variety of heavy metals in vinasse has also limited its agricultural use because of the negative effect on plants in the discharge areas (España-Gamboa et al., 2011). In fact, environmental legislation, e.g., U.S. EPA (2010), prohibits inappropriate disposal of vinasse into rivers, lakes, oceans, and soils without a prior conditioning treatment.

A variety of technologies, many based upon recovery of the effluent via microbial pathways, are continually being evaluated in order to mitigate the pollution potential of vinasse. Application of microbial treatments for improving vinasse quality is feasible due to the high concentration of biodegradable organic carbon (Silva et al., 2011). Also, alternative use of vinasse as a substrate for producing metabolites of biotechnological interest by microbial fermentation represents a promising practice in terms of reducing volume of effluent while significantly reduces the production costs (Aguiar et al., 2010; Silva et al., 2011; Bhattacharyya et al., 2012; de Lima and Rodríguez de Souza, 2014).

The actinobacterium *Streptomyces* sp. MC1 has been widely recognized for its ability to remove toxic compounds from diverse systems (Colin et al., 2012; Polti et al., 2014). The ability of this strain to produce molecules of high biotechnological value such as bioemulsifiers has also been reported (Colin et al., 2013a). In the field of bioremediation, bioemulsifiers can be used as washing agents to increase displacement and removal of organic and inorganic pollutants from soils and sediments (Aniszewski et al., 2010; Bustamante et al., 2012; Colin et al., 2013b, 2014). However, large-scale use of bioemulsifiers is currently restricted because expensive substrates are being used for formulation of the fermentation media (Colin et al., 2014). To overcome this limitation, greater emphasis can be placed on the use of agro-industrial wastes as low-cost substrates (Colin et al., 2013b, 2014). Based upon this background, this paper presents a first study on the ability of *Streptomyces* sp. MC1 to mitigate the pollution potential of a vinasse sample. Alternative use of the raw effluent as a substrate for bioemulsifier production by this strain was also evaluated.

2. Materials and methods

2.1. Sampling and physico-chemical characterization of vinasse

The sugarcane vinasse samples used in the present study were obtained from a distillery in the province of Tucumán, Argentina, which uses molasses as a raw material for ethanol production. Samples were taken from a container that receives the still-hot vinasse, and samples were then bottled for subsequent refrigeration at 4 °C. The physico-chemical analysis of raw vinasse was performed following the Standard Methods of Examination of Water and Wastewater (A.P.H.A et al., 2012): pH by the electrometric method and conductivity by the electrical conductivity method; total solids by drying at 103–105 °C, biological oxygen demand by the 5-d BOD test; determination of phosphates by the ascorbic acid method; and total nitrogen by the Kjeldahl method. The organic matter concentration was estimated using the dry combustion method (Read, 1921) and the potassium content by flame photometry. The presence of metals including Cr, Mn, Fe, Cu, Zn, As, Cd, Hg, and Pb was analysed using inductively coupled plasma mass spectrometry (ICP-MS) (Ammann, 2007). Finally, effluent color was determined by spectrophotometric measurement at 455 nm (de Souza et al., 2013).

2.2. Maintenance and culture conditions for the microorganism

Streptomyces sp. MC1 (PROIMI Collection, NCBI accession number: AY741287) isolated by Polti et al. (2007), was used in this work. The strain was maintained on Starch-Casein agar slants (SC agar) containing (g L^{-1}): starch, 10.0; casein, 1.0; K_2HPO_4, 0.5; and agar, 12.0. A spore standardized suspension of *Streptomyces* sp. MC1 (concentration of 1×10^5 CFU mL^{-1}) harvested from SC agar was inoculated in Erlenmeyer flasks containing vinasse diluted in distilled water at final concentrations that ranged from 1.0% to 50.0% v/v. These culture media were labelled as $M1_{1.0}$ to $M1_{50}$ and were prepared either without adjustment of the initial pH or with adjustment of the pH to 7.0 using NaOH prior to sterilization. All cultures were incubated at 30 °C on an orbital shaker (150 rpm) for 72 h.

The vinasse concentration used in subsequent assays was selected based upon maximum biomass concentration detected in the culture media M1. Biomass concentration was determined according to methodology described by Colin et al. (2013a). Finally, the growth kinetics of *Streptomyces* sp. MC1 at the selected vinasse concentration was monitored in order to detect the beginning of the stationary phase. At that point in time the culture supernatant was harvested and the physico-chemical parameters of the treated vinasse were again determined according at the same technical procedures described in item 2.1.

2.3. Phytotoxicity assay

The pollution potential of the raw and treated vinasse, either in the absence of or in the presence of soil, was evaluated by phytotoxicity assay, using seeds of *Lactuca sativa* (lettuce) as a bioremediation indicator. To this end, thirty lettuce seeds were grown in Petri dishes containing sterile filter paper (Wattman No. 1) moistened with 2 mL of raw or treated vinasse. Lettuce seeds grown in the dishes containing 15 g of a natural garden soil were moistened with 15 mL of raw or treated effluent. All Petri dishes were incubated in darkness for 5 d under controlled environmental conditions (22 °C, 70% relative humidity). At the end of the incubation time, the number of germinated plants (G), the root length (RL), and the hypocotyl length (HL) were measured using a millimetre scale. The vigour index of the seedlings (VI) was calculated based upon Eq. (1) (Ajithkumar et al., 1998):

$$VI = [(RL + HL) \times G]/10 \quad (1)$$

2.4. Design of a vinasse-based culture medium to produce bioemulsifiers

Bioemulsifier production was initially checked in the supernatants of the culture media labelled as M1. As an alternative, the strain was cultivated in a defined medium whose composition was described by Colin et al. (2013a), but using vinasse (0.1, 1.0, 10, 20, 30, 40, and 50%, v/v) as the carbon source, instead of glucose as in the original medium. The culture media where vinasse was used as the carbon source were labelled as $M2_{0.1}$ to $M2_{50}$. The pH was adjusted to 7.0, and bioemulsifier production was estimated following the methodology described by Cooper and Goldenberg (1987) with minimal modifications. To this end, 1 mL of a hydrocarbon such as kerosene was added to an equal volume of supernatant harvested from the M2 cultures, then mixed with a vortex for 2 min. After being left to stand for 24 h, the emulsification index (E_{24}) was calculated based upon Eq. (2):

$$E_{24} = (he \times 100)/ht \quad (2)$$

Where "he" is height of the emulsified layer and "ht" is the total height of the liquid column.

2.5. Statistical analyses

The statistical analyses were performed using Infostat (version 2004) and Minitab (version 14) software for Windows. The results were presented as mean ± standard deviation, with the assays carried out in triplicate. The statistical significance values for the means were evaluated using one-way analysis of variance. Subsequent comparisons were performed using Tukey's post-hoc test. Differences were accepted as significant when $p < 0.05$. The main effects and interactions were studied for a 2^2 full factorial design in order to evaluate the germination and development of the *L. sativa*. The independent variables and their levels are presented in Table 1, where the "+ and −" notation is used to represent the high and low levels of each variable.

3. Results and discussion

3.1. Effect of pH, vinasse concentration, and incubation time on the bacterial growth

Sugarcane vinasse usually contains a substantial fraction of potentially biodegradable sugars and organic acids. However, the presence of various types of toxic substances in the effluent can inhibit microbial growth (España-Gamboa et al., 2012).

As expected, analysis of the vinasse revealed its acidic nature (pH = 4.5). In this connection, no growth was detected when the strain was cultivated in vinasse without an initial pH adjustment. In fact, the actinobacteria have been reported as being sensitive to acid pH, presenting their optimal growth at pH values from 6.5 to 8.0 (Solans and Vobis, 2011). After adjustment to pH 7.0, however, significant bacterial growth was observed for all the concentrations tested (Fig. 1A). It is important to emphasize this high vinasse tolerance as shown by *Streptomyces* sp. MC1, including at concentrations where growth of other microorganisms is usually inhibited. Pramanik et al. (2012), e.g., reported on the inhibitory effects of vinasse on the growth of the bacterium *Haloarcula marismortui* at concentrations above 10%. Omar et al. (2002) found that nitrogen-fixing bacterial strains such as *Azospirillum brasiliense*, *Bacillus polymyxa*, and *Azotobacter chroococcum* were only able to utilize concentrations of 6–15% of vinasse obtained from an Egyptian distillery. More recently, Santos et al. (2008) reported the inhibition of a variety of fungal strains by a 15% concentration of vinasse collected from Spanish breweries.

Table 1
Effects of independent variables and their levels on germination and development of *Lactuca sativa*.

Run	Independent variables		Experimental result			
	A (Soil)	B (Vinasse)	RL (cm)	HL (cm)	G	VI
1	−	−	NG	NG	NG	NG
2	+	+	4.4	14.0	27.0	50.0
3	−	+	2.1	3.1	24.0	12.5
4	−	−	NG	NG	NG	NG
5	+	−	3.3	11.4	19.0	28.0
6	+	−	3.5	10.9	23.0	33.0
7	+	+	4.8	13.2	29.0	52.0
8	−	+	2.8	3.7	23.0	14.9

The "−" notation is used to represent "soil absent or raw vinasse" while the "+" notation is used to represent "soil present or treated vinasse". RL (root length), HL (hypocotyl length), G (germinated plants), VI (vigour index), NG (not germinated).

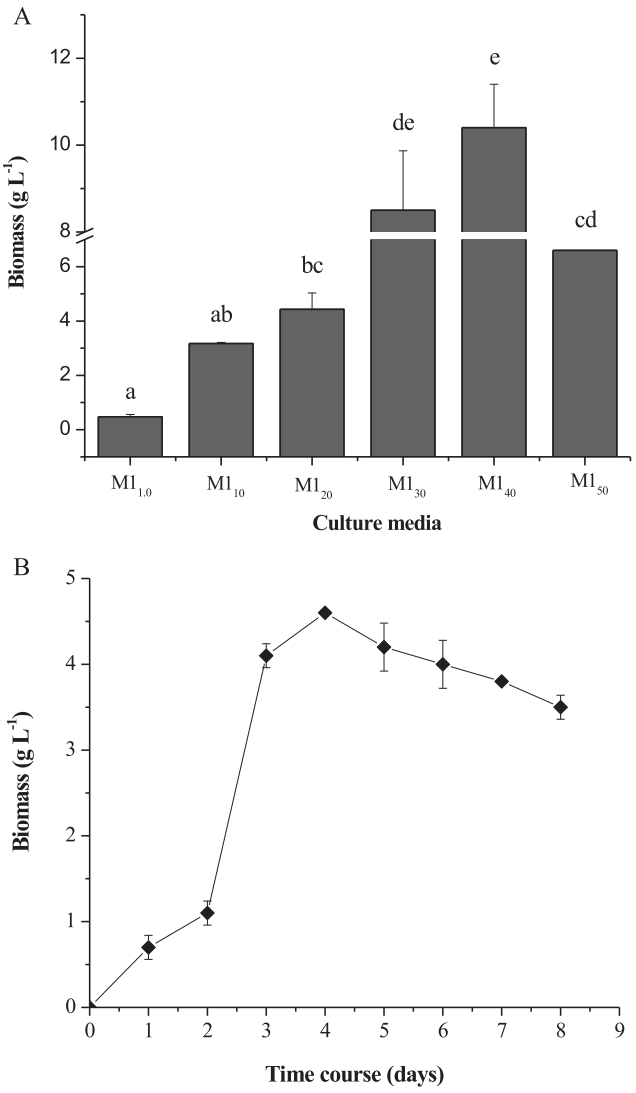

Fig. 1. Biomass of *Streptomyces* sp. MC1 determined: (A) at a variety of vinasse concentrations ($M1_{1.0}$ to $M1_{50}$) after adjustment of the initial pH, (B) at the selected vinasse concentration ($M1_{40}$). Error bars represent the standard deviation calculated from three independent experiments. Bars with different letters (a–e) are significantly different ($p < 0.05$).

Under our assay conditions, maximum bacterial growth was detected at a final vinasse concentration of 40% (Fig. 1A). To save water resources, which permit a reduction in the handled volumes, the growth kinetics of *Streptomyces* sp. MC1 was then monitored at this vinasse concentration ($M1_{40}$) (Fig. 1B). Bacterial biomass increased progressively in the $M1_{40}$ culture medium until the 4th d of cultivation, where the beginning of the stationary phase was detected. Then, supernatant was harvested at that point in time in order to estimate the effectiveness of the microbial treatment.

3.2. Assessment of the effectiveness of Streptomyces sp. MC1 for vinasse treatment

A comparative analysis of the main physico-chemical parameters of the raw effluent and effluent treated with *Streptomyces* sp. MC1 was then performed. The high conductivity exhibited by raw vinasse decreased from 16 to 10 mS cm^{-1}, while color decreased by about 20% after the 4th d of cultivation with this strain. The level of

total solids was also reduced from 28,546 to 22,034 mg L^{-1} after bacterial growth. It must be taken into account that the chemical composition of vinasse can be quite variable and depends upon the raw material, the type of distillate being obtained, and the fermentation process employed. However, vinasse commonly contains high amounts of organic matter and a predominance of potassium among the cations (de Lima and Rodríguez de Souza, 2014). In relation to this, under the current assay conditions the organic matter and BOD were reduced by 54% and 53%, respectively, towards the end of the culture period (Fig. 2).

A variety of aerobic and anaerobic processes have been tested for improving the quality of vinasse. Anaerobic digestion is, e.g., a process with high biotechnological value which removes about 90% of the BOD for vinasse. However, a desirable level of BOD removal is only achieved by using a microbial consortium formed by methanogenic bacteria and archaea. The conventional process requires an average period of time equivalent to 30–40 d (Baez-Smith, 2006). The preliminary results presented in this present study appear highly promising since over 50% of the BOD in the vinasse was removed by using a single microorganism, *Streptomyces* sp. MC1. This implies that the pollution potential of this effluent was reduced by more than 50% in a time period as short as 4 d.

Also, we detected a high initial potassium concentration in the raw vinasse, which was slightly removed after 4 d of treatment with the strain (Fig. 2). This is similar to the responses reported when anaerobic processes are applied for vinasse treatment, where potassium removal is not often favoured (España-Gamboa et al., 2012).

Since vinasse effluent has a high content of nitrogen and phosphorus, it rarely requires the addition of an external source to promote microbial growth. Fig. 2 shows the content of these elements in the vinasse, both before and after treatment with *Streptomyces* sp. MC1. It can be seen that phosphate was reduced by 33%, while nitrogen content decreased by only 16% towards the end of the incubation period. A similar nitrogen removal was reported by Ryznar-Luty et al. (2008) during aerobic degradation of vinasse using a mixed culture of bacteria from the genus *Bacillus*.

Agro-industrial wastes such as vinasses usually contain a diverse variety of heavy metals in variable concentrations. Therefore, content of certain metals in raw vinasse, and the potential for *Streptomyces* sp. MC1 to remove them was finally evaluated. Under our assay conditions, the presence of Mn and Fe was detected in the raw effluent, with a removal level of the 95% and 62%, respectively, after 4 d of microbial treatment (Fig. 2). Metals such as Zn, As, and Pb were also detected, with all of these found to be completely removed towards the end of culture period (Fig. 2).

Although some heavy metals are required in trace amounts to enable sufficient cellular growth, a diverse of biological processes can be inhibited by even low concentrations of some metals (Colussi et al., 2009; Saidi, 2010). Therefore, treatment of wastewater contaminated with heavy metals is usually recommended prior to its discharge into the environment. Application of microbial processes for removal of metals has been recognized as a potential alternative to existing physico-chemical technologies (Sameera et al., 2011; Colin et al., 2012; Suresh Kumar and Thatheyus, 2013). In this connection, our research group has reported on significant advances regarding the Cr(VI)-removal mechanisms operating in *Streptomyces* sp. MC1 (Polti et al., 2007, 2009, 2010, 2011a, 2011b, 2014). However, results presented herein are the first advances on this strain's potential for removing Mn, Fe, Zn, As, and Pb. Based upon these findings, further studies will be required in order to elucidate the possible detoxification mechanisms operating in *Streptomyces* sp. MC1 for removing these metals.

3.3. Assessment of the pollution potential of the treated microbiologically vinasse using L. sativa seedlings as a bioindicator

In the present work, we evaluated the pollution potential of raw vinasse and vinasse treated with *Streptomyces* sp. MC1, both in the absence and in the presence of soil, using *L. sativa* seedlings as a bioremediation indicator.

Qualitative observations suggest that the presence of soil and application of treated vinasse, improves germination and development of the seedlings (Fig. 3). The quantitative results of these experiments are shown in Table 1, while the estimated effects analysis for the A and B factors on the RL, HL, G, and VI parameters are presented in Table 2. As expected, the presence of soil had a marked positive effect on the HL and G parameters, and consequently on the VI values (Table 2). A positive effect on seed germination (G) as observed by application of treated vinasse is also reflected by an increase in the VI values (Table 2). These findings suggests an improve in the agricultural quality of treated effluent, compared to raw vinasse. Finally, interaction effects between the A

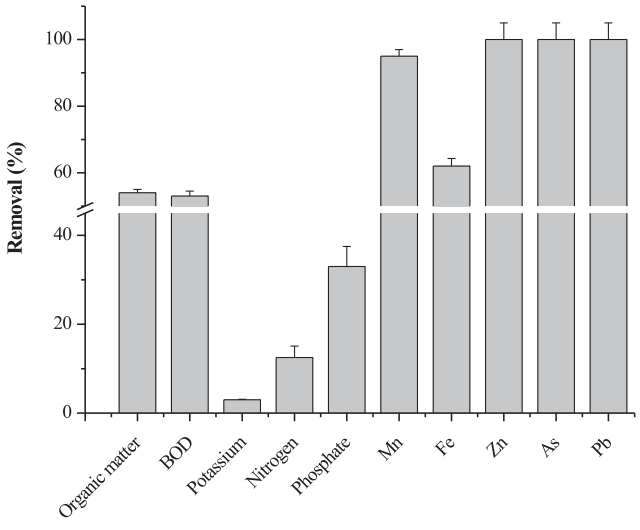

Fig. 2. Removal percentages for a 40% vinasse sample ($M1_{40}$) after treatment with *Streptomyces* sp. MC1 for 4 d.

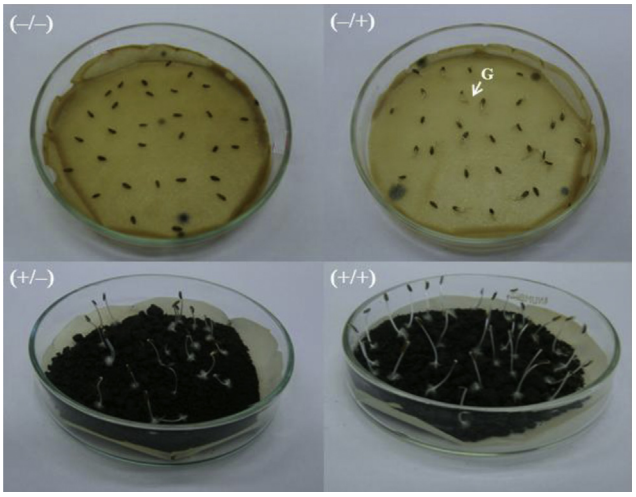

Fig. 3. Effect of soil presence and vinasse treatment on germination and development of seeds of *Lactuca sativa* (lettuce): (–/–) soil absent/raw vinasse; (–/+) soil absent/treated vinasse, G = Germination; (+/–) soil present/raw vinasse; (+/+) soil present/treated vinasse.

Table 2
The main effects and interactions of independent variables (A and B) on RL, HL, G and VI parameters, determined at 5th d of assay.

Term	RL (cm)		HL (cm)		G		VI	
	Effects	*T*-values (*p*-values)	Effects	*T*-values (*p*-values)	Effects	*T*-values (*p*-values)	Effects	*T*-values (*p*-values)
Soil (A)	2.775	13.36 ($p < 0.05$)	10.675[a]	38.19 ($p < 0.05$)	12.750[a]	11.30 ($p < 0.05$)	33.900[a]	23.00 ($p < 0.05$)
Vinasse (B)	1.825	8.79 ($p = 0.001$)	2.925	10.46 ($p < 0.05$)	15.250[a]	13.31 ($p < 0.05$)	17.100[a]	11.60 ($p < 0.05$)
A B	−0.625	−3.01 ($p = 0.040$)	−0.475	−1.70 ($p = 0.164$)	−8.250	−7.20 ($p = 0.002$)	3.400	2.31 ($p = 0.082$)

RL (root length), HL (hypocotyl length), G (germinated plants), VI (vigour index).
[a] Most relevant effects.

and B factors only slightly affected the parameters measured in the seedlings.

In recent years, the high cost of fertilizers and concerns about environmental protection have provided great incentives for studies related to recycling of the large quantities of organic wastes produced as by-products of the sugar and alcohol agro-industries. The use of organic residues such as vinasse in agriculture can contribute to the conservation of natural resources by recycling carbon and mineral elements (de Mello Prado et al., 2013). However, use of vinasse as a fertilizer is controversial because this practice has also been associated with possible depletion of soil fertility (Soler da Silva et al., 2013). For example, it is reported that exposure to even trace levels of some heavy metals present in vinasse could represent a risk for many living cells, including those of microorganisms, plants and mammals (Saidi, 2010; Hua et al., 2012). Also, has been demonstrated that certain metals can increase the toxicity of others by synergistic effects (Colussi et al., 2009). Therefore, a treatment of vinasse could be productive prior to final disposal in order to reduce its pollutant potential (de Mello Prado et al., 2013). Various studies suggesting that the environmental impacts of vinasse could be reduced by aerobic and anaerobic microbiological treatments (de Mello Prado et al., 2013; Rajagopal et al., 2014). The assay conditions applied in the present study have effectively demonstrated an improvement in the germination percentage and development of seedlings associated with application of treated vinasse instead of the raw effluent. This response may be related to the ability of *Streptomyces* sp. MC1 to achieve a desirable level of removal of BOD and other potential pollutants in a short time period. However, the response to toxicity testing is highly dependent on the organism used as the bioindicator, *L. sativa*, in our particular case. We therefore have plans to evaluate the agricultural quality of effluent subjected to treatment with *Streptomyces* sp. MC1 using a variety of crops of regional interest as indicator organisms.

3.4. Bioemulsifier production using vinasse as substrate

In previous studies, our research group has reported on the ability of *Streptomyces* sp. MC1 to produce bioemulsifiers in a synthetic medium (Colin et al., 2013a). In the present work, the potential of this strain to produce bioemulsifiers using vinasse as a substrate was also evaluated. Under our assay conditions, bioemulsifier production could not be detected when the strain was incubated in the M1 culture media. However, when vinasse was used as a carbon source (M2 culture media), a significant emulsifying activity in the supernatants was detected. This finding could be related to the positive effect of some components of M2 (e.g., phosphate ions) on biosynthesis of the emulsifier (Colin et al., 2013a). The bacterial biomass and bioemulsifier production (E_{24}) measured in the M2 media are shown in Fig. 4. While biomass significantly increased at vinasse concentrations up to 10% ($M2_{10}$), maximum bioemulsifier production was detected at a final concentration of 1.0% ($M2_{1.0}$), with an E_{24} value of 63.0 ± 0.5%. It is important to note that no emulsifying activity could be detected at a vinasse concentration higher than 10%, probably due to the high concentration of total solids.

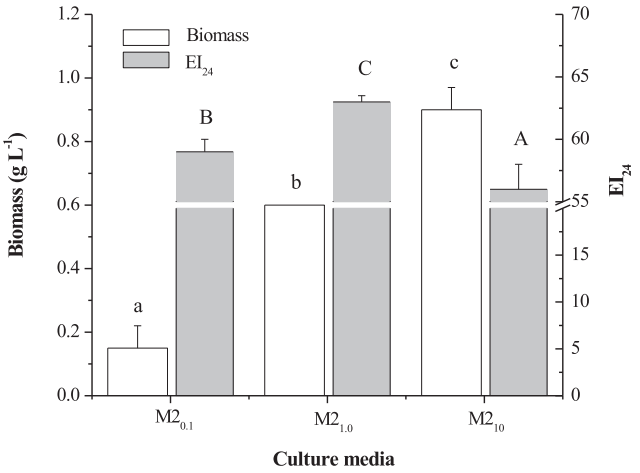

Fig. 4. Biomass and emulsification index (E_{24}) of the supernatants of *Streptomyces* sp. MC1 harvested from the M2 culture media at 24 h of incubation. Error bars represent the standard deviation calculated from three independent experiments. Bars with different letters (a–b) for biomass and (A–C) for E_{24} are significantly different ($p < 0.05$).

It is assumed that the most appropriate treatment for recovery and re-valuation of an effluent by a microbial pathway will depend upon the product obtained, in terms of its potential applications and market acceptance. Liquid effluents with a high load of organic matter can be used as raw material for fermentation processes (Aguiar et al., 2010; Bhattacharyya et al., 2012; Aimaretti et al., 2013; Saharan et al., 2014) while the volume of effluent is reduced. Microbial action on the organic matter in vinasse can therefore produce not only potential soil fertilizers but also other valuable products such as bioemulsifiers. Like us, de Lima and Rodriguez de Souza (2014) reported on the use of vinasse as a carbon substrate for a fermentation process using *Bacillus subtilis* PC, where an emulsification index of 51% was detected. In contrast, Guerra de Oliveira and Garcia-Cruz (2013) have reported that the cell-free broth obtained after cultivation of *Bacillus pumilus* using vinasse as carbon source did not show any emulsifying activity. These varying responses may depend upon the metabolism of each microorganism, but may also reflect variability in the composition of the vinasses used as substrates. Further studies may therefore be required in order to evaluate the potential of *Streptomyces* sp. MC1 for producing bioemulsifiers using vinasses of variable origin.

4. Conclusions

Application of a single microorganism, *Streptomyces* sp. MC1, for vinasse treatment seems highly promising. Mitigation of the pollution potential of the treated effluent compared to crude

vinasse was reflected in a significant increase in the vigour index of *L. sativa* seedlings used as bioindicador. The ability of this strain to produce bioemulsifiers using raw vinasse as a low-cost substrate was also demonstrated. This alternative use of vinasse could represent an effective means of ensuring the sustainability of the production process for bioemulsifiers. However, new studies are currently in progress in order to evaluate this strain's potential for the vinasse treatment and the bioemulsifier production at a more realistic scale.

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