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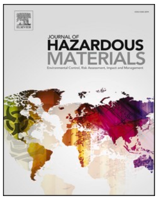


Toluene bioconversion into ectoines by halophile mixed microbial cultures

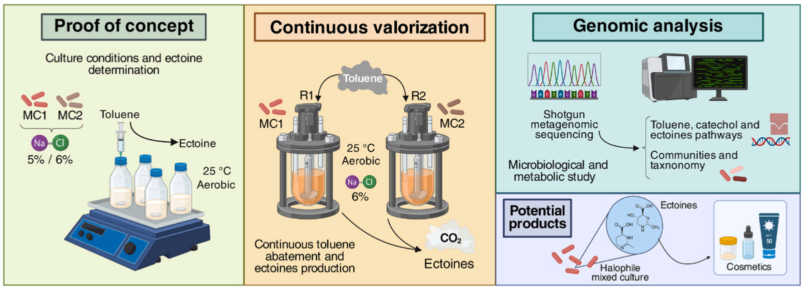
Nicolás Díaz-Moreno [a,b], Raquel Lebrero [a,b,*], Sara Cantera [a,b]

[a] Institute of Sustainable Processes, University of Valladolid, Dr. Mergelina s/n, Valladolid 47011, Spain
[b] Department of Chemical Engineering and Environmental Technology, University of Valladolid, Dr. Mergelina s/n, Valladolid 47011, Spain

HIGHLIGHTS

- First demonstration of continuous toluene bioconversion into ectoines.
- Ectoine and hydroxyectoine reached values of 71.2 mg L^{-1} under continuous operation.
- Metagenomics revealed taxa and pathways for toluene valorization into ectoines.
- Community functional synergies enabled pollutant degradation and valorization.

GRAPHICAL ABSTRACT



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ABSTRACT

Toluene, which has been listed in the Pollutant Release and Transfer Register (PRTR) of many countries, is one of the most emitted pollutants to the atmosphere. This study demonstrates for the first time a new perspective in toluene treatment based on its continuous bioconversion into high-value chemicals, specifically ectoine and hydroxyectoine, which hold considerable commercial relevance in the cosmetic industry with market prices reaching 1000 € kg^{-1}. Specific ectoine and hydroxyectoine contents of 27.3 mg g_{TSS}^{-1} were achieved together with toluene elimination capacities of 7.2 ± 1.9 g m^{-3} h^{-1} and a maximum biomass concentration of 1.8 g L^{-1}. Ectoine synthesis predominated initially, later shifting toward hydroxyectoine, reaching a combined amount of 71.2 mg L^{-1} (ectoine:hydroxyectoine 32:68) by the end of the assay. Metagenomic analysis revealed key pathways and taxa involved in toluene degradation and ectoine and hydroxyectoine synthesis. Members of *Paenibacillus*, *Rhodococcus* and *Microbacterium* were identified as possessing the enzymes required for toluene degradation via the TOL pathway, while *Gordonia*, the most abundant genus, was primarily associated with the degradation of intermediates such as benzoate, muconate, or oxoadipate derivatives and their bioconversion into ectoine. These findings revealed a potential metabolically diverse consortium with functional complementarities, where metabolic synergies overcome species-specific limitations and promote the elimination and subsequent valorization of toluene into high-value products fostering sustainable industrial innovation.

1. Introduction

Air-pollution has emerged as one of the central concerns for the World Health Organization (WHO) in recent years [1]. Among atmospheric pollutants, volatile organic compounds (VOCs) have attracted the attention of the scientific community due to their toxicity and

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* Corresponding author at: Institute of Sustainable Processes, University of Valladolid, Dr. Mergelina s/n, Valladolid 47011, Spain.
E-mail addresses: nicolas.diaz@uva.es (N. Díaz-Moreno), raquel.lebrero@uva.es (R. Lebrero), sara.cantera@uva.es (S. Cantera).

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polluting potential [2]. These organic substances are responsible for a wide range of health problems, including respiratory diseases and mutagenic, teratogenic and carcinogenic effects on the human body [3,4]. VOCs are also environmentally harmful not only because their ubiquity and persistence, but also due to their role in tropospheric ozone formation, stratospheric ozone degradation, secondary aerosols generation, and their detrimental impact on biodiversity [5]. The main anthropogenic sources of VOCs are combustion engines, paints, solvents, chemical and petrochemical industries [6]. Among VOCs, the most representative compounds are benzene, toluene, ethylbenzene and xylenes (BTEX), as they account for more than 60 % of non-methane emissions [7]. Toluene, in particular, stands out as one of the major atmospheric pollutants emitted into the atmosphere due to its high volumes, volatility and its inherent toxicity [8]. This has led to its inclusion in the Pollutant Release and Transfer Register (PRTR) in many countries. Indeed, in urban areas, ambient toluene concentrations typically range from 5 to 150 µg m^{-3}, with even higher levels in areas close to emission sources [9].

A wide range of technologies have been developed for the treatment of VOCs, which can be broadly classified according to whether they aim to recover the VOC or degrade them into simpler, non-toxic compounds, primarily CO_2. In particular, biodegradation technologies offer a highly energy-efficient and sustainable alternative to traditional physicochemical methods [10]. However, most research on BTEX bioremediation focuses on the biodegradation process itself, rather than exploring the potential for bioconversion into valuable products that support circular recycling strategies. To develop a truly competitive biological alternative to mature physicochemical technologies, exploring this valorization aspect is crucial. Although some valuable products have been explored in BTEX valorization, such as aromatic derivates, fine chemicals or polyhydroxyalkanoates, high-value chemicals with interest for the pharma/biotech markets have rarely been addressed in BTEX bioconversion technologies. In this context, toluene valorization into ectoines presents an unexplored promising route for the cost-effective and circular elimination of gaseous contaminants.

Ectoine (CAS No.: 96702–03–3) and its hydroxylated derivate, hydroxyectoine (CAS No.: 165542–15–4), hereafter referred to as ectoines, are compatible solutes that enable halophilic and halotolerant microorganisms to cope with osmotic stress under high salinity and temperature conditions [11]. Due to their structural properties, ectoines help stabilizing proteins, nucleic acids and other macromolecules, protecting them from oxidative damage, dehydration, and thermal or saline stress [12,13]. In human applications, ectoines have demonstrated remarkable protective effects as anti-inflammatory and antioxidant molecules [14]. Moreover, ectoines are being explored as anticancer and antimicrobial chemicals to treat emerging diseases [11,15]. These multifunctional characteristics have positioned ectoines as one of the most valuable microbial-derived products, with a market price of approximately 1000 € kg^{-1} [16]. A large number of halophilic bacteria have been identified as capable of synthesizing ectoines, including those naturally occurring in the genera *Marinococcus*, *Halomonas*, *Streptomyces*, *Paracoccus*, or *Halobacillus* [17], among many others, or even recombinant ones, such as *Escherichia coli* ET01 through the introduction of the ectABC operon from *Halomonas venusta* ZH [18]. However, current industrial-scale production primarily uses the pure strain *Halomonas elongata*, which is cultivated in high salinity environments with sugars as the primary carbon source [12,19]. However, recent research has aimed at enhancing sustainability and cost-effectiveness of ectoine production, exploring the use of industrial byproducts as alternative carbon and energy sources [20]. In this sense, coupling ectoine synthesis with the biodegradation of toluene offers a dual advantage: it improves the techno-economic viability and sustainability of the biodegradation process and reduces reliance on conventional sugar-based substrates for bacterial growth.

The use of co-cultures or mixed microbial communities offers clear advantages for BTEX bioconversion, as metabolic synergies between microbial species can accelerate substrate degradation, improve ectoine biosynthesis, and support higher biomass yields, particularly during long-term operations or under suboptimal environmental conditions. These synergies may also enable the sequential breakdown of complex contaminants like toluene, while simultaneously fulfilling the metabolic requirements of ectoine-producing strains [21]. Despite the potential of mixed microbial communities for BTEX bioconversion into valuable chemicals, no studies to date have systematically explored the microbial interactions or identified the key functional players involved in this process. Under this premise, the aim of the present study was to demonstrate, for the first time, the valorization of toluene into ectoines using halotolerant mixed cultures under both batch and continuous operation. Additionally, it uncovered the main microbial contributors and physiological strategies driving toluene degradation and ectoine production.

2. Materials and methods

2.1. Inocula preparation and culture medium

The composition of the Ammonium Mineral Salt (AMS) medium was as follows: 1.0 g L^{-1} (5.7 mM) K_2HPO_4, 1.0 g L^{-1} (0.4 mM) $MgSO_4$-$7H_2O$, 0.5 g L^{-1} (9.3 mM) NH_4Cl, 1.0 g L^{-1} (9.9 mM) KNO_3, 0.1 g L^{-1} (0.7 mM) $CaCl_2$-$2H_2O$. The medium was supplemented with trace elements (0.01 mg L^{-1} $CuCl_2$, 0.9 mg L^{-1} $FeCl_2$, 0.06 mg L^{-1} $ZnCl_2$, 0.01 mg L^{-1} $NiCl_2$, 0.06 mg L^{-1} $CoCl_2$, 0.06 mg L^{-1} $MnCl_2$, 0.06 mg L^{-1} H_3BO_3, 0.03 mg L^{-1} Na_2MoO_4, 0.04 mg L^{-1} Na_2SeO_3, 0.01 mg L^{-1} Na_2WO_4) as well as vitamins (0.02 mg L^{-1} biotin, 0.2 mg L^{-1} thiamin, 0.2 mg L^{-1} nicotinamid, 0.1 mg L^{-1} p-aminobenzoic acid, 0.1 mg L^{-1} pantothenic acid, 0.5 mg L^{-1} pyridoxamine, 0.1 mg L^{-1} cyanocobalamine, 0.1 mg L^{-1} riboflavine). The concentration of NaCl was also adjusted based on the requirements of the assay, ranging between 3 % (AMS3 %), 5 % (AMS5 %) and 6 % (AMS6 %). 1,4-Piperazinediethanesulfonic acid (PIPES) was also added at a concentration of 30 g L^{-1} as a buffering agent to maintain the pH at 7 throughout the duration of the operation.

All the reagents were acquired from PanReac AppliChem (Barcelona, Spain) or Merck (Madrid, Spain). Liquid toluene ≥ 99.5 % was purchased from Sigma-Aldrich (St. Louis, MO, USA), while ethanol ≥ 99 % for ectoines extraction was supplied by PanReac AppliChem (Barcelona, Spain). Ectoine and hydroxyectoine standards were purchased from Sigma-Aldrich (St. Louis, MO, USA) at a purity of 95 %.

The medium was finally autoclaved at 1.5 atm and 121 ºC for 20 min in a Raypa AES Classic Line autoclave (Terrassa, Spain). The halotolerant mixed cultures (MC1 and MC2) were collected from two independent bioreactors previously subjected to continuous BTEX supply. Cultures were inoculated at 5 % (v/v) in 120 mL serum vials with 50 mL of AMS3 % and glucose at 4 g L^{-1}. They were incubated in an IKA KS 4000i orbital shaker (Barcelona, Spain) at 25 °C and 150 rpm for 5 days. Upon growth, MC1 and MC2 were independently transferred to autoclaved 1.2 L Schott Duran Afora V-62897 bottles (Mainz, Germany) sealed with bromobutyl septa. These bottles contained 200 mL of AMS3 % and 5 mL of the inoculum at 0.5 g L^{-1}. In this case, glucose was replaced by toluene at ~4 g m^{-3} as the sole energy and carbon source. Toluene was added to the bottles using a 10 µL Hamilton 800 Series liquid syringe (Reno, NV, USA). Growth at 3 % salinity using toluene was maintained for 1 week at 25 °C and 250 rpm under continuous agitation using a Thermo Fisher Scientific Cimarec i Multiple 6 magnetic stirrer plate (Waltham, MA, USA).

2.2. Toluene valorization and salt acclimation in batch bioreactors

Two batch assays were performed for each mixed culture (MC1 and MC2), one with AMS5 % (assays MC1.5 and MC2.5) and other with AMS6 % (assays MC1.6 and MC2.6) (Table 1). In each experiment, 5 sterile 1.2 L batch bioreactors were used, containing 200 mL of AMS medium. Each condition was tested with 4 replicates (n = 4), inoculated at 5 % (v/v) with the MC1 and MC2 communities previously enriched at 3 % NaCl. To assess its biodegradation potential, approximately 4.5 μL of liquid toluene was added as the sole carbon and energy source, resulting in a gas-phase concentration of ~4 g m^{-3}. Negative controls without biomass served to rule out physicochemical degradation of the pollutants. Assays were maintained at 25 °C under magnetic stirring at 250 rpm. After toluene depletion, toluene was replenished to enable multiple removal cycles over a period of 14 days.

During the experiments, gas-phase toluene concentrations were analyzed daily. At the end of the experiment, optical density at 600 nm (OD_{600}) and total suspended solids (TSS) were measured and biomass samples were taken for ectoine analysis. MC1.6 and MC2.6 enriched biomass was used to test the continuous conversion of toluene into ectoines at 6 % NaCl in a stirred tank bioreactor.

2.3. Toluene valorization under continuous conditions

The optimization of the valorization process was performed in an autoclaved 0.5 L bioreactor controlled by an Applikon MiniBio system (Delft, Netherlands) and equipped with Applikon AppliSense pH and temperature probes (Fig. 1). The toluene gas stream was prepared by continuous addition of toluene to an air stream through a KD Scientific Legato 100 syringe pump (Holliston, MA, USA) and a 5 mL Hamilton 1005 RN liquid syringe (Reno, NV, USA). The gas flow rate was controlled at 40 mL min^{-1}, using an Aalborg rotameter (Orangeburg, NY, USA), corresponding to a calculated gas residence time of 10 min. Prior to entering the bioreactor, the gas stream was homogenized in a 0.5 L mixing chamber to ensure consistent composition. The bioreactor was operated at a constant temperature of 25 °C and agitated at 150 rpm with a Rushton six-blade turbine. The system was operated with a working volume of 0.4 L of AMS6 %. The bioconversion process was evaluated through two independent experiments (Table 1), R1 inoculated with MC1.6 and R2 inoculated with MC2.6, to determine the most effective inoculum for toluene valorization into ectoines.

In R1, the continuous bioreactor was inoculated with MC1.6 and operated for 14 days with a toluene continuous supply of 4.0 ± 0.2 g m^{-3}, resulting in a toluene inlet load of 24.1 ± 1.3 g m^{-3} h^{-1}. The dilution rate of the liquid phase was maintained at 0.025 h^{-1} through a daily exchange of 10 mL of fresh AMS6 %. This volume was employed for the measurement of OD_{600}, TSS and ectoines concentration. Temperature and pH were maintained at 22.6 ± 1.0 ºC and 7.0 ± 0.1, respectively.

Subsequently, the bioreactor was inoculated with MC2.6 (R2) and operated under similar conditions, with a toluene continuous supply of 4.3 ± 0.3 g m^{-3} and a corresponding toluene inlet load of 25.9 ± 1.9 g m^{-3} h^{-1}. The dilution rate and biomass sampling were the same as those used in R1. Temperature and pH were maintained at 23.4 ± 0.7 ºC and 7.0 ± 0.1, respectively.

2.4. Analytical procedures

Toluene was analyzed by collecting 250 μL from bottles headspaces (batch assays) or from sampling points (continuous bioreactor) with a 250 μL Hamilton gas syringe. Samples were manually injected into an Agilent 8860 GC-FID (Santa Clara, CA, USA) with a HP-5 column (30 m × 320 μm × 0.25 μm). The conditions were maintained at 150, 80 and 250 ºC for the injector, oven and detector, respectively. In this case, helium was used as carrier gas at 2 mL min^{-1}. O_2 and CO_2 were determined using a 100 μL Hamilton gas syringe and injecting the samples into a Bruker 430 GC-TCD (Palo Alto, CA, USA) with a CP-Molsieve 5 A column (15 m × 0.53 μm × 15 μm) and a CP-PoraBOND Q column (25 m × 0.53 μm × 10 μm). The temperatures of the injector, column and detector were maintained at 150, 45 and 175 °C, with helium as the carrier gas.

TSS were analyzed by filtration of 5–10 mL of culture broth with filters of 0.22 μm previously weighed. Then, filters were dried for 24 h at 80 ºC, cold in a desiccator and weighted to calculate TSS. A Shimadzu UVmini-1240 spectrometer (Kyoto, Japan) was used to measure OD_{600}. A calibration curve correlating OD_{600} and TSS was prepared to estimate TSS from the OD values for both MC1 ($y = 1.2988x$, $R^2=0.93$) and MC2 ($y = 0.663x$, $R^2=0.99$).

Ectoines extraction and analysis followed the protocol described by Cantera et al. [22]. Briefly, 2 mL of culture were centrifuged in an Eppendorf at 10,000 rpm for 5 min to obtain a biomass pellet. The process was repeated to concentrate the biomass from a total of 4 mL of culture. The samples were kept frozen at −20 °C. The ectoine extraction process was initiated by adding 2 mL of 70 % ethanol and resuspending the biomass. The content was transferred to a conical Eppendorf with zirconia to perform 3 homogenization breaks for 60 s each, followed by a centrifugation at 11,000 rpm for 2 min. The supernatant was filtered through 0.22 μm and introduced into HPLC vials for measurement. The analysis was carried out in a HPLC LC_2050_C (Shimadzu, Japan) coupled with a Dual λ absorbance detector at 210 nm and 35 °C. The

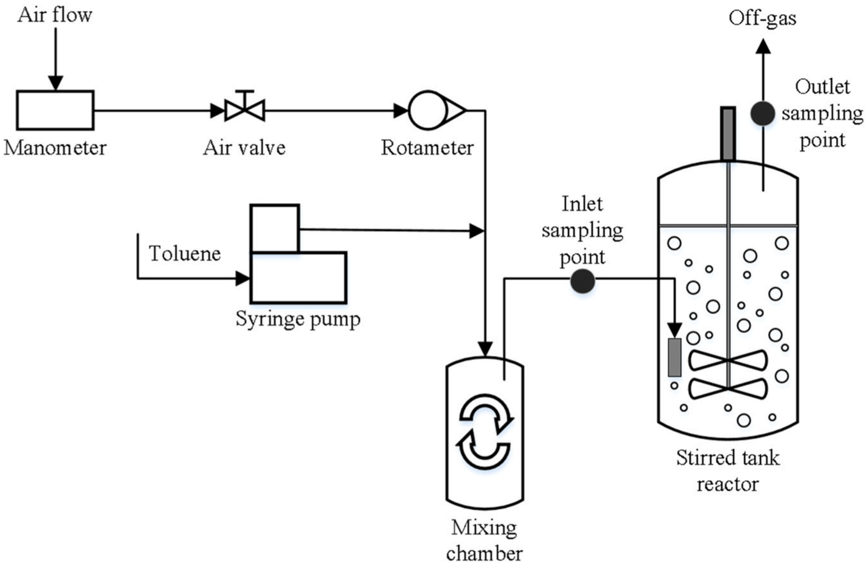

Fig. 1. Experimental set-up of the continuous gas bioreactor system.

Table 1
Experimental conditions of the different valorization tests.

Operation mode	**Batch**				**Continuous**	
Culture	**MC1.5**	**MC1.6**	**MC2.5**	**MC2.6**	**R1**	**R2**
Toluene concentration	3.9 ± 0.1 g m^{-3}				4.0 ± 0.2 g m^{-3}	4.3 ± 0.3 g m^{-3}
Toluene load	0.2 ± 0.0 g m^{-3} h^{-1}				24.1 ± 1.3 g m^{-3} h^{-1}	25.9 ± 1.9 g m^{-3} h^{-1}
System	1.2 L bottle bioreactor				0.5 L stirred tank reactor	
Working (liquid) volume	0.2 L				0.4 L	
Temperature	25 °C				22.6 ± 1.0 °C	23.4 ± 0.7 °C
pH	7				7.0 ± 0.1	
Agitation	Magnetic stirring 250 rpm				Rushton six-blade turbine 150 rpm	

column was a Spherisorb Amino (NH_2) column (Waters, USA) with a mobile phase of acetonitrile/H_2O 75/25 (%) at a flow rate of 0.6 mL min^{-1}. Unlike the well-established Bligh and Dyer [23] method, this technique omits chloroform, significantly improving its environmental and economic sustainability.

Statistical analyses were conducted using SPSS 26.0 (IBM, USA). Significant differences were assessed by ANOVA. Differences were considered significant at $p < 0.05$.

2.5. Genomic analysis

Shotgun metagenomic analysis was performed to analyze the microbial community and determine the main taxa present. To this aim, samples of biomass were taken by the end of the R1 operation, since R2 failed to sustain growth. DNA was extracted and sent for Illumina shotgun sequencing (NovaSeq X Plus-PE150) to Novogene (Germany) according to manufacturer's protocol. The obtained genomes from each

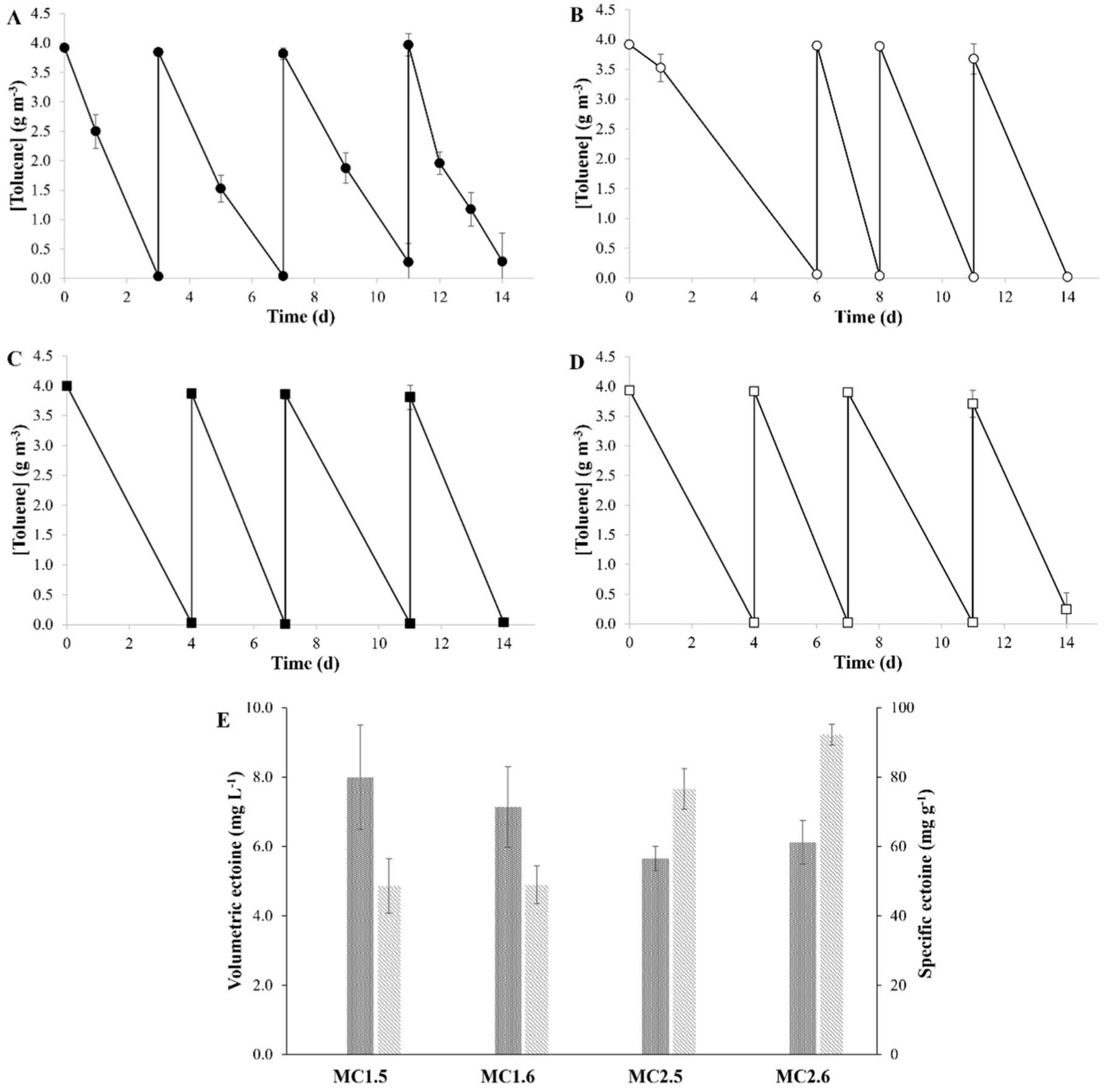

Fig. 2. **A)** Toluene elimination cycles in MC1.5 batch assays (black circles); **B)** in MC1.6 batch assays (white circles); **C)** in MC2.5 batch assays (black squares); and **D)** in MC2.6 batch assays (white squares). Error bars represent the standard deviation (n = 4). **E)** Ectoine production of MC1 and MC2 in batch experiments at different salinities. Volumetric ectoine (dotted columns) and specific ectoine (striped columns).

sample were quality checked and assembled according to Marcos-Rodrigo et al. [24]. Taxonomic analysis was conducted by aligning unigene sequences against bacterial, fungal, archaeal, and viral sequences from NCBI's NR database (https://www.ncbi.nlm.nih.gov/) using a cut-off e-value of 10^{-5}. MEGAN software was used to retain species annotation information during taxonomic classification. Functional annotation of assembled metagenomes was conducted using DIAMOND (v0.9.9) [25], aligning unigenes against functional databases, including KEGG Orthology (http://www.kegg.jp/kegg/), eggNOG (http://eggnogdb.embl.de/#/app/home), and CAZy (http://www.cazy.org/), with a cut-off e-value of 10^{-5} [26]. Functional abundance differences were assessed using ANOSIM, while comparative analyses of metabolic pathways and functional differences were performed via MetaStat and LefSe [27]. This whole genome shotgun project has been deposited in GenBank-NCBI under Bioproject number PRJNA1294334.

3. Results and discussion

3.1. Toluene valorization and salt acclimation in batch bioreactors

MC1 (MC1.5 and MC1.6) and MC2 (MC2.5 and MC2.6) demonstrated substantial toluene biodegradation capacity in batch bioreactors, completing 4 removal cycles at each salinity tested. Since the carbon and energy source, toluene in this case, was the limiting factor, all of them showed the same toluene removal rate of 1.2 ± 0.2 g m^{-3} d^{-1}, regardless of salinity (Fig. 2). Toluene loss in the abiotic control due to physicochemical degradation or sample collection was 0.03 ± 0.02 g m^{-3} d^{-1}. Regarding biomass, MC1.5 exhibited significantly higher TSS concentration by the end of the assay (0.16 ± 0.01 g L^{-1}) compared to MC2.5 (0.07 ± 0.001 g L^{-1}). A similar trend was observed at 6 % salinity, with TSS values of 0.15 ± 0.01 g L^{-1} in MC1.6 and 0.07 ± 0.001 g L^{-1} in MC2.6. Therefore, for each mixed culture, no appreciable differences in TSS were observed between the two salinity conditions, while MC1 appeared to be better adapted for growth during toluene degradation. These results highlighted differences in the microbial capabilities of each consortium when metabolizing toluene as the sole carbon and energy source. In particular, the superior biomass growth performance of MC1.5 and MC1.6 may be attributed to increased culture-specific activity in toluene catabolism.

Some halotolerant and halophilic bacteria have been previously shown to have the capability to degrade BTEX, such as *Marinobacter*, *Halobacillus,* different *Bacilus* spp. and some halophilic members from the genus *Pseudomonas* [28,29]. The toluene concentration removed in this study, ~16 mg L^{-1} after 14 days, using the halotolerant consortia MC1 and MC2, was comparable to those reported for individual halotolerant degraders, such as *Bacillus* spp., which degraded 15 mg L^{-1} of BTEX (including toluene) at 5 % of salinity after 10 days [30] or the haloalkaliphilic strains of *Pseudomonas*: HA10, HA12 and HA14 which degraded 50 mg L^{-1} of toluene under 7 % of NaCl at pH 9 in 12 days [31]. Notably, other mixed microbial cultures have been shown to degrade up to 120 mg L^{-1} of toluene within 5 days under extreme salinity conditions (20 % of NaCl) [32]. However, the available bibliography on this topic remains scarce. Despite the recognized metabolic versatility of halophilic microorganisms, capable of utilizing a broad range of BTEX and producing high-value metabolites, no study to date has systematically evaluated toluene degradation under different salinities and its potential bioconversion into valuable osmolytes, such as ectoine and hydroxyectoine.

In this regard, MC1.5 produced 48.6 ± 17.8 mg g_{TSS}^{-1} of ectoine, with no significant increase observed at 6 % NaCl (48.9 ± 5.5 mg $g_{TSS}^{-1}$of ectoine). In contrast, both MC2.5 and MC2.6 exhibited higher ectoine production and a positive salinity-dependent response, reaching 76.6 ± 5.9 mg g_{TSS}^{-1} and 92.2 ± 2.9 mg g_{TSS}^{-1} of ectoine, respectively (Fig. 2), which demonstrated a considerable difference in culture behavior between MC1 and MC2, despite growth over the same number of cycles and identical toluene concentrations supplied as the carbon and energy source.

Although MC2 exhibited higher ectoine contents per unit of biomass, MC1 achieved superior volumetric ectoine production due to its higher biomass growth. In addition, volumetric ectoine production by MC2 increased at higher salinity levels, a trend not observed in MC1. In both cases, hydroxyectoine was not detected in the assays, likely due to the metabolic cellular state, since it is generally absent in the early exponential phase and increases at the expense of ectoine during late exponential growth. Based on these complementary traits, both consortia were selected for continuous process experiments to assess their overall productivity and identify the most efficient biocatalyst.

3.2. Toluene valorization into ectoines under continuous conditions

Both inocula were tested in continuous bioreactors at 6 % NaCl, since it was the salinity that promoted the highest ectoine specific concentration in batch assays. This allowed to assess the capacity of MC1 and MC2's communities in sustained toluene valorization into ectoine. Although MC2.6 was effective on toluene degradation in batch conditions, it failed to cope with continuous mode in R2. Following inoculation, the initial biomass concentration was 0.1 g L^{-1}, however growth remained limited, stabilizing at 0.3 ± 0.1 g_{TSS} L^{-1} until the end of the experiment. A slight increase in CO_2 production was observed, with an outlet concentration of 1.1 ± 0.1 g m^{-3} and a mineralization rate of 44.9 ± 15.3 %. Despite this, toluene removal remained low at 8.8 ± 6.0 % throughout the assay, with a maximum of 19.6 % reached on day 4. From day 5 onwards, removal rates plateaued and remained roughly stable throughout the 14-day test period with average elimination capacities of 2.3 ± 1.9 g m^{-3} h^{-1}. The limited improvement in removal capacity may be attributed to an insufficient abundance of toluene-degrading communities to sustain continuous abatement. Consequently, community members may have been unable to tolerate the high toluene concentrations, leading to their decline, a loss of metabolic functionality, and eventual cell decay (See Supplementary Materials).

In the case of R1, the initial biomass concentration in the bioreactor after inoculation was 0.2 g L^{-1}. The culture followed a common growth curve, characterized by a prolonged lag phase of more than 5 days, followed by an exponential phase of 4 days (Fig. 3A). Once biomass stabilized, toluene removal efficiency during the stationary phase reached 30.0 ± 1.8 %, corresponding to a toluene elimination of 7.2 ± 1.9 g m^{-3} h^{-1} and a specific toluene consumption of 7.4 ± 3.7 mg g_{TSS}^{-1} h^{-1}. Although higher toluene degradation rates have been widely reported for specialized consortia in dedicated VOC removal systems, salinity can lead to reduced toluene removal efficiency due to high osmotic pressure under energy-limited conditions. For instance, *Bacillus stercoris* exhibited a decrease in toluene removal efficiency from 90 % to 26 % as the salinity increased from 0 % to 5 % at an initial toluene concentration of 400 mg L^{-1} [33].

Under continuous operation, CO_2 production was 15.7 ± 9.6 mg g_{DCW}^{-1} h^{-1} with an outlet concentration of 2.6 ± 0.9 g m^{-3} (Fig. 3B). Therefore, the resulting mineralization rate was 65.0 ± 2.4 %, consistent with typical values reported in literature, which range from 34 % to 91 %, depending on environmental conditions and pollutant concentrations [34]. Regarding biomass growth, the maximum TSS reached 1.8 g L^{-1} by the end of the experiment, with an average TSS of 1.0 ± 0.5 g L^{-1} during the stationary phase.

Ectoine production increased progressively in parallel with biomass accumulation. The maximum ectoine concentration, 26.9 mg L^{-1}, was reached on day 10, while the highest specific ectoine content of 27.3 mg g_{TSS}^{-1} was achieved on day 8 (Fig. 3C). As shown in the graph, hydroxyectoine production increased during the final days of the experiment, concomitant with a decline in ectoine concentration, likely due to its conversion into hydroxyectoine through enzymatic hydroxylation. The highest hydroxyectoine content was observed on day 14 (48.4 mg L^{-1}),

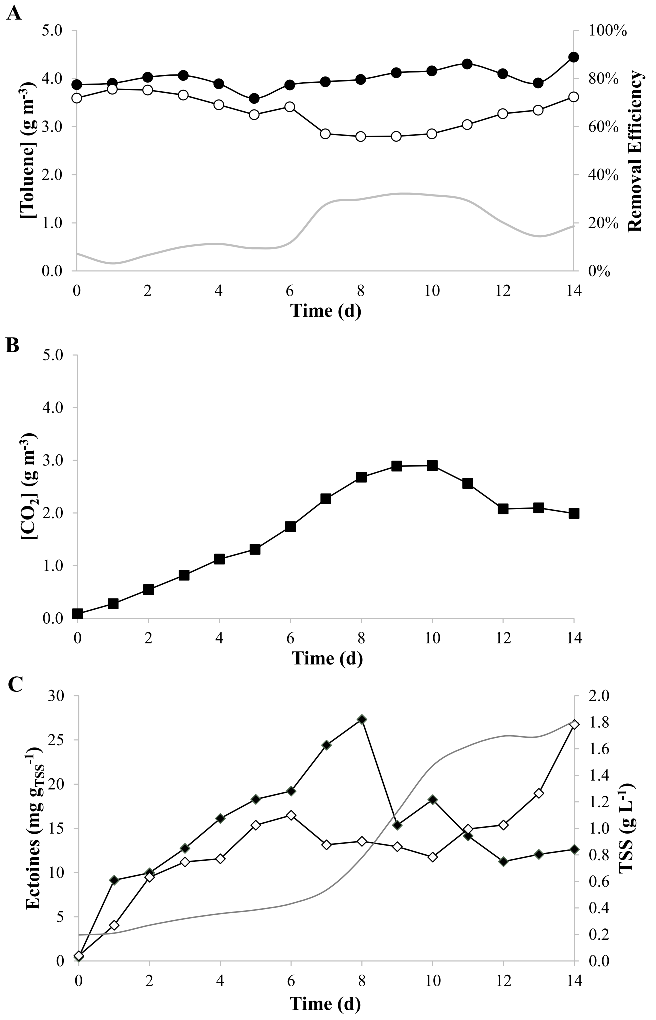

Fig. 3. Operational parameters during the continuous operation of R1. **A)** Inlet (black circles) and outlet (white circles) toluene concentrations and toluene removal efficiency (continuous line); **B)** Outlet CO_2 concentration (black squares); **C)** Specific ectoine (black diamonds) and hydroxyectoine (white diamonds), and TSS concentration (grey continuous line).

with a corresponding specific production of 26.7 mg g_{TSS}^{-1} (Fig. 3C). This phenomenon, in which ectoine and hydroxyectoine are shown to be dependent on the growth phase, was previously demonstrated by Tao et al. [35]. While ectoine predominates during the initial growth phases, the synthesis of hydroxyectoine significantly increases during the late exponential and stationary phases at the expense of ectoine. This is because the expression of the genes *ectA*, *ectB*, and *ectC*, which are responsible for ectoine synthesis, occurs much earlier after an osmotic shock compared to the gene *ectD*, which converts ectoine into hydroxyectoine. In addition, the availability of restricted or suboptimal carbon sources has been shown to promote hydroxyectoine accumulation over ectoine, suggesting that its synthesis is favored under conditions of metabolic or energetic stress [24]. In terms of concentration, close values of ectoine, 20–52 mg g_{DCW}^{-1}, were achieved by Rodero et al. [19] employing a continuous 20 L bubble column bioreactor at 6 % salinity with CH_4 and a methanotrophic bacterial consortium. Moreover, similar values were also recorded for hydroxyectoine, 44.8 mg g_{DCW}^{-1}, when using H_2, CO, and CO_2 as the sole carbon and energy sources, with 3 % NaCl, in *Hydrogenibacillus schlegelii* [24]. However, heterotrophic bacteria, such as *Halomonas salina* DSM 5928, have been reported to produce higher amounts of ectoine, reaching 357.5 mg g^{-1}, growing at 3 % salinity in a 10 L fermenter [36]. Considering the results from this study, although the volumetric productivity of ectoines was relatively low, the specific content achieved is highly promising. This outcome was primarily attributed to the limited toluene mass transfer in the bioreactor, which was insufficient to ensure complete toluene removal. Consequently, the implementation of advanced configurations, such as airlift or Taylor-flow reactors, to enhance carbon mass transfer could significantly improve the yield. In parallel, higher toluene bioavailability would increase the efficiency of toluene and intermediates removal and ensure a contaminant-free final product. In addition, it should be noted that only intracellular ectoine was considered in this study. Furthermore, the subsequent ectoine extraction and purification steps are expected to ensure the complete removal of any trace levels of toluene, thereby guaranteeing a non-toxic final product.

3.3. Microbial community and physiological traits of toluene valorization

Shotgun metagenomic analysis determined that R1´s culture was dominated mainly by species of the genus *Gordonia* (57.7 ± 3.0 %). In fact, *G. polyisoprenivorans* was identified as the main species (22.6 ± 2.6 %). The culture was also composed by other genera, such as *Cellulosimicrobium* (5.8 ± 0.7 %) and *Paenibacillus* (3.9 ± 0.7 %). Among these, *C. cellulans* (2.3 ± 0.3 %) and *P. glucanolyticus* (0.2 ± 0.0 %) were the most representative species. Other minor genera that could have had an impact on the metabolic capabilities of the culture are *Ralstonia*, *Bosea*, *Rhodococcus*, *Achromobacter*, *Microbacterium* or *Pseudomonas* (Fig. 4).

Metabolic analysis of the metagenome revealed the presence of genes encoding enzymes involved in the degradation pathways of toluene and its intermediates, as well as in the metabolic routes for benzoate, catechol, and ectoine synthesis.

The aerobic degradation of toluene can follow four different degradation routes (Fig. 5), depending on the first degradation product obtained, which can be *p*-cresol, *m*-cresol, *o*-cresol or benzyl alcohol.

The main toluene degradation pathway (TOL) requires the expression of genes for toluene monooxygenases (*tmo*, *xylM*, *xylA*), benzyl-alcohol dehydrogenase (*xylB*) and benzaldehyde dehydrogenase (*xylC)*, which degrade toluene to benzoate [37]. In this study, only members belonging to the *Rhodococcus* genus, such as *R. wratislaviensis* or *R. opacus*, were identified with all genes necessary for the TOL pathway. However, *Microbacterium* also displayed the *xylC* gene for benzaldehyde dehydrogenase synthesis and, together with *Paenibacillus glucanolyticus* and *Stenotrophomonas,* the genes required for benzyl-alcohol dehydrogenase, suggesting their potential for the degradation of aromatic compounds such as toluene. Despite being the most abundant genus, *Gordonia* did not carry genes encoding any of the TOL enzymes. Literature evidence for the presence of benzyl alcohol dehydrogenases in these communities has been limited to the genus *Rhodococcus*, specifically to *R. opacus* TKN14 and *R. erythropolis* PR4 [37].

The toluene p-monooxygenation pathway (TMO) requires the expression of the genes encoding toluene monooxygenase (*tmo*), 4-cresol dehydrogenase (*pchC*, *pchF*) and 4-hydroxybenzaldehyde dehydrogenase (*pchA*) (Fig. 5). In this research, *Microbacterium murale*, *Microbacterium* sp., and *Achromobacter denitrificans* were identified with the toluene monooxygenase genes for this pathway. Indeed, toluene 4-monooxygenase genes were already reported in *Microbacterium esteraromaticum* SBS1–7 [38]. However, this could not be confirmed with bibliography related to *Achromobacter*. As in the previous pathway, *Gordonia* was not identified as possessing any of the corresponding enzymes in our analysis, although the sequenced genome of *G. polyisoprenivorans* VH2 harbored a cluster coding for the toluene-4-monooxygenase (*tmoA*, *tmoB* and *tmoC*) of this route [39].

Following the TOL and TMO pathways, the resulting benzoates are further metabolized to CoA intermediates and pyruvate via benzoate degradation pathways. Analysis of the metagenome revealed that the genera *Rhodococcus*, *Ralstonia*, *Stenotrophomonas*, *Bosea* and *Gordonia* possess the metabolic potential to degrade both benzoate and 4-

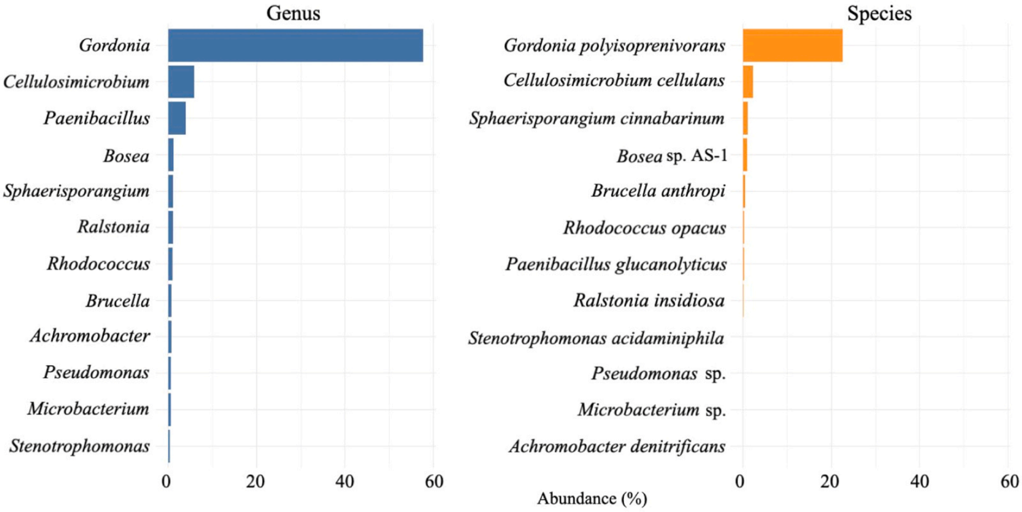

Fig. 4. Relative abundance of the main genera and major identified species of R1.

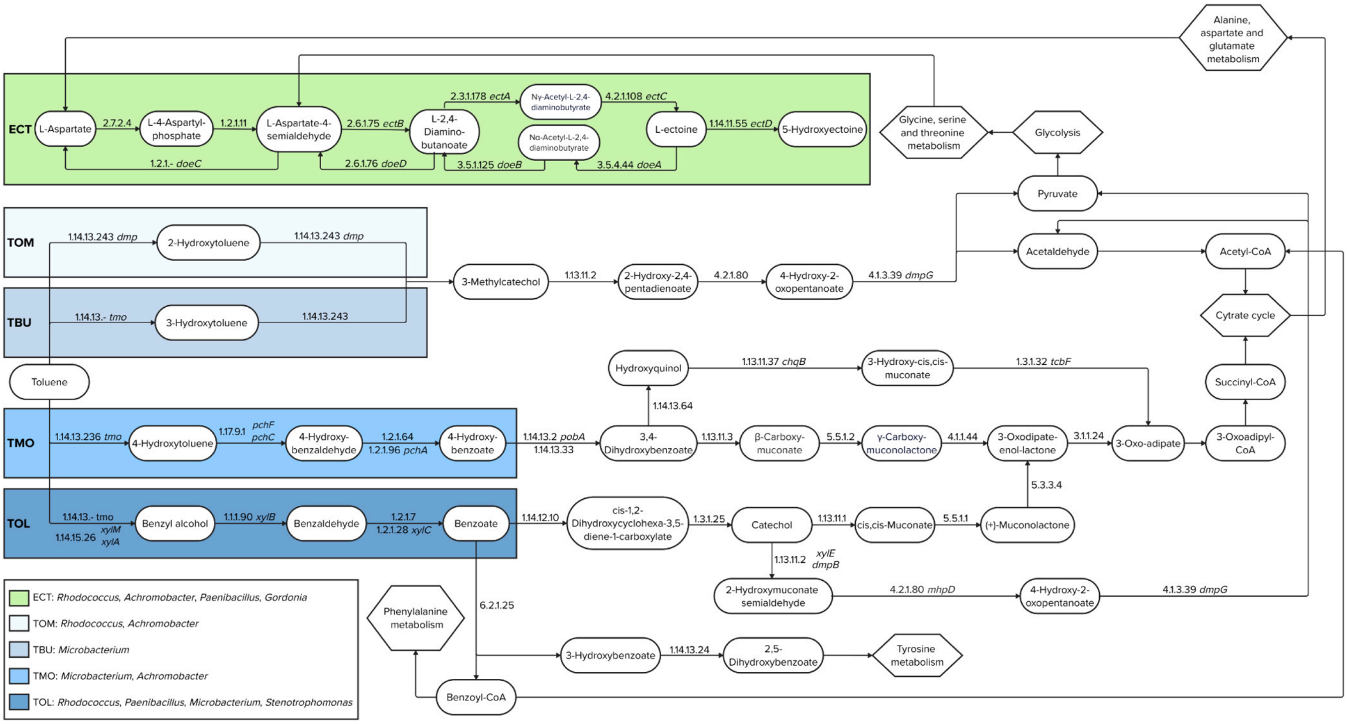

Fig. 5. Diagram of the metabolic pathways detected in MC1. Pathways for BTEXS degradation, conversion to metabolic intermediates and ectoine synthesis are shown: p-toluene monooxygenation (TMO), toluene deoxygenation (TOL), m-toluene monooxygenation (TBU), o-toluene monooxygenation (TOM), and ectoine synthesis and degradation (ECT). The main identified genes include: toluene monooxygenases (*tmo*, *xylM*, *xylA*), benzyl-alcohol dehydrogenase (*xylB*), benzaldehyde dehydrogenase (*xylC)*, 4-cresol dehydrogenase (*pchC*, *pchF*), 4-hydroxybenzaldehyde dehydrogenase (*pchA*), p-hydroxybenzoate 3-monooxygenase (*pobA*), hydroxyquinol 1,2-dioxygenase (*chqB*), maleylacetate reductase (*tcbF*), toluene 2-monooxygenase (*dmp*), catechol 2,3-dioxygenase (*dmpB*, *xylE*), 2-keto-4-pentenoate hydratase (*mhpD*), 4-hydroxy 2-oxovalerate aldolase (*dmpG*), diaminobutyrate-2-oxoglutarate transaminase (*ectB*), L-2,4-diaminobutyric acid acetyltransferase (*ectA*), L-ectoine synthase (*ectC*), ectoine hydroxylase (*ectD*), ectoine hydrolase (*doeA*), N2-acetyl-L-2,4-diaminobutanoate deacetylase (*doeB*), L-2,4-diaminobutyrate transaminase (*doeD*), and aspartate-semialdehyde dehydrogenase (*doeC*).

hydroxybenzoate via distinct central aromatic degradation routes (Fig. 5), as indicated by the presence of p-hydroxybenzoate 3-monooxygenase gene (*pobA*). However, only *Bosea* and *Microbacterium* possessed the complete gene set required for the conversion of hydroxyquinol to 3-oxoadipate, including both hydroxyquinol 1,2-dioxygenase (*chqB*) and maleylacetate reductase (*tcbF*).

The toluene o-monooxygenation pathway (TOM) (Fig. 5) involves the conversion of toluene into 2-hydroxytoluene (*o*-cresol) and subsequently 3-methylcatechol, a process catalyzed by the enzyme toluene 2-monooxygenase (*dmp*). In this study, members of the genus *Rhodococcus,* as well as *Achromobacter denitrificans*, were found to possess the toluene 2-monooxygenase genes required for this pathway. However, aside from reports indicating that *Achromobacter* is often a dominant taxon in biofilters treating BTEX compounds [40], limited additional information on its role in TOM-mediated degradation is available. In parallel, the toluene m-monooxygenation pathway (TBU) converts toluene to 3-methylcatechol via 3-hydroxytoluene (*m*-cresol). Within our metagenomic dataset, only members of the genus *Microbacterium* had the genes coding for toluene monooxygenase (*tmoABCDEF*). Although not identified in this study, members of the genus *Ralstonia*, such as *Ralstonia picketti*, have been well studied for their capacity to degrade BTEX via the TBU pathway [41,42].

Following both the TOM and TBU pathways, the degradation of 3-methylecatechol to acetyl-CoA proceeds through a series of enzymes responsible for aromatic ring cleavage (Fig. 5). Genes encoding one or more of these enzymes, such as catechol 2,3-dioxygenase (*dmpB*, *xylE*), were detected in many of the identified microbial communities, including *Gordonia*, *Rhodococcus*, *Paenibacillus*, *Microbacterium*, *Bosea*, and *Achromobacter*. Notably, in this metagenome, *Gordonia* also harbored genes for 2-keto-4-pentenoate hydratase (*mhpD*) and 4-hydroxy 2-oxovalerate aldolase (*dmpG*), suggesting its potential to utilize intermediates from the degradation of toluene and other aromatic compounds.

The biosynthesis of ectoine is mediated by three core enzymes encoded by the genes *ectA*, *ectB* and *ectC* [15]. Some bacterial species can further convert ectoine to hydroxyectoine via ectoine hydroxylase (*ectD*) [11,43]. In addition, ectoine degradation is controlled by the *doe* gene cluster (*doeA*, *doeB*, *doeC*, *doeD*) which channels ectoine back to L-aspartate. In this research, members of the genera *Achromobacter*, *Paenibacillus* and *Gordonia* carried the *ectC* gene encoding L-ectoine synthase, whereas only *Gordonia* harbored *doeA*, responsible for ectoine hydrolysis. Regarding hydroxyectoine production, *Rhodococcus*, *Achromobacter* and *Paenibacillus* were found to possess the *ectD* gene for ectoine hydroxylase. Although the presence of the ectoine and hydroxyectoine biosynthetic genes were previously confirmed in members of the genera *Achromobacter*, *Rhodococcus and Paenibacillus* [44-46], information on ectoine production in *Gordonia* remains scarce, with only a predicted biosynthetic cluster reported in *Gordonia alkanivorans* strain YC-RL2 [47] and canonical ectoine synthesis identified through transcriptomic analyses associated with stress adaptation in *Gordonia* sp. strain BP1o [48]. In contrast, our study identified the complete ectoine biosynthetic gene cluster (*ectABC*) in this genus.

This analysis confirmed the presence of *Paenibacillus*, *Rhodococcus* and *Microbacterium* in R1 and demonstrated their genomic potential to degrade toluene mainly via the TOL pathway, though they may also participate in toluene degradation through additional alternative metabolic pathways, such as TOM for *Rhodococcus* or TBU and TMO for *Microbacterium*. *Gordonia*, the most abundant genus in the community, was primarily associated with the degradation of intermediate compounds, such as benzoate, muconate or oxoadipate derivatives. This suggests that *Gordonia* members, together with other dominant genera detected, harbor the genetic potential to metabolize toluene-derived intermediates, potentially indicating a synergistic role within the consortium. However, these conclusions are based on qualitative presence/absence of genes for each genus, and future complementary approaches, such as transcriptomics, proteomics or co-culture-based validation, would be necessary to assess the level of contribution and activity of each genus. Regarding ectoines biosynthesis, *Achromobacter*, *Paenibacillus*, *Gordonia* and *Rhodococcus* exhibited the genomic potential for their synthesis. However, hydroxyectoine production could not be confirmed for the *Gordonia* genus. Although a complete characterization of all genes required for the full pathway could not be achieved, as well as some genera lacked detectable genes, altogether the results point to a metabolically diverse community exhibiting functional complementarities. This supports the view that this culture could act as a true microbial consortium capable of degrading toluene and producing ectoines, where metabolic synergies compensate for species-specific limitations. Future transcriptomic analyses would be instrumental in resolving the active metabolic pathways and identifying the populations most actively involved under the studied conditions. To further enhance process performance, the selection of highly productive microbial communities through the gradual increase of salinity and toluene exposure, combined with long-term operation in continuous bioreactors, could lead to higher biomass concentrations and, consequently, increased ectoine recovery.

4. Conclusions

This study introduces an innovative halophilic bioprocess for the continuous conversion of toluene into ectoines using mixed halophilic microbial cultures. These communities displayed efficient, time-modulated production of ectoine and hydroxyectoine, even at low toluene levels (4 g m^{-3}). Metagenomics revealed *Paenibacillus*, *Rhodococcus*, and *Microbacterium* in R1, with potential to degrade toluene mainly via the TOL pathway. *Gordonia*, the dominant genus, was linked to metabolizing intermediates such as benzoate, muconate, or oxoadipate derivatives. Genes for ectoine biosynthesis were found in *Achromobacter*, *Paenibacillus*, *Gordonia*, and *Rhodococcus*, while hydroxyectoine synthesis was confirmed in *Paenibacillus*, *Rhodococcus*, and *Achromobacter*. This metabolically diverse community acts as a true microbial consortium, where synergies overcome species-specific limitations. *Gordonia* is hypothesized to metabolize toluene-derived intermediates and contribute to ectoine synthesis, a potential role for this genus which requires more in depth research. Beyond mechanistic insight, this work demonstrates a circular bioeconomy strategy turning pollutants into high-value products, lowering costs, and advancing sustainable innovation.

CRediT authorship contribution statement

Nicolás Díaz-Moreno: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis. **Raquel Lebrero:** Writing – review & editing, Supervision, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Sara Cantera:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization.

Environmental implications

Volatile organic compounds (VOCs) are major contributors to air pollution, photochemical smog, and adverse health effects. The proposed alternative provides an environmentally sustainable solution for VOC abatement in waste gas streams compared to conventional physicochemical treatments. By promoting biological degradation and potential resource recovery, this approach contributes to cleaner air, circular carbon management, and compliance with increasingly stringent emission standards, supporting the transition toward greener industrial practices.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2026.141045.

Data availability

Data will be made available on request.

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