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Original Research Article

Furfural Biodegradation in Consortium through *Bacillus licheniformis*, *Microbacterium* sp. and *Brevundimonas* sp.

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ABSTRACT

Furfural (Furan-2-carbaldehyde) is a potentially toxic substance. Indigenous bacterial strains naturally adapted to that contaminant were previously isolated from the effluent of a tannin industry and identified as *Microbacterium sp., Bacillus licheniformis* and *Brevundimonas sp.* The aim of this work was to determine the maximum concentration of furfural that can be degraded by bacterial consortium metabolism. The colonies were activated in peptone solution and then in a mineral medium with glucose as co-substrate, adding the bacterial consortium and different concentrations of furfural. They were incubated in a shaker during 72 hours at 30 ºC and 200 rpm. Bacterial growth was monitored by measuring absorbance at 610 nm. The concentration of furfural was analyzed by High Performance Liquid Chromatography. The consortium was able to grow in furfural concentration of up to 4516 mg L^{-1}. Up to 2723 mg L^{-1} can be degraded in 24 hours. According to these results, a considerable furfural consumption increase is possible with a consortium.

KEYWORDS

Furfural, Contaminant, Bacterial consortium, Biodegradation, Bacillus licheniformis, Brevundimonas, Microbacterium.

INTRODUCTION

Furfural is a heterocyclic aldehyde derived from furan. It is an oily, pale yellow liquid [1] with a tendency to darkening - photochemical oxidation - in the presence of light and oxygen [2]. It is a recalcitrant and toxic substance to various living organisms and severely inhibits the enzymatic metabolism of many microorganisms. In any case, furfural can be eliminated by using the capacity of some bacteria to metabolize it [3].

Widely used by chemical industries, furfural acts as an intermediate product for the manufacture of plastics, paint solvents, furan resin and adhesives, among other applications. However, despite its production and exportation benefits, it is known to cause serious environmental problems during its production process [4].

Many technologies are now available to treat effluents that have been contaminated with hazardous materials such as furfural. Among these options, biodegradation stands out, gaining increasing attention owing to its efficient biotechnological approach to restore contaminated environments [5]. It involves the use of microorganisms to degrade environmental pollutants.

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In this regard, some studies have engineered furfural metabolizing strains for the biological detoxification of the furan aldehydes by cloning or adapting the furfural metabolic pathway from bacteria such as *C. basilensis* [3], *P. putida* [6] and *E. coli* strains [6].

The genus *Microbacterium* is part of the Phylum *Actinobacteria*, a group of Gram-positive bacteria present in high concentrations in soils and aquatic sediments. Not only can they grow and subsist in very diverse substrates but also, they can metabolize complex organic matter [8], playing an important role in the recycling of substances. It has also been demonstrated that *Actinobacteria* play an important ecological role thanks to their ability to eliminate xenobiotic compounds such as pesticides [9] and heavy metals [10], among other substances. Khan *et al.* [10] determined that the group of *Actinobacteria* was one of the most abundant microorganisms in the bacterial community structure in anaerobic digesters of a full-scale municipal wastewater treatment plant. Haleyur *et al.* [12] used *Microbacterium sp*. in a consortium as a successful Polycyclic Aromatic Hydrocarbons (PAHs) degrader.

By contrast, *Bacillus* genus microorganisms are large-sized bacilli (4 – 10 µm), Gram-positive, strict aerobic or encapsulated facultative anaerobes. Bacteria are ubiquitous in soils, some of them are species of economic value due to their production of compounds such as enzymes, antibiotics and surfactants used for various industrial purposes. The ability of *Bacillus* species of forming highly resistant endospores gives them a very important competitive advantage in an environment such as soil. In addition to that, they can adapt to sudden temperature changes because of an inducible heat shock gene that includes chaperone proteins and proteases [13]. The *Bacillus* spores are generally formed under limited nutrients conditions [14]. Different studies demonstrated the ability of some *Bacillus* species to form bacterial consortiums able to degrade oil [15], lubricating oils residues [16] and petroleum hydrocarbons in crude oil contaminated soil [16]. Felshia *et al.* [18] identified strains of *Bacillus licheniformis* which demonstrate phenol degradation up to 2000 mg L^{-1}. Yan *et al.* [19], found a bio-catalytic reduction of furfural to furfuryl alcohol by using *Bacillus coagulans,* a furfural tolerant strain, and glucose as co-substrate.

Brevundimonas are a genus of proteobacteria, Gram-negative, non-fermentative aerobic bacilli. *Brevundimonas* species are ubiquitous in the environment and some clones have been recovered from hydrocarbons contaminated soils [20]. The genus is known for the degradation of a variety of phenolic contaminants [21] and some of its species can make use of diesel oil as the sole source of carbon and energy [22]. Some species have been associated with a potential and activity of polyaromatic hydrocarbons degradation increase [23]. Basuki [24] identified strains of *Brevundimonas diminuta* capable of significantly degrading aliphatic, aromatic and polycyclic hydrocarbons of short and long chains.

In previous studies, sludge bacteria were isolated from the tannin effluent treatment plant and 500 mg L^{-1} of furfural was used as a selection pressure [25]. The unidentified consortium was able to grow and degrade furfural up to 600 mg L^{-1} in 20 hours [26].

The microorganisms identified by the Polymerase Chain Reaction amplification method (PCR) and sequencing of the 16S rRNA (Ribosomal Nucleic Acid) gene were, among others, *Microbacterium sp*., *Bacillus licheniformis* and *Brevundimonas sp*. Degradation of furfural by the bacteria was determined individually, achieving a removal of up to 1300 mg L^{-1} of this compound.

The aim of this paper was to determine the ability of these bacteria to act in consortium and remove furfural at high contaminant concentrations.

MATERIALS AND METHODS

Strains of *Microbacterium sp*., *Bacillus licheniformis* and *Brevundimonas sp*., previously isolated, identified by PCR-amplification and 16S-rRNA-gene-sequencing method and properly conserved, were used for furfural degradation tests.

Inoculum preparation

The growth of bacterial strains was carried out in two stages: meat peptone solution inoculation and subsequent minimal medium (M9) inoculation. The solutions and materials used were previously autoclaved at 1.5 bar and 112 °C for 15 minutes.

The first inoculum was made in three 250 mL Erlenmeyer flasks (one for each strain), containing 49 mL of meat peptone to a 1% m/v, 0.5 mL of NH_4Cl 1 M (JT Baker Inc., 99.7% purity), 0.5 mL of NaH_2PO_4 1 M (Mallinckrodt Chemical Works of 99.0% purity) each. Colonies were sown from a solid medium (nutritive agar) with 24 hours of development (Figure 1).

These enrichments were incubated at 30 ºC using a DLAB orbital shaker (SK-0330-PRO) at 200 rpm, in darkness for 48 h.

Subsequently, a second inoculum was carried out in a minimal mineral medium consisting of 32 mL of sterile deionized water, 10 mL of M9, prepared with 34 g of sodium monoacid phosphate (Na_2HPO_4) (Mallinckrodt Chemical Works, 99.8% purity), 15 g of potassium diacid phosphate (KH_2PO_4) (Mallinckrodt Chemical Works, 99.0% purity), 2.5 g of sodium chloride (NaCl) (Alcor, 99.9% purity) and 0.5 g of ammonium chloride ((NH_4Cl) (JT Baker Inc., 99.7% pure), adjusted to 1 liter. It was then planted with 2.5 mL of glucose ($C_6H_{12}O_6$) (Cicarelli, 99.0%) at 20% m/v, 0.1 mL of 1M magnesium sulfate ($MgSO_4$) (Cicarelli, 98.0% purity) and 5 µL of calcium chloride ($CaCl_2$) 1M (Merck, 99.5% pure).

5 mL of meat peptone inoculum was added and incubated at 30 °C on a DLAB orbital shaker (SK-0330-PRO) at 200 rpm, for 24 hours.

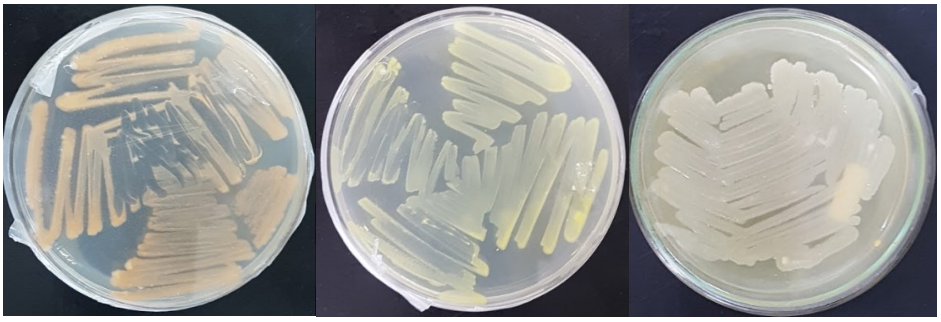

Figure 1 Left: Bacillus licheniformis; center: Microbacterium sp.; right: Brevundimonas sp.

Biodegradation tests

A triplicated culture was made. Erlenmeyer flasks of 250 mL were prepared with: 34 mL of sterile distilled water, 10 mL of M9, 0.1 mL of magnesium sulfate 1 M (Cicarelli, 98.0% purity), 5 µL of calcium chloride 1 M, 1 mL of glucose at 20% m/v.

The consortium was inoculated as follows: 1.7 mL of *Microbacterium sp*. inoculum, 1.7 mL of the inoculum of *Bacillus licheniformis* and 1.7 mL of the inoculum of *Brevundimonas sp*.; each one of them at 0.7 optical density at 610 nm (OD_{610}), thus estimating a total amount of bacteria of 106 Colony Forming Units (CFU). Eight furfural concentrations between 722 and 4517 mg L^{-1} were tested.

An abiotic control was performed at the same time under the same cultivation conditions.

The flasks were incubated at 30 °C at 200 rpm for 72 h using a DLAB orbital shaker (SK-0330-PRO). Bacterial growth was monitored by measuring culture turbidity at 610 nm with a PerkinElmer UV / VIS Lambda 25 spectrophotometer.

Furfural concentration was analyzed every 24 hours by High Performance Liquid Chromatography (HPLC) using a Shimadzu CBM 20A device with UV SPD 20A detector at 278 nm wavelength, and with a C18 reverse phase column, at 40 °C, with 1 mL min^{-1} flow rate. In addition, an injection volume of 20 µL acetonitrile – water (17.5: 82.5) mixture was used as mobile phase [27].

RESULTS AND DISCUSSION

In order to determine the furfural inhibitory concentration for the consortium, 722, 964, 1365, 1488, 1749, 1990, 2724, 4517 mg L^{-1} were tested. Furfural consumption was detected in all trials. Figure 2 shows that bacterial consortium formed by the strains of *Microbacterium sp.*, *Brevundimonas sp.* and *Bacillus licheniformis* is capable of metabolizing the contaminant in 72 hours, at least up to 4517 mg L^{-1} yet, in the latter case, with lower furfural consumption. From 1749 mg L^{-1} to 2724 mg L^{-1}, total contaminant degradation was not achieved in 24 hours. Conversely, furfural was completely degraded in 48 hours in this range of concentration. A reduction in consumption was observed at high concentration of pollutant (greater than 4500 mg L^{-1}), but full degradation was not observed in three days.

Farías *et al.* [28] isolated the *Bacillus licheniformis* strain and tested it with furfural concentrations of 1400 mg L^{-1}. As a result, it was demonstrated that this strain tolerated this condition and degraded the pollutant by 11.8% in 3 days.

Instead, in the study by Zheng *et al.* [2], *Bacillus cereus sp*. showed that at a furfural concentration of 500 mg L^{-1}, the best furfural degradation ability was 35% in 7 days.

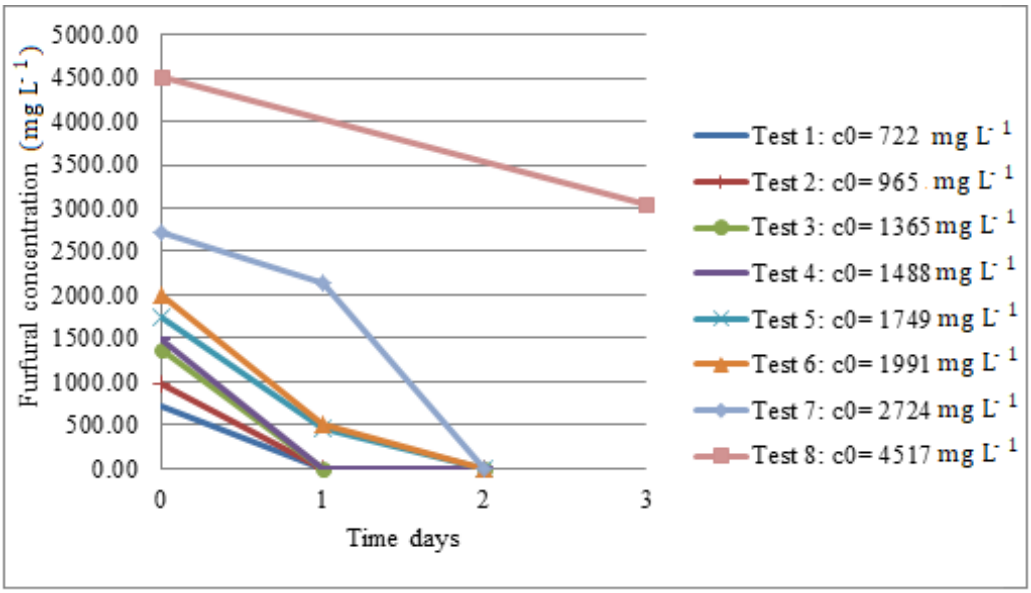

Figure 2 Furfural degradation by bacterial consortium vs Time, incubation conditions 30 º C at 200 rpm; c0 indicates initial furfural concentration

Figure 3 shows furfural´s removal percentage at 72 hours, related to the abiotic test. The average abiotic degradation was 28%. In all cases, a significantly higher degradation is observed in biotic essays. The degradation of 100% of the pollutant was achieved up to concentrations of 2723 mg L^{-1} and 11% effective (biotic least abiotic) at concentrations of 4516 mg L^{-1}. It was observed, nonetheless, that it was difficult for the consortium to grow and the furfural to be metabolized at higher concentrations. 11 % of the contaminant was removed in 72 hours, which suggests that it may be possible to achieve the complete degradation in longer periods.

Boopathy [5] found a growth limit for *Methanococcus sp.* at 2400 mg L^{-1} of furfural concentration and 100% degradation at lower concentrations but in a 5 days incubation period. Zheng *et al.* [29] isolated *Bacillus subtilis* strain DS3, which is capable of growing on furfural as sole carbon source. Despite the similarities in the results, degradation percentages were lower (about 30%) in longer periods (7 days).

Degradation of furfural in abiotic tests can be observed, which may suggest that this decrease could be a photochemical degradation of furfural as mentioned by Zheng *et al.* [2] and not a result from bacterial action. Similarly, Bariani *et al.* [30] and Steinbach *et al.* [31],

report furfural degradation reactions that lead to autoxidation when it is exposed to air. Nevertheless, bacterial degradation is clearly observable.

The bacterial growth was then monitored from the absorbance measured at 610 nm.

In previous studies (data not shown), the capacity of both *Brevundimonas sp*. and *Microbacterium sp*. to grow in a furfural environment was determined. About 1400 mg L^{-1}, OD_{610} readings showed less than 0.15 after 3 days. It can be inferred that these microorganisms have not been able to metabolize furfural in that time.

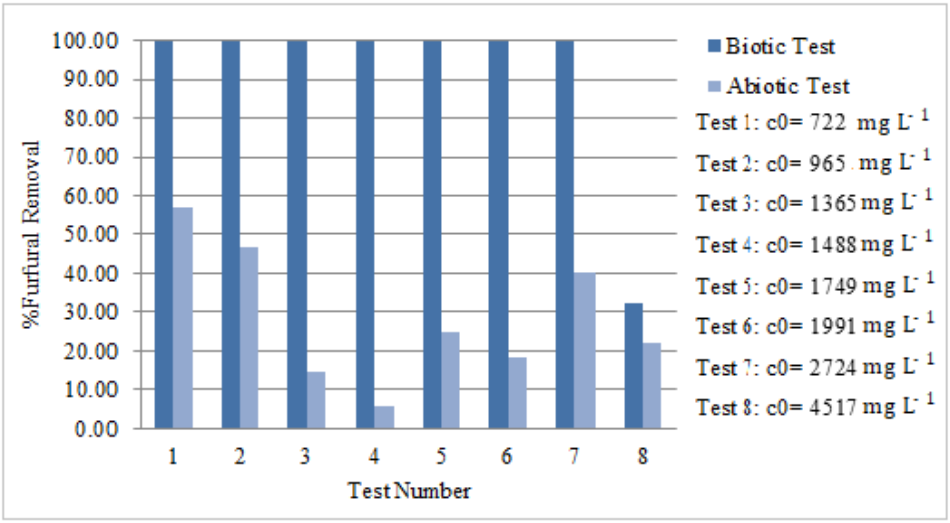

Figure 3 Furfural removal percentage at 3 days in biotic and abiotic test, incubation conditions 30 º C at 200 rpm; c0 indicates initial furfural concentration

The capacity of each microorganism alone to grow in a furfural environment and use it as the sole carbon and energy source was determined by some authors.

Table 1 shows the impact of furfural concentration in individual bacterial growth during days. It displays a complete inhibition in individual strains with furfural initial concentration at 1433 mg L^{-1}, specifically on *Bacillus licheniformis* strain. Both, *L. mesenteroides* and *L. pseudomesenteroides* have achieved growth at 2200 mg L^{-1} of furfural with OD_{610} levels between 0.46 and 0.62 in 3 days. Similar results were obtained with *Z. mobilis* at 1900 mg L^{-1}. As regards *Bacillus cereus sp.* and *Methanococcus sp.,* they only achieved 0.30 OD_{610} in 7 days. Our consortium could grow at 2724 mg L^{-1} of furfural. It can be seen in the high OD_{610} level (1.81) in only 3 days.

Several studies showed significant differences between microbial consortium and individual strains concerning advantage in their degradative capacities or in various biotechnological processes. Thus, Ding *et al*. [32] indicated the degradation effects for Chemical Oxygen Demand determined by the Dichromate Method (CODCr) of single isolated strain, mixed strains, and microbial consortium originally screened from viscose fiber wastewater. Microbial consortium showed the optimum degradation rate of CODCr. Jing *et al.* [33] made a comparison between individual strains and the microbial consortium for biopolymer production in different treatment processes. The experimental results showed that the microbial consortium could produce more biopolymers than individual strains, and the reason might be attributed to the synergetic action of strains. Jing *et al.* [34] compared the degradation of phenanthrene between individual predominant strains and the microbial consortium in different treatment processes. The microbial consortium showed a significant improvement of phenanthrene degradation rates in either static or shaken culture.

Table 1 Furfural growth inhibition; OD values of individual strains vs consortium

Bacteria	Furfural initial concentr. (mg L^{-1})	Absorbances				
		day 0	day 1	day 2	day 3	day 7
B. licheniformis (Farías *et al.*, 2019)	1400	0.11	0.10	0.10	0.10	-
Microbacterium sp. (Echeverría *et al.*, 2018)	1600	0.10	0.10	0.11	0.14	-
Z. mobilis (Hunter and Manter, 2014)	1900	0.05	0.40	0.60	0.60	-
L. mesenteroides (ibid.)	2200	0.09	0.45	0.55	0.62	-
L. pseudomesenteroides (ibid.)	2200	0.09	0.30	0.42	0.46	-
Bacillus cereus sp. (Zheng *et al*., 2015)	2000	-	-	-	-	0.32
Methanococcus sp. (Boopathy *et al.,* 1993)	2000	0.02	0.03	0.10	0.16	0.27
Consortium	2700	0.14	0.23	1.81	-	-

Boopathy [5] indicated that furfural is a germicide. This means that it inhibits the growth of some biological systems. This effect has also been reported by Moussavi *et al.* [35] who made a degradation test in a continuous inflow cyclic biological reactor (CBR) and could remove over 98% of furfural at inlet furfural concentration up to 1200 mg L^{-1}. The studies carried out by Crigler *et al.* [6] characterized the genes involved in the aerobic bacterial metabolism of furfural in two species, *Pseudomonas putida* Fu1 and *Cupriavidus basilensis* HMF14.

Even though at higher concentrations, a difficulty in the consortium growing and furfural metabolizing was observed, the removal of 11% of the contaminant in 72 hours, previously described, suggests the possibility of its degradation in longer periods. Hunter and Manter [36] showed that the furfural treatments slowed the growth rates of three *L. mesenteroides* and *L. pseudomesenteroides* strains, but they obtained better degradation values (between 55% to 75% at 4500 mg L^{-1} of furfural).

Figure 4 represents 24 h bacterial growth, according to different furfural concentrations. The bacterial consortium growth was monitored by measuring culture turbidity at 610 nm. Up to 1991 mg L^{-1}, the bacterial consortium was able to grow rapidly in just 24 hours (OD_{610} > 0.6). However, the influence of furfural concentration on bacterial growth can be seen due to the turbidity decrease in solutions with increasing pollutant concentration. Consequently, decreasing slopes at 4517 mg L^{-1} were observed. The results could give an idea of the amount of viable cells needed to degrade furfural at those particular concentrations.

Although, Guarnieri *et al.* [37] obtained through genetic engineering, a *P. putida* KT2440 strain capable of growth on furfural as a sole carbon and energy source up to 1000 mg L^{-1}, that bacteria needed more than 15 hours to reach 0.6 OD_{610}, while our consortium could grow in similar concentrations of furfural, achieving this OD_{610} in only 8 hours, indicating a higher development index.

Table 2 shows the percentages of furfural removal by bacterial consortium in 24 hours for different initial concentrations. For lower concentrations, (722 mg L^{-1}, 965 mg L^{-1}, 1365 mg L^{-1} and 1488 mg L^{-1}) the degradation was 100%. This shows consistency with the increase in bacterial growth (Figure 4), while for 1749 mg L^{-1}, 1991 mg L^{-1} it was 74%. Finally, for higher concentrations (2724 mg L^{-1} and 4517 mg L^{-1}) degradation values were only 21% and 11%, respectively.

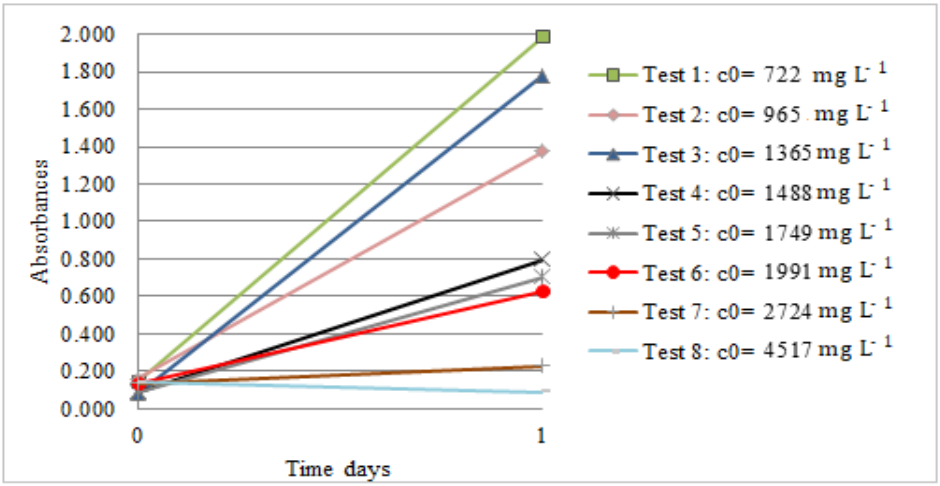

Figure 4 610 nm absorbance measurements at the initial time and day 1 at different furfural concentrations, incubation conditions: 30 º C at 200 rpm; c0 indicates initial furfural concentrations.

Table 2 Furfural concentrations on days 0 and 1 and removal percentage for the 8 tests

Test	Concentration (ppm)		% Furfural Removal
	day 0	day 1	
1	722	0	100
2	965	0	100
3	1365	0	100
4	1488	0	100
5	1749	459	74
6	1991	512	74
7	2724	2147	21
8	4517	4030	11

Boopathy *et al.* [38] worked with strains of enteric bacteria. They found similar results, both in biodegradation percentages and bacterial growth, at 960 mg L^{-1} of furfural. Hunter and Manter [36] showed that the furfural treatments slowed the growth rates of three *L. mesenteroides* and *L. pseudomesenteroides* strains, but they obtained better degradation values (between 55% to 75% at 4500 mg L^{-1} of furfural).

CONCLUSION

It was shown that a bacterial consortium formed by the strains of *Microbacterium sp.*, *Brevundimonas sp.* and *Bacillus licheniformis* can use furfural as a carbon source more effectively than the individual strains. In only 48 hours bacterial consortium removes 100% of furfural in concentrations up to 2723 mg L^{-1}. Thus, it could be suggested as an adequate method to eliminate such contaminants.

Furfural concentration influences consortium growth, and prevents the exponential growth of microorganisms during the first 24 hours from 2000 mg L^{-1}.

At high concentrations, such as 4500 mg L^{-1} of the contaminant, it was verified that there was no exponential bacterial growth within 72 hours and that the percentage of contaminant removal proved to be low.

Future work could focus on immobilizing bacteria techniques and bioreactor assays to improve furfural biodegradation.

ABBREVIATIONS

PAHs	Polycyclic Aromatic Hydrocarbons
PCR	Polymerase Chain Reaction
rRNA	Ribosomal Nucleic Acid
M9	Minimal medium
OD_{610}	Optical Density at 610 nm
CFU	Colony Forming Units
HPLC	High Performance Liquid Chromatography
CODCr	Chemical Oxygen Demand determined by the Dichromate Method
CBR	Cyclic Biological Reactor

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