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Biodegradation of Di-isononyl phthalate by a consortium of saline soil bacteria: Optimisation and kinetic characterisation

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Abstract

Di-isononyl phthalate (DINP) is one of plasticizers most employed in the production of plastic materials, and belongs to the most important environmental contaminants. In this work, a consortium of saline soil bacterial (SSB) capable of degrading DINP is presented. The genera of SSB-consortium were *Serratia* sp., *Methylobacillus* sp., *Achromobacter* sp., *Pseudomonas* sp., *Stenotrophomonas* sp., *Methyloversatilis* sp., *Delftia* sp. and *Brevundimonas* sp. Response surface methodology (RSM) study was employed to optimise and evaluate the culture conditions to improve the biodegradation of DINP. The optimal conditions were a pH 7.0, 31°C and an initial DINP concentration of 500 mg L^{-1}, resulting in almost complete biodegradation (99%) in 168 h. DINP degradation followed a first-order kinetic model, and the half-life was 12.76 h. During the biodegradation of DINP, 4 derived-compounds were identified: monoisononyl phthalate, methyl nonyl phthalate, iso-nonanol, and dimethyl phthalate. The metabolite profiling indicated that DINP was degraded through simultaneous pathways of de-esterification and β-oxidation. Results suggest that the SSB-consortium could be useful for efficient biodegradation of the DINP-contaminated environments.

***Keywords*:** Biodegradation; Consortium; Degradation; Endocrine disruptors; Plasticizers; Phthalates.

Key points

1. DINP degradation is mediated by de-esterification and β-oxidation processes.
2. Temperature and the concentration of the substrate are key factors for DINP biodegradation
3. The SSB-consortium has the ability to biodegrade 99% of DINP (500 mg L^{-1}).

Introduction

Phthalic acid esters (PAEs) are a class of compounds employed as additives and plasticizers in several industrial applications and industrial products to improve their mechanical properties such as plasticity, strength and versatility (Xu et al. 2017; Li et al. 2019). Phthalate production has been reported to have reached 8 million tons worldwide (Jabesa and Ghosh 2016; Zhang et al. 2018). PAEs are referred as endocrine disruptors and their hepatotoxic, teratogenic and carcinogenic effects have been extensively documented (Katsikantami et al. 2016; Tang et al. 2016; Zhao et al. 2016). Thus, the U.S. Environmental Protection Agency and the European Union have listed phthalates as top priority contaminants (Zhang et al. 2018). From PAEs, the Di-isononyl phthalate (DINP) has become one of the most used plasticizers in the plastic industry. DINP is a mix of PAEs with 9-carbon alkyl chains of different lengths and branching distributions (Koch and Angerer 2007). DINP is used to replace di-(2-ethylhexyl) phthalate (DEHP) in several PVC products since it appears to have less toxic effects than DEHP (Hines et al. 2012; Chiang et al. 2020). However, other studies have found similar negative effects on reproduction when comparing DINP to DEHP (Chiang and Flaws 2019; Chiang et al. 2020). Therefore, DINP was classified as priority pollutant and endocrine-disruptor compound as well.

Since phthalates are not chemically bound to plastics, they can be released into different environmental matrices during their production and use (Jin et al. 2013; Zhao et al. 2016). Consequently, phthalates are found in several environmental matrices such as soil, water bodies, sediments, landfill leachates, and even in the food and the atmosphere (Maitra 2016; Feng et al. 2018; Li et al. 2019). Concentrations of up to 500 mg L^{-1} of PAEs have been reported in industrial wastewater (Ahmadi et al. 2017).

Phthalates can be degraded by abiotic processes like photolysis and chemical hydrolysis (Jin et al. 2013; Boll et al. 2020). However, microbial biodegradation is the best-known process for degrading PAEs from the environment. Several bacteria able to degrade phthalates isolated from different environmental matrices have been reported (Benjamin et al. 2015; Wang et al. 2017). They have the ability to use PAEs as only carbon source and energy, acting alone or as consortium (Ren et al. 2018). The most reported genera are:

Acinetobacter sp., *Arthrobacter* sp., *Pseudomonas* sp., *Gordonia* sp., *Agrobacterium* sp., *Bacillus* sp., *Rhodococcus* sp., *Enterobacter* sp., *Burkholderia* sp., *Cyanobacteria*, among others (Babu and Wu 2010; Wu et al. 2010; Wang et al. 2012; Jin et al. 2013; Jin et al. 2015a; Liu et al. 2020). Existing evidence indicates that degradation and mineralization is faster using mixed cultures rather than monoaxenic cultures (He et al. 2013), because complete mineralization of PAEs involves a broad diversity of enzymes and cellular activities. In the environment, the complete degradation of phthalates is mainly carried out by the metabolic synergy of several microorganisms (Wu et al. 2010; Gao and Wen 2016).

In this study, a consortium of saline soil bacterial (SSB) was designed from bacterial isolates that were recognized as having the potential to use PAEs as only carbon source. The optimal culture conditions such as temperature, pH, and initial DINP concentration for DINP degradation by the SSB-consortium were evaluated using the response surface methodology. Also, the kinetic characterization and degradation pathways of DINP were proposed from the intermediates detected by gas chromatography/mass spectrometry (GC/MS).

Materials and Methods

Chemicals

DINP was acquired from Sigma-Aldrich (USA) with purity >99%. The stock of DINP (10 g L^{-1}) and Tween® 20 (10 g L^{-1}) were prepared in methanol and water, respectively, and kept at 4°C. Methanol and dichloromethane were HPLC grade (Sigma-Aldrich, Inc.). Chemicals used to prepare the culture media were analytical reagent grade and purchased from Sigma-Aldrich, Inc.

Bacterial collection and formulation of DINP-degrading SSB-consortium

21 bacterial isolates were obtained from soil samples from a Saltworks site (Zancarrón community, Santo Domingo municipality, San Luis Potosi. Mexico. 23° 23´40", 102° 11´46.5").

The identification of DINP tolerant isolates was evaluated using the spot-plate screening technique. DINP tolerance test was performed in solid mineral salt medium (MSM) plus DINP at 0, 500 or 1000 mg L^{-1}. The MSM contained per litter: $CaCl_2$ 0.02 g, $(NH_4)_2SO_4$ 2.0 g, $MgSO_4$ 0.16 g, KH_2PO_4 4.5 g, K_2HPO_4 5.85, 1 mL of trace elements solution ($MgSO_4$ 0.010 g, $Na_2MoO_4 \cdot 2H_2O$ 0.00036 g, Na_2SeO_3 0.0002 g, $NiCl_2 \cdot 6H_2O$ 0.00034 g, $FeCl_3 \cdot 4H_2O$ 0.015 g, $CoCl_2 \cdot 6H_2O$ 0.0007 g, $CuCl_2 \cdot 2H_2O$ 0.0002 g, $ZnCl_2$ 0.023 g, $MnCl_2 \cdot 4H_2O$ 0.030 g) and agar 15 g. The medium was supplemented with yeast extract (0.3%) as a co-substrate to stimulate the bacterial growth. Two microliters of each bacterial isolate were spot-cultivated onto MSM agar plate and incubated at 28°C for 72 hours. Bacteria able to grow at 1000 mg L^{-1} of DINP were chosen as DINP-tolerant isolates and afterwards employed to formulate the SSB-consortium for further experiments.

Enrichment culture of the SSB-consortium

The enrichment culture technique for DINP degradation was similar to that described by Wu et al. (2010). Initially, the enrichment culture of the SSB-consortium was performed in a 50 mL Erlenmeyer flask with 25 mL of MSM plus 100 mg L^{-1} DINP. Then, the culture was serially transferred to fresh medium supplemented with 100, 250, 500, 750 or 1000 mg L^{-1} of DINP as only carbon substrate. Tween® 20 (500 mg L^{-1}) was added to improve the solubility of DINP in the medium (Jin et al. 2015a; Xu et al. 2017). All transfer cultures were initiated at an optical density at 595 nm (OD_{595}) of 0.1 and incubated for 5 days at 31°C and 180 rpm. The culture that grew and tolerated the highest concentration of DINP was selected as the final enrichment, and used for the subsequent biodegradation experiments. The SSB-consortium is available upon request to the corresponding author.

Taxonomic identification of the SSB-consortium (16S Illumina sequencing)

The community analysis of the SSB-consortium was conducted through a 16S microbiota study. The total genomic DNA was extracted with Wizard®Genomic DNA Purification Kit. The V3 hypervariable region of the 16S rRNA gene was amplified by PCR using the V3-388F and V3-533R primers (Huse et al. 2008) and Illumina adapters in the MiniSeq System (Illumina, Inc.) under standard conditions (300 cycles, 2x150 pair-

end). The DNA extraction, amplification by PCR and data processing were carried out at the Microbial Genomics Laboratory (CIAD- Unit for Aquaculture, Mazatlán, Sinaloa, Mexico) (García-López et al. 2020). The sequencing data have been deposited in NCBI BioProject PRJNA691434 with the SRA submission accession SRR13415824 **(Table 1)**.

Optimisation of DINP biodegradation conditions

RSM analysis based on a Central Composite Design (CCD), was used to optimise the temperature, pH and initial concentration of DINP to improve the percentage of degradation of DINP by the SSB-consortium. The values of the independent variables are shown in **Table 2**.

A quadratic polynomial model **(Eq. 1)** was suggested to explain the effects of independent variables on the dependent variable based on the data obtained in the experimental design.

$$Y_i = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ii} X_i^2 + \Sigma\beta_{ij} X_i X_j \quad \textbf{(1)}$$

Where Y_i is the corresponding response, β_0 is the intercept term, β_i is the linear coefficient, β_{ii} is the squared coefficient and β_{ij} is the interaction coefficient, whereas X_i and X_j are the variables (Zhao et al. 2018).

The experimental design and analysis of variance (ANOVA) was employed to determine the interaction between independent variables (temperature, pH and DINP concentration) and the dependent variable. The statistical analysis and response surface analysis were performed with Design-Expert® Software (Minneapolis, MN).

Biodegradation kinetics of DINP by the SSB-consortium

The SSB-consortium was inoculated in MSM plus 500 mg L^{-1} DINP at 31°C and 180 rpm for 72 h. Subsequently, the bacteria were collected and washed two times with PBS 0.1 mM (pH 7.0) and resuspended in MSM. Cell suspension with an OD_{595} of 0.1 was employed to inoculate the medium for degradation assays.

Biodegradation kinetics were carried out in 200 mL MSM plus 500 mg L^{-1} DINP as the only carbon source under the optimum culture conditions for 168 h. Tween® 20 (500 mg L^{-1}) was added to enhance the solubility of DINP (Jin et al. 2015a; González et al. 2020). Non-inoculated culture was used as control. Samples were collected every 24 h to quantify the residual DINP and cell growth was followed as optical density at OD_{595}. Residual DINP in the samples was extracted as previously reported by Zhang et al.,(2018) and measured by GC/MS. Experiments were carried out in triplicates.

The biodegradation of DINP (%) was determined from the difference in concentration in the medium according to **Eq. 2**:

$$DINP\ Biodegradation\ (\%) = (C_0 - C_t)/C_0 * 100 \quad \textbf{(2)}$$

Where C_0 is the initial concentration of DINP in the medium, C_t is the final concentration of DINP at time interval t (Zhao et al. 2018).

To evaluate the influence of the initial concentration of DINP on its degradation kinetics, differents concentrations (250, 500, 750 mg L^{-1}) were evaluated and fixed to a first-order kinetic model **Eq. 3**:

$$Ln\ C = -kt + A \quad \textbf{(3)}$$

Where C is the concentration of DINP (mg L^{-1}), *k* is the first-order constant (h^{-1}), t is the time (h), and A is a constant (Zhang et al. 2018; Zhao et al. 2018).

Then the half-life time ($t_{1/2}$) of the DINP biodegradation was evaluated according the following **Eq. 4:**

$$t_{1/2} = Ln\ 2/k \quad (4)$$

Analytical methods

Optical density at 595 nm (OD_{595}) was measured with a microplate reader (iMark™, *Bio-Rad* Laboratories, Inc.). For residual DINP quantification and identification of its metabolites, 10 mL of culture were collected from the culture media daily during 168 h of incubation. Then, compounds were extracted with dichloromethane from the samples as reported by Zhang et al., (2018). The organic phase was collected and evaporated to dryness and resuspended in 1 mL of methanol. DINP quantification and metabolite identification were performed by GC/MS 7820A/5977E (Agilent Technologies, Inc.) using a HP-5MS column (30 m x 250 µm x 0.25 µm) with helium as carrier gas at a flow rate of 1.0 mL min^{-1}. The chromatographic conditions reported by González et al. 2020 were used. The identification of the metabolites was done by comparing their mass spectra to those obtained in the NIST 14 library of the MS database (Tang et al. 2016).

Results

Identification and characterisation of the SSB-consortium.

Twenty-one bacteria from a collection of saltwork site-isolates were cultured in MSM-agar plates at high concentrations of DINP (500-1000 mg L^{-1}) using yeast extract as co-substrate. From those, 18 bacteria grew in 500 mg L^{-1} DINP, and only 11 grew with 1000 mg L^{-1} DINP. For the design of the SSB-consortium, only the microorganisms that grew on and tolerated 1000 mg L^{-1} DINP were selected. When DINP was the only

source of carbon in the culture medium of the enrichment assay, the bacterial growth capacity and tolerance decreased. Previous reports have indicated that the use of co-substrates, such as yeast extract, in the culture medium could promote and enhance the cell growth and phthalate degradation (Li et al. 2019). After several transfers of the enrichment culture using fresh medium, a consortium capable of growing up to 500 mg L^{-1} of DINP was obtained and used for further DINP biodegradation experiments.

Bacteria present in the SSB-consortium were taxonomically identified through microbiome 16S rRNA sequencing **(Fig. 1)**. The results indicated that the bacterial community of the SSB-consortium is composed by seven families, the main families were *Enterobacteriaceae* (60.91%), *Methylophilaceae* (28.23%), *Burkholderiaceae* (7.12%), *Pseudomonadaceae* (3.11%), representing 99.37% of the reads. The families *Xanthomonadaceae* (0.45%), *Rhodocyclaceae* (0.11%), and *Caulobacteraceae* (0.07%), were also found but at relatively low abundance. The analysis at genus level revealed that the SSB-consortium is composed by 8 bacterial genera, based on their relative abundance the most predominant genera were *Serratia* sp. (60.91%), *Methylobacillus* sp. (28.23%), *Achromobacter* sp. (7.05%) and *Pseudomonas* sp. (3.11%). The relative abundance of *Stenotrophomonas* sp., *Methyloversatilis* sp., *Delftia* sp. and *Brevundimonas* sp. were 0.45%, 0.11%, 0.07%, 0.07%, respectively.

Optimisation of operational conditions for DINP biodegradation

Based on a Central Composite Design a RSM analysis was used to assess and optimise the influence of the cultivation variables: temperature (X_1), pH (X_2) and initial DINP concentration (X_3) on the DINP biodegradation. The results of the % of DINP degradation obtained by the CCD are shown in **Table 3**. From the regression analysis of the experimental data a quadratic polynomial model was obtained to describe the degradation of DINP **(Eq. 5)**.

$$Y_{DINP} = 97.44 - 11.71X_1 + 2.33X_2 - 23.48X_3 - 4.26X_1X_2 + 19.13X_1X_3 - 4.26X_2X_3 - 31.94X_1^2 - 32.94X_2^2 - 15.82X_3^2 \quad \textbf{(5)}$$

Where Y is the predicted % biodegradation of DINP by the SSB-consortium, X_1, X_2, X_3 are the coded variables that correspond to temperature, pH and initial DINP concentration, respectively.

Table 4 shows the ANOVA analysis performed to assess the importance of the adjusted model together with the effects of the variables with their linear, quadratic and interactive terms associated with the degradation of DINP. Moreover, to verify the suitability of the model and the statistical significance of the terms, a *p*-value <0.05, was used. The model *p*-value of 0.0006 indicated that the model was statistically significant (Cisneros et al. 2017). The accuracy and reliability of the model were assessed according to the determination coefficient (R^2= 0.9567), indicating that the DINP biodegradation model could explain the 95.67% variability of the dependent variable, and the good fit between the model and the experimental biodegradation data, indicating that the treatment was highly significant.

The percentage of biodegradation in the response surface graphs and contour graphs **(Fig. 2)** directly showed the influence of the initial concentration of DINP, temperature and pH. The results showed a degradation range from 0.13 to 97.88% **(Table 3)**. The regression analysis indicated that linear and quadratic terms of temperature (X_1) and initial DINP concentration (X_3) had significant impact ($p < 0.05$) in the degradation of DINP, while the linear term of pH (X_2) and their interaction terms (X_1X_2 and X_2X_3) had no significant effects ($p > 0.05$) as shown in **Table 4**, indicating that the pH (in the evaluated range) was not a key factor in the biodegradation of DINP. The maximum theoretical DINP degradation was 97.52% and the optimal culture conditions were pH 7.0, temperature of 31°C, and the initial concentration of 500 mg L^{-1} DINP **(Table 3)**.

A final set of DINP biodegradation experiments using the predicted optimal culture conditions was implemented. DINP degradation was 98.88%, which was similar to that predicted by the model, demonstrating the suitability of the obtained model to optimise the conditions of DINP biodegradation. Maximum % of DINP biodegradation was reached after 168 h of cultivation.

The effect of the variables pH, temperature and initial DINP concentration on the % of degradation was evaluated using ANOVA (**Table 4**). Temperature had a significant effect (p <0.0232) and it was a key factor in the DINP degradation. The surface and contour plots **(Fig. 2 a-d)** show the interaction of temperature with pH and the initial concentration of DINP, indicating that temperature had an important role in the biodegradation of DINP. At 25°C, 20-30% of DINP degradation were observed, whereas at 37°C there was no degradation. The pH in the range of 6.0-8.0 was not statistically significant (p >0.5829), indicating that it did not play an important role in DINP biodegradation, thus a pH 7.0 was chose as the optimal condition. On the other hand, the initial concentration of DINP showed to be statistically significant (p <0.0007), the results showed that the SSB-consortium had the capacity to degrade up to 500 mg L^{-1} of DINP, whereas higher concentrations inhibited the growth of the consortium.

Biodegradation kinetics of DINP

To determine the kinetic parameters of DINP biodegradation by the SSB-consortium, a set of cultures were carried out in MSM under optimal culture conditions of 31°C, pH 7, and 500 mg L^{-1} DINP (**Fig. 3**). Optical density increased from 0.1 to 1.73 ±0.06 in the presence of DINP in 144 h, while no increase was observed in the control cultures without the DINP **(Fig. 3a)**. As observed in **Fig. 3b,** 99% of the DINP was degraded in 144 h in the inoculated cultures**,** whereas no change of DINP concentration was observed in the cell-free cultures. These results indicated that the SSB-consortium used DINP as the only source of carbon and energy, to confirm this asseveration, additional experiments were carried out by inoculating the SSB-consortium in MSM without DINP but containing Tween 20, and no growth was observed after 168 h of cultivation. Tween 20 did not show toxicity at the used concentration (500 mg L^{-1}), on the other hand it helped in the assimilation of the DINP by its emulsifying property, as previously reported (Jin et al. 2015a; Xu et al. 2017).

To evaluate the biodegradation rate of DINP by the SSB-consortium, DINP was used at different initial concentrations (250, 500 and 750 mg L^{-1}) in MSM. As shown in **Fig. 4,** DINP biodegradation followed a first-

order kinetics and the parameters are summarized in **Table 5**. In the cultures with the initial concentration of 750 mg L^{-1} DINP, no degradation was observed, indicating an inhibitory effect. DINP was rapidly biodegraded when the initial concentration was 250 mg L^{-1} compared to 500 mg L^{-1} **(Fig. 4)**. First-order degradation constants (*k*) were 0.0629 h^{-1} and 0.0543 h^{-1}, whereas the half-life times ($t_{1/2}$) were 11.01 and 12.76 h for 250 and 500 mg L^{-1}, respectively. The $t_{1/2}$ corresponds to the time it takes to decrease the DINP concentration in half (Benjamin et al. 2015; Zhao et al. 2018).

Identification of DINP degradation intermediates and metabolic pathways

To determine the biodegradation pathways of DINP by the SSB-consortium, their intermediates were identified by GC/MS. A total of four intermediates were detected **(Fig. 5** and **Table 6)**. At the beginning of the cultivation only DINP (retention time, RT: 9.6 – 10.6 min) was detected **(Fig. 5a)**. Throughout the time of cultivation, DINP decreased and at the same time, 4 new compounds were detected at the 72 h of culture (**Fig. 5b)**. These compounds were monoisononyl phthalate (MiNP; RT: 8.0 - 8.5 min), methyl nonyl phthalate (MNP; RT: 8.11 min), iso-nonanol (INA; RT: 3.8 - 4.5 min), and dimethyl phthalate (DMP; RT: 5.68 min). Where MiNP, MNP and INA were the most abundant. As observed in **Fig. 5c** the relative abundance of metabolites continued gradually changing and DINP was almost completely degraded at 120 h. MiNP and MNP decreased and the peaks of DMP and INA increased, showing the SSB-consortium was able to transform DINP and its metabolites into more simple compounds. At the end of the culture (168 h) no peaks of DINP, MiNP, MNP were detected, indicating that they were completely degraded, and only traces of Iso-nonanol and DMP remained (**Fig. 5d**). No intermediates were detected in the control samples (without the inoculum) and only DINP was detected.

Based on the metabolites detected by GC/MS, β-oxidation also occurred as a simultaneous pathway in the first stage of the biodegradation. DINP was transformed into MiNP and INA by de-esterification, whereas MNP and DMP were produced by β-oxidation. Then, the products were degraded into phthalic acid (PA). Since PA was not observed, it could indicate that it was quickly incorporated and metabolized into the central

carbon pathways to CO_2 and H_2O (**Fig. 6**). Li et al., 2018 reported a similar effect during the biodegradation of DEHP with the consortium LF, phthalic acid was not detected.

Discussion

In this study, a SSB-consortium formulated with bacterial isolates from a saltworks site, was able to degrade DINP and use it as the only carbon and energy source. SSB-consortium was mainly constituted by four bacterial genera *Serratia* sp., *Methylobacillus* sp., *Achromobacter* sp. and *Pseudomonas* sp. Nowadays, there are many reports on the ability of *Achromobacter* sp. and *Pseudomonas* sp. to degrade organic contaminants, including phthalate esters, bisphenol A, phenols, and other aromatic compounds (Xu et al. 2005; Benjamin et al. 2015; Jin et al. 2015b; Liu et al. 2015; Li et al. 2018). However, there are few reports on degradation of phthalates by the genera *Serratia* sp. (Chuang Li 2012) and *Methylobacillus* sp. (Maitra 2016), which represent the higher percentage of relative abundance in the consortium. Previous studies indicate that the use of bacterial consortia is more suitable for bioremediation than pure bacterial strains (Wang et al. 2017; Li et al. 2018), indicating that the SSB-consortium is an ideal candidate for bioremediation of sites contaminated by phthalate esters.

Results showed that temperature and initial DINP concentration play a significant role on the degradation process, while pH (range evaluated) was not a key factor in the biodegradation of DINP. Several studies noted a similar statistical effect, indicating that bacteria may be able to adapt and degrade over wide pH ranges (Xu et al. 2005; Li et al. 2019). The biodegradation results obtained by the SSB-consortium are promising, so far, there is only one report of DINP biodegradation by Park et al., (2008). They reported 100% degradation of 500 mg L^{-1} DINP in 10 days by *Sphingobium chungbukense*, while the consortium used here, attained total degradation in less than 7 days. Optimal conditions reported were similar in both studies (pH 7.0), and temperature of 30 and 31°C, respectively. These results suggest that the use of bacterial consortia is more efficient for the DINP degradation.

The results for DINP biodegradation kinetic indicated the SSB-consortium used DINP as the sole source of carbon and energy and followed a first-order kinetics. The ability of the SSB-consortium was outstanding, to biodegrade DINP (500 mg L^{-1}), resulting in an almost complete elimination (99%) in 168 h with a *k* of 0.0543 h^{-1}, whereas the half-life time ($t_{1/2}$) was 12.76 h. These results are comparable with previous studies reporting the degradation of phthalates with long and short alkyl-chains. For instance, Li et al., (2018) found that the LF consortium could degrade 500 mg L^{-1} DEHP completely with a *k* of 0.0404 h^{-1}, while the SSB-consortium degraded the same amount of DINP with a higher degradation constant (0.0543 h^{-1}), suggesting that the SSB-consortium is more efficient than consortium LF. Zhang et al., (2018) described the completely degradation of 500 mg L^{-1} DEHP by *Bacillus mojavensis* B1811 with a *k* of 0.0149 h^{-1}. Lu et al., (2009) reported a degradation constant of 0.0062 h^{-1} and $t_{1/2}$ of 4.63 days for a mixture of PAEs (di-n-butyl phthalate, diethyl phthalate and dimethyl phthalate) at a concentration of 600 mg L^{-1} using a *Rhodococcus* sp. L4. Feng et al., (2002) described the degradation of DEHP by *Pseudomonas flourescens* FS1, where they found that the *k* was 0.0408 h^{-1}. These results showed that the SSB-consortium could biodegrade DINP more efficiently compared to the other systems, being a viable alternative for bioremediation of phthalates with long alkyl-chains such as DINP.

The presence of MiNP and iso-nonanol suggests that de-esterification was carried out, whereas the presence of MNP indicates that the biodegradation of DINP also occurred through β-oxidation. One of the main steps in the degradation of phthalates involves the hydrolysis of the ester bonds through esterases, resulting in generation of the monoesters that are subsequently hydrolysed to phthalic acid (PA) and alcohols (Liang et al. 2008; Zhang et al. 2018; Li et al. 2019). Both pathways (β-oxidation and de-esterification) in the biodegradation of long alkyl-chains phthalates have been described previously (Benjamin et al. 2015; Ren et al. 2018; Li et al. 2019; Bai et al. 2020). To our knowledge, only one de-esterification had been proposed for the biodegradation of DINP by *Sphingobium chungbukense* (Park et al. 2008), whereas in this work, DINP degradation by the SSB-consortium was performed by de-esterification and β-oxidation simultaneously. This may be due to the use of bacterial consortia, previous studies indicated that the use of consortiums improve the degradation efficiency of environmental pollutants (He et al. 2013; Wang et al. 2017; Li et al. 2018).

Thus, SSB-consortium degraded the DINP faster than that reported using a single bacterium, therefore the SSB-consortium is a potential candidate to bioremediate effluents contaminated by DINP.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions MAPC and ALR planned the work. MAPC performed the experiments work. VEBH provided technical help during the experiments. MAPC, VEBH and ALR drafted the manuscript. ALR supervised the whole experiments. All authors reviewed and accepted the final version of the manuscript.

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Tables

Table 1. Bacterial diversity of the SSB-consortium sample for NCBI BioProject PRJNA691434

BioSample	Bacteria	Relative Abundance (%)	# Spots	% GC content	NCBI SRA accession
SSB-consortium ID: SAMN17293086	*Serratia* spp.	60.91%	14,553	52.68	SRR13415824
	Methylobacillus sp.	28.23%			
	Achromobacter sp.	7.05%			
	Pseudomonas sp.	3.11%			
	Stenotrophomonas sp.	0.45%			
	Methyloversatilis sp.	0.11%			
	Delftia sp.	0.07%			
	Brevundimonas sp.	0.07%			

Table 2. Levels of independent variables used in the CCD for the optimization of DINP degradation.

Independent variables (units)	Symbol	Code levels of variables				
		-α	**-1**	**0**	**1**	**+α**
Temperature (°C)	X_1	21	25	31	37	41
pH	X_2	5.3	6	7	8	8.6
DINP concentration (mg L^{-1})	X_3	79.5	250	500	750	920.4

Table 3. Matrix of the Composite Central Design and the response: % degradation of DINP.

Run	Levels of independent variables			Response
	Temperature (°C)	pH	Concentration of DINP (mg L^{-1})	DINP degradation (%)
1	25	6	250	62.30
2	37	8	250	2.00
3	25	6	750	0.67
4	31	7	500	97.60
5	37	6	250	0.80
6	25	8	250	95.28
7	31	7	500	97.30
8	37	6	750	0.93
9	25	8	750	0.67
10	37	8	750	0.13
6	31	7	79.55	97.11
12	31	7	920.45	0.92
13	41.1	7	500	2.40
14	31	8.68	500	0.40
15	20.9	7	500	6.20
16	31	7	500	97.88
17	31	5.32	500	0.80
18	31	7	500	97.30

Table 4. ANOVA of the % of DINP degradation obtained by Central Composite Design.

Source	Sum of Squares	Df	Mean Square	F Value	*p*-value
Block	18.10	1	18.10		
Model	34549.43	9	3838.83	17.17	0.0006
X_1-Temperature	1873.36	1	1873.36	8.38	0.0232
X_2-pH	74.08	1	74.08	0.33	0.5829
X_3-DINP Concentration	7528.64	1	7528.64	33.68	0.0007
X_1X_2	145.18	1	145.18	0.65	0.4468
X_1X_3	2926.13	1	2926.13	13.09	0.0085
X_2X_3	145.18	1	145.18	0.65	0.4468
X_1^2	12574.78	1	12574.78	56.25	0.0001
X_2^2	13636.53	1	13636.53	61.00	0.0001
X_3^2	3145.43	1	3145.43	14.07	0.0072
Residual	1564.82	7	223.55		
Lack of Fit	1564.61	5	312.92	2935.48	0.0003
Pure Error	0.210	2	0.110		
Cor Total	36132.35	17			

Table 5. Kinetic equations of DINP biodegradation at different initial DINP concentrations.

Initial Concentration (mg L^{-1})	Kinetic equations	$t_{1/2}$ (h)	R^2
250	Ln C= -0.0629t + 7.6241	11.01	0.997
500	Ln C= -0.0543t + 10.9140	12.76	0.987
750	-	-	-

Table 6. Identification of DINP biodegradation metabolites by GC/MS*.

Metabolites	Molecular ion (*m/z*)	Retention Time RT (min)
Diisononyl phthalate	149, 280.9, 135	9.6 -10.6
Monoisononyl phthalate	149, 163, 150	8.0 - 8.5
Methyl nonyl phthalate	163 , 149 , 181	8.118
Iso-nonanol	56, 43, 70	3.8 - 4.5
Dimethyl phthalate	163, 77, 92	5.692

*GC Column HP-5MS (30 m x 250 µm x 0.25 µm).

Figure captions

Fig. 1 Identification of the bacterial diversity of the SSB-consortium. (a) at family level; (b) at genus level.

Fig. 2 Contour and 3D response surface plots showing the influence of pH, temperature, and initial DINP concentration on the % of DINP biodegradation by the SSB-consortium.

Fig. 3 Growth of the SSB-consortium and DINP biodegradation kinetics started at 500 mg L^{-1} DINP: a) Evaluation of cell growth in MSM with or without DINP as the only carbon source, and b) degradation of DINP by the SSB-consortium under optimal conditions (31°C, pH 7 and 500 mg L^{-1}) in MSM. Non-inoculated MSM was employed as control (black squares). Error bars indicate the standard error of the three replicates.

Fig. 4 Biodegradation of DINP at different initial concentrations performed by the SSB-consortium in MSM at 31°C and pH 7. DINP concentrations used were: ▲; 250 mg L^{-1}, ●; 500 mg L^{-1} and ○; 750 mg L^{-1}.

Fig. 5 GC/MS chromatograms of DINP biodegradation by the SSB-consortium: a) 0 h, b) 72 h, c) 120 h, and d) 168 h of bacterial growth.

Fig. 6 Proposed pathways for DINP degradation by the SSB-consortium. The dotted box indicates that the metabolite was not detected in this study.