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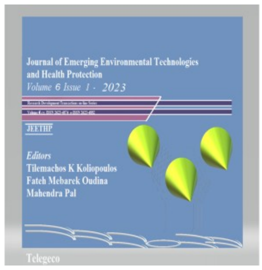

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Vinasse a source of compounds with antimicrobial activity

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Abstract

The objective of this work is the isolation and investigation of the antibacterial activity of bacteria and compounds present in vinasse. Vinasse is a big problem in our country, due to the large amount of organic matter present in it and the volume in which it is generated. The inadequate disposal of sugarcane vinasse has received much attention since a long time ago, due to environmental problems associated to this practice and this is one of the organic wastes with the greatest polluting effect on flora and fauna. In this work was investigated the antibacterial efficiency of ethanolic extract of vinasse from Argentine, and bacteria isolated from this waste, against pseudomonas aeruginosa, Escherichia coli and Candida albicans. All the isolated were assigned as presumed lactic acid bacteria, and the screening of the antimicrobial activity demonstrates that only 2 supernatants possess antibacterial activity against E. coli and Ps. aeruginosa, whereas ethanolic extract of vinasse showed antimicrobial activity against E. coli, Ps. aeruginosa and C. albicans Vinasse extract was more effective to inhibit the biofilm formation of E. coli and Pseudomonas aeruginosa. On the other hand, the addition of the extract combined with shaking on bacterial biofilm previously formed, removed the biofilm by both bacteria studied and the removal percentage increases with vinasse concentrations and with the stirring time. The biofilm formed by Pseudomonas aeruginosa was more resistant at treatment compared with that biofilm formed by E.coli. The possible use of proposed treatment allows the vinasse to stop being a polluting agent and to become an abundant and raw material as antibacterial agent.

Keywords: Microbiology, vinasse, bacteria, antibacterial agent

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1. INTRODUCTION

The environmental impacts of the industry cause a direct impact on the population, either through the emission of particles, polluting gases and solid or liquid waste that hinder the environmental sanitation of ecosystems (Ibarra-Camacho & León-Duarte, 2018). Vinasse is the final by-product of the biomass distillation, mainly for the production of ethanol, from sugar crops (beet and sugarcane), starch crops (corn, wheat, rice, and cassava), or cellulosic material (harvesting crop residues, sugarcane bagasse, and wood). The inadequate disposal of sugarcane vinasse has received much attention since a long time ago, due to environmental problems associated to this practice and this is one of the organic wastes with the greatest polluting effect on flora and fauna (Christofoletti *et al*.,2013). Vinasses, which hold the remaining soluble matter after the fermentation-distillation process of sugarcane molasses as well as the non-volatile fermentation byproducts, is one of the most recalcitrant wastes (Fitzgibbon *et al.,*1995). The production of sugarcane bioethanol generates large amount of vinasse, a polluting effluent that has no final disposal;so vinasse is a big problem due to the large amount of organic matter present in it, and the volume in which it is generated. For these reasons is very important to find alternative treatments or uses.

In last decades there were a lot of investigations about sugar can vinasse reutilization, such as energy production,the effects on soil physical, chemical and biological properties,its influence on seed germination or its use as biostimulant (Cortez *et al*2007, Szymanski *et al,* 2010, Jiang *et al* 2012, Laime *et al*., 2011, Albuquerque *et al.,*2020, Carpio *et al.,*2021). Recently, some authors reported that the long-term disposal of vinasse in sugarcane fields could challenge crop management, such as the performance of traditional herbicides, by changing soil properties, vinasse affected soil properties by increasing pH, electrical conductivity, and soil organic carbon (Portocarrero *et al.,*2023). Moreover, vinasse has been explored for the culture of microalgae (de Carvalho *et al*., 2022), or yeasts (Díaz-Vázquez *et al*., 2022).

Recently, it has demonstrated that ethanolic extract of vinasse is effective to inhibit the growth, gene expression, and biofilm formationof *vibrio parahaemolitycus* (Tep-ubon *et al.,*2020; U-taynapun*et al.*, 2022).Bacteria predominantly exist in matrix-enclose dmulticellular communities associated with various surfaces, called biofilms. Bacteria in biofilms are extremely resistant to antibacterial agents, producing serious problems. *Pseudomonas aeruginosa*is a ubiquitous, opportunistic and fairly persistent pathogen in the environment; it can effectively persist in water and soil living with a minimum nutritional requirement and tolerating diverse physical media (Mesquita *et al*., 2023). *Pseudomonas aeruginosa*is a primordial microorganism that causes nosocomial infections and is one of the main pathogens causing infections predisposing to multi-resistance due to the intra-hospital environment (Olaechea *et al.*, 2010). *Pseudomonas aeruginosa*is one of the major pathogens causing healthcare-associated infections. Its capacity of adaptation, dissemination, intrinsic resistance to antimicrobials and of acquiring new mechanisms through mobile genetic elements, make the treatment of infections by this microorganism a challenge for the clinician. *Escherichia coli* is a natural inhabitant in the intestinal tracts of humans and warm-blooded animals. Animal fecal matter is the primary source of pathogenic bacteria on meat products (Cody*et al., 1999*)and the presence of this specie provide absolute proof of fecal contaminations. Some strains of *E. coli* can cause diarrhea, urinary tract infections, inflammations and peritonitis in immune-suppressed patients as children and elderly people *(*Karin*et al,*2005*;*Burchand *et al.,* 1997*)*. As a consequence, the absence of *E. coli* in foods can be used to asses it the sanitary quality (Hitchin*et al.,* 1998*)*.On the other hand, vulvovaginal candidiasis is a fungal infection of high incidence that causes pain, irritation and pruritus in the affected area. 75% of women suffer at least one episode throughout their lives. The most common etiological agent is *Candida albicans*. Treatment consists mainly of the use of antifungal azoles, which cause numerous side effects and the appearance of resistant strains.

The aim of this work was investigated the antibacterial efficiency of ethanolic extract of vinasse from Argentine, and bacteria isolated from this waste, against *pseudomonas aeruginosa* ATCC 27853,*Escherichia coli* ATCC 25922and *Candida albicans*IBL001.

2. MATERIALS AND METHODS

Obtention of ethanolic extract of vinasse

The vinasse used was produced in 2022, in Tucumán province located in the northwest region of Argentine. The vinassewas extracted using ethanol as the extraction solvent following. Firstly, 100 g of vinasse was dry and then it was mixed with ethanol with a ratio of 1:5 (w/v) during 24 h, finally, the mixture was filtered through a Whatman N_o1 filter paper and the ethanol was removed by evaporation. For further study, the ethanolic vinasse extract was dissolved in ethanol 1:5, 1:4, 1:3 and 1:2 v/v),then filtered through the membrane filter pore size 0.22µm and kept at -20°C.

Isolation and preliminary identification of Bacteria.

100ml of vinasse was mixed with 900 ml of MRS supplemented with 1% w/v cycloheximide (MRS-c).The agar-MRS-c plates were incubated in microarofilia at 30°C for 3 days. Colonies with typical macroscopic characteristics of lactic acid bacteria (LAB)were selected and transferred to broth MRS medium, pH 5,5. These colonies were later purified by successive transplants on agar MRS for their subsequent phenotypic identification. Plates were exposed to 3% H_2O_2(v/v) to reveal positive catalase colonies. Selected isolates considered as LAB on the basis of their results for Gram staining, cell morphology, motility, spore formation and catalase reaction were tested for fermentative catabolism of glucose, gas production from glucose in Gibson medium. The glucose fermentation in MRS broth containing glucose 1% (v/v), bromocresol purple (0.04 g/L) and inverted Durham tube was carried out. The formation of ammonia from arginine was tested in medium that contained in g/L: L-arginine,3; triptone,4; yeast extract,4; glucose,1; peptone,6; tween 1; pH=6.5). Molecular identification was carried out in those isolated with antimicrobial activity against all selected microorganisms. All isolated were grown in broth MRS medium, pH 5.5 and incubated at 30°C.

Microorganism, culture conditions and inoculum preparation.

Three microorganisms from the American Type Culture Collection were used, *Escherichia coli*ATCC 25922, *pseudomonas aeruginosa* ATCC 27853and *Candida albicans* isolated frompatient with vulvovaginal candidiasis. All of the microorganisms were aerobically grown in Nutrient broth medium (Britania, Argentina) at 37°C and then were sub-cultured twice.

***Determination of antimicrobial activity*.**

Screening of the antimicrobial activity.

The antimicrobial activity of four dilutions of ethanolic extract of vinasse (1:2,1:3, 1:4 and 1:5)andthe cell-free culture supernatants of the 11 isolated bacteria (Vin-1 to Vin-11) was evaluated using agar diffusion test against *E. coli,Ps. Aeruginosaor Candida albicans* with a final concentration of 10^5CFU/mL, followed the method described in previously work (Rodríguez-Vaquero *et al*., 2007). Isolated bacteria were grown in MRS broth, the CFS were obtained by centrifugation (4000 g, 4°C, for 20 min) and subsequent sterilized through 0.22 μm pore-size filters (Sigma, USA). The plates were incubated 24 h at 37°C and the results were expressed as the diameter of the areas of inhibition. The test was carried out in triplicate.

***Biofilm formation inhibition*.**

The inhibition of the biofilm formation was investigated in ethanolic extract of vinasse (1:2, 1:3, 1:4 and 1:5).Briefly, 200 µL of the overnight culture and 50 µL of extract was added to the wells of sterile flat bottom 96-well polystyrene microtiter plates and incubated for 36h at 20 °C for biofilm formation. Then, the non-adherent cells were taking off and the wells washed twice with distilled water in order to remove all non-adherent cells, and 200 µL of 0.01% (w/v) Crystal Violet (CV), were added to the wells for 30 min in darkness. The stained biofilms were rinsed with distilled water and extracted with 200 µL of 96% ethanol. The amount of biofilm was quantified by measuring the OD 595 nm of dissolved CV using the microplate reader.

Cell viability.

The viability of selected bacteria in presence of ethanolic extract of vinasse was determined in nutrient media, using vinasse extract dilutions. The final concentration of each bacteria,in nutrient broth contained serial dilutions of the waste, was approximately 5×105cfu mL-1. Bacterial growth was followed directly by aerobically incubation for 24h at 37ºC in shaker, counting the number of viable cells in solid medium. The number of viable cells were at final incubation was compared with the number of final cells of the control (without vinasse addition) and the reduction was calculated as the difference between them. All experiments were carried out at least in triplicate.

Removal of formed bacteria biofilm:

The antibacterial activity of dilutions of ethanolic extract of vinasse was evaluated on a biofilm formed in polystyrene microtiter plates. Briefly, 200 µL of the overnight culture of *E. coli* or*Ps. aeruginosa* was added to the wells of sterile flat bottom 96-well polystyrene microtiter plates and incubated for 36 h at 20 °C for biofilm formation, Then, the wells washed twice with distilled and 200 µL of a solution with ethanolic extract of vinasse (1:2, 1:3 and 1:4) in sterile distilled water was added to the well, incubated during 1 hour and then shaked by 1, 2 or 3 minutes at 250 rpm. Finally, the wells were washed twice with distilled water and 200 µL of0.01% (w/v) CV, were added to the wells for 30 min in darkness. The stained biofilms were rinsed with distilled water and extracted with 200 µL of 96% ethanol. The amount of biofilm was quantified by measuring the OD 595 nm of dissolved CV using the microplate reader. A control without ethanolic extract of vinasse added was carried out.

3. RESULTS

All the isolated were assigned as presumed lactic acid bacteria because they were Gram positive, catalase negative, non-spore forming, non-motile bacteria, the ability of growth at different NaCl concentration and pH. The group of lactic acid bacteria isolates from vinasse on the basis of their physiological and biochemical characteristics are observed in table 1.

Table1. Group of lactic acid bacteria isolates from vinasse on the basis of their physiological and biochemical characteristics.

Group	**I (N=18)**	**II (N=2)**
Gram	+	+
Catalase	-	-
Morphology	Rod	Rod
Metabolism	Heterof	Homof.
Glucose fermentation	+	+
Growth at NaCl: 2, 4, 6, 8 %	+	+
Growth at pH: 4,0- 5,5 - 6,5	+	+
Ammonio from arginine	-	-

(+): positive, (-): negative. All strains were non-motile and non-sporing. The data were determined in three independent experiments.

The screening of the antimicrobial activity demonstrates that the only supernatant with antibacterial activity were Vin-10 and Vin-11 supernatant, and the inhibition only was observed in *E. coli*and *Pseudomonas aeruginosa.*The antimicrobial activity of ethanolic extract of vinasse was showed in table 2.

Table 2. Antimicrobial activity of ethanolic extract of vinasse.

EEV	*Pseudomonas aeruginosa* 27853	*E. coli* 25922	*Candida albicans*
1/2	24,00* ± 0,50	19,00 ± 0,50	21,00 ± 0,50
1/3	16,00 ± 0,50	13,00 ± 0,50	18,00 ± 0,50
1/4	3,00 ± 0,50	-	-
1/5	-	-	-

* Inhibition zone in mm. (-) negative. The data were determined in three independent experiments.

The inhibition of the biofilm formation was investigate in vinasse extract was observed in table 3.

Table 3. Inhibition of biofilm formation with vinasse extract.

	Pseudomonas aeruginosa 27853	*E.coli* 25922	*Candida albicans*
EEV 1/2	48,00* ± 0,90	39,00 ± 0,80	10,00 ± 0,20
EEV 1/3	27,00 ± 0,50	20,00 ± 0,60	-
EEV 1/4	10,00 ± 0,20	2,00 ± 0,30	-
EEV 1/5	-	-	-

*Percentaje of inhibition (%)

Results showed that vinasse extract was more effective to inhibit the biofilm formation in *E. coli*and *Pseudomonas aeruginosa* than *Candida albicans.*

The viability of *E.coli* and *Pseudomonas aeruginosa* in presence of ethanolic extract of vinasse was observed in Figure 1.

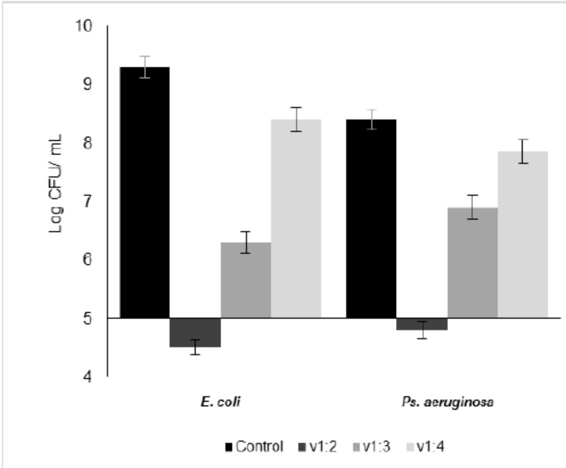

Figure 1. Number of final cells of *E.coli* and *Pseudomonas aeruginosa,*in medium with vinasse extract addition. The data were determined in three independent experiments.

E.coli and *Ps.aeruginosa* growthlo were than their controlsin media added with vinasse extract at 1:3 and 1:4 dilution, but in presence of vinasse extract(1:2 dilution), the death of inoculated cells of *E.coli* and *Ps.aeruginosa* was observed.

The anti-biofilm activity of ethanolic extract of vinasse was evaluated on a biofilm formed in polystyrene microtiter plates. Table 4 shows the percentage of biofilm removed from the wells, after washing with vinasse for 30, 45 and 60 seconds, with shaking.

Table 4. Biofilm removed after extract treatment

EEV	Time (sec)	*Pseudomonas aeruginosa* 27853	*E. coli* 25922
1/2	60	12% *	18%
	120	18%	22%
	180	20%	29 %
1/3	60	5%	7%
	120	9%	12%
	180	13%	15%

*Percentaje of biofilm remotion (%)

Results showed that the addition of the extract combined with shaking removed the biofilm formed by both bacteria studied, partially. The removal percentage increases with increasing vinasse concentration and with the stirring time. The biofilm formed by *Pseudomonas aeruginosa* was more resistant at treatment compared with that biofilm formed by*E.coli.*
The inhibition of biofilm formation by both bacteria, with the addition of vinasse extract was higher than the removal of formed bacteria biofilm.

4. CONCLUSIONS

All the isolated were assigned as presumed lactic acid bacteria, and the screening of the antimicrobial activity demonstrates that only 2 supernatants possess antibacterial activity against *E. coli*and *Ps. aeruginosa,* whereas ethanolic extract of vinasse showed antimicrobial activity against *E. coli*, *Ps. aeruginosa* and *C. albicans* Vinasse extract was more effective to inhibit the biofilm formation in *E. coli* and *Pseudomonas aeruginosa.* On the other hand, the addition of the extract combined with shaking on bacterial biofilm previously formed, removed the biofilm by both bacteria studied and the removal percentage increases with vinasse concentration and with the stirring time. The biofilm formed by *Pseudomonas aeruginosa* was more resistant at treatment compared with that biofilm formed by *E.coli.* The possible use of proposed treatment allows the vinasse to stop being a polluting agent and to become an abundant and raw material as antibacterial agent.

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