Screening And Genome Sequencing of a di-*n*-butyl Phthalate Degrading Bacterium J2 Isolated From Peanut Field Soil

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Abstract

Di-*n*-butyl phthalate (DBP) is commonly used plasticizers in agricultural plastic films, and is a priority pollutant due to its toxicity to human health. A newly isolated strain J2, which used DBP as its sole carbon source, was screened from peanut filed soil by continuous enrichment cultivation. Based on morphological, physiological characteristics and 16S rRNA gene sequence analysis (GenBank accession No. OK598965), it was identified as *Priestia* sp. J2. The research results revealed the optimal conditions for DBP degradation as 35 °C and pH 8.0. The strain could effectively degrade 97.6% DBP within 5 days. Substrate tests showed that strain J2 could utilize shorter side-chained PAEs, but could not utilize long-chained PAEs. The whole genome comprises a complete chromosome of 5,067,299 bp and four plasmids of 147,924 bp, 75,940 bp, 11,604 bp, 11,333 bp (GenBank accession No. CP086208-CP086212). This genome harbors 5,585 predicted protein-encoding genes, 130 tRNA genes, and 42 rRNA genes. Gene annotation analyses showed a DBP-degrading gene contained an open reading frame of 930 bp, and the enzyme was named Est-J2-1. The amino acid sequence of the Est-J2-1 exhibited no significant homology with those of reported DBP-degrading enzymes, suggesting the enzyme is a novel enzyme. The gene of Est-J2-1 was found to be located on the chromosome. This study provided strain resource for DBP removal from farmland and other environments.

Introduction

Plastic films can maintain soil moisture, regulate soil temperature and restrict weed growth to promote crop yield, which play an indispensable role in modern agricultural production (Sun et al. 2020). Phthalate acid esters (PAEs) are widely added as plasticizers in the production process of plastic films (Zhang et al. 2021). PAEs are easy to accumulate and migrate in soil and water environment (Vikelsøe et al. 2002; Le et al. 2021). As environmental hormones, they can affect human reproduction and induce genetic aberrations (Surhio et al. 2017). Some PAEs, such as di-*n*-butyl phthalate (DBP), diethyl phthalate (DEP), Dioctyl Phthalate (DOP), have been classified as priority pollutants in USA, European Union and China. DBP has been widely used in agricultural plastic films, mainly as plasticizer. Because DBP is only physically bound to plastic structures, it tends to escape into soil, water and other environments. Therefore, DBP is be detected in various soil, groundwater, and other environmental samples. In addition, DBP can be absorbed by crops into the food supply, threatening human and animal health (Wang et al. 2016).

DBP removal methods include hydrolysis, photo degradation, and biodegradation. However, because of the inefficiency of the first two methods, biodegradation plays a major role in the DBP degradation. Biodegradation is considered a promising and environmentally friendly method to eliminate DBP. Many microorganisms capable of degrading DBP were be screened, including *Rhodococcus ruber* (Li et al. 2006), *Pseudomonas aeruginosa* (Tang et al. 2021), *Bacillus subtilis* (Xu et al. 2020), *Arthrobacter* sp. (Liu et al. 2020), *Pleurotus ostreatus* (Ahuactzin-Pérez et al. 2018). Only a few identified enzymes involved DBP degradation have been reported. A phthalate esters hydrolase DphB was identified through a metagenomic library isolated from wastewater, which could degrade DBP (Ding et al. 2015). A DBP-

degradation enzyme was found from *Sulfobacillus acidophilus* DSM10332, named EstS1t (Zhang et al. 2014). DBP hydrolase was found in *Acinetobacter* sp. M673 (Wu et al. 2013).

In this study, a DBP-degrading strain J2 from peanut field soil was screened and identified. Strain J2 could utilize DBP to grow, with high degradation activity. We investigated the DBP degradation characteristics of strain J2, and further sequence the genome to study the DBP-degrading genes.

Materials And Methods

Reagents, samples and cultivation medium

DBP, DEP, DOP, dimethyl phthalate (DMP), diisooctyl phthalate (DIOP) and diisononyl phthalate (DINP) were purchased from Sigma-Aldrich (USA). The methanol was HPLC grade from Merck. The rest of chemical reagents were analytical grade, obtained from China National Medical Medicine Group. The soil samples of peanut field were collected from Laixi Experimental Station of Shandong Peanut Research Institute in Qingdao, Shandong Province, China. All samples were stored at 4 °C before analysis. The mineral salt medium (MSM) was prepared as previously described (Wu et al. 2011). DBP was added, and the solution was sterilized at 120 °C for 15 min.

Enrichment, screening and identification of DBP-degrading bacteria

DBP-degrading bacteria were screened from peanut field soil by continuous enrichment cultivation. 1 gram soil sample was inoculated to 200 ml of solution with 200 mg/L DBP. After 7 days, 1000 µl of the culture added to 200 ml solution with 1000 mg /L DBP. After 7 days, 100 µL samples were spread onto MSM agar with DBP under sterile conditions, and then the plates were incubated at 28 °C for 5-10 days until colonies appeared. Single colony was isolated and subsequently transferred to MSM agar for three times, and the pure culture was named J2.

The morphology of strain J2 was observed on LB agar plates after 16 h at 28 °C. The hydrolysis of casein, starch, gelatin, Tween 40, Tween 80 and gelatin was studied as described in previous reports (Romanenko et al. 2013). The strain J2 was identified by 16S rRNA gene sequencing. 27F and 1492R primers were used for PCR amplification (Frank et al. 2008). PCR amplification products were analyzed using 0.8% agarose gel, and were then purified. The fragments were linked into pMD19-T, and then were transformed into competent cells. The DNA sequencing was performed using an DNA sequence analyzer ABI 3720 (Applied Biosystems, United States) by Shanghai Personalbio. The 16S rRNA gene sequence was analyzed in Ezbiocloud database. The phylogenetic tree was then constructed using MEGA X (Kumar et al. 2018).

Substrate utilization experiments

In order to study strain J2 to degrade PAEs, the bacterium was inoculated into MSM containing 0.3 g/L of one of PAEs: DBP, DMP, DEP, DOP, DIOP, DINP. Uninoculated media supplemented with each substrate were

used negative controls. The suspension was cultured on a rotating shaker at 28 °C. 5 days later, substrate utilization was measured using optical density method.

Biodegradation of DBP by strain J2

Strain J2 was first cultured in LB medium at 28 °C for 24 h. Strain J2 was harvested and washed three times with phosphate buffer, and then was suspended in the phosphate buffer. Inoculated 1% bacterial solution into 50 mL MSM with 1000 mg/L DBP. In order to study the optical conditions of degradation, the degradation of 1000 mg/L DBP by strain J2 was investigated under pH 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0, and different temperature (18, 28, 35, 40, 45 °C). After 5 days, the residue remaining DBP was investigated using HPLC.

Analysis of DBP biodegradation using HPLC

The samples were mixed with ethyl acetate at a 1:1 vol ratio. The aqueous phase was separated using centrifugation at 8,000 g. The aqueous solution samples were extracted twice. The ethyl acetate was evaporated by a rotary evaporator (Buchi, Switzerland). The residue was dissolved in 10 ml methanol, and then filtered. Finally, HPLC was used to analyze the residual concentration of DBP with C18 column. The experiment was carried out at a column temperature of 30 °C, with a mixture of methanol and water at a ratio of 90:10 (v/v). The concentrations of residual DBP were quantified by comparing the integral values of the peak area with the calibration standard curve. DBP degradation efficiency was evaluated, based on the initial and final concentrations of DBP in the test solution.

Genome sequencing

The whole genome of strain J2 was sequenced by using PacBio RS II Single Molecule Real Time (SMRT) and Illumina sequencing platform, provided by Shanghai Majorbio Biopharmaceutical Technology Co., LTD. For PacBio sequencing, 20 µg DNA of strain J2 was spun in a Covaris G-tube. The DNA fragments were then purified, repaired at the end, and linked with SMRTbell sequencing adapters. The sequence library was constructed by 0.45 x volumes of Agencourt AMPure XP beads. An insertion library of about 10 kb was sequenced on one SMRT cell. For Illumina sequencing, more than 1 µg DNA samples were used for sequencing. According to the manufacturer's protocol, genomic DNA samples were cut into 400-500 bp fragments. The libraries were sequenced on Illumina HiSeq X Ten machine with 2 × 150 bp.

Annotation of Genomic Information

CDS, transfer RNA (tRNA) genes and ribosomal RNA (rRNA) genes were predicted by Glimmer (Delcher et al. 2007), Trnascan-SE (Lowe et al. 1997) and rRNAmmer (Lagesen et al. 2007), respectively. The CDSs were annotated based on six databases, including NR, Swiss-Prot, Pfam, GO, COG and KEGG, using several sequence alignment tools (Besemer et al. 2001; Kanehisa et al. 2004; Tatusov et al. 2003). DNAMAN software was used for multiple sequence alignment of esterase.

Results And Discussion

Identification of DBP-degrading bacteria

The bacteria were screened from peanut field soil collected from Laixi Experimental Station of Shandong Peanut Research Institute. After continuous enrichment culture, strain J2 could utilize DBP to grow, with high degradation activity. Therefore, the strain J2 was selected for further study. The colonies of strain J2 appeared on LB agar plates were circular, creamy white, raised (Fig. 1). It is a gram-positive bacterium. The physiological and biochemical of strain J2 are shown in Table 1. It was able to hydrolyze gelatin, casein, starch, urea, but not to hydrolyze Tween 80. It could use glucose, arabinose, mannose, maltose, mannitol and N-Acetyl-glucosamine, but could not assimilate citric acid, proline, malic acid or phenylacetic acid. The 16S rRNA gene of strain J2 was 1513 nt (Fig. 2), and has been submitted to GenBank with the accession number OK598965. Through 16S rRNA gene sequencing and a BLAST homology search, strain J2 shared 16S rRNA gene similarity of 99.93%, 99.80%, 98.78%, 98.17%, 97.76%, 96.07% with *P. aryabhattai* B8W22^T, *P. megaterium* NBRC 15308^T, *P. flexus* NBRC 15715^T, *P. qingshengii* G19^T, *P. paraflexus* RC2^T, *P. koreensis* DSM 16467^T. Phylogenetic analysis clearly indicated that strain J2 was a member of the genus *Priestia* (Fig. 3). In conclusion, the morphological, physiological and genetic sequence analyses identified strain J2 as belonging to the genus *Priestia*. Therefore, the strain was named *Priestia* sp. J2.

Table 1
Biochemical and physiological characteristics of strain J2

Item	Result	Item	Result
Gelatin	+	Mannose	+
Casein	+	Citric acid	-
Starch	+	L-proline	-
Tween 40	-	Malic acid	-
Tween 80	-	Phenylacetic acid	-
Arginine	-	Maltose	+
Glucose	+	N-Acetyl-glucosamine	+
Arabinose	+	Mannitol	+

Substrate utilization tests

The substrate utilization test results indicated that the strain has differing abilities for degrading PAEs. The strain could grow well in media containing DBP, DMP and DEP, but could not grow well in DOP, DIOP or DINP (Table 2). DBP, DMP and DEP were shorter alkyl-chained PAEs, but DOP, DIOP and DINP were

longer alkyl-chained PAEs. These results indicated that the side chain of substrates has a significant effect on the ability of strain J2 to degrade PAEs, which is consistent with the previous studies. *Sphingobium* sp. TJ could use DMP, DBP, DEP and MBP, but could use DOP and DIOP (Jin et al. 2013). *Pseudomonas* sp. YJB6 could use in the media of DBP, DMP and DEP, but could not grow better in the media of DEHP (Feng et al. 2021). *Microbacterium* sp. USTB-Y could assimilate DMP and DEP, but could not use DOP, DEHP and PA (Zhao et al. 2021).

Table 2

Substrate utilization test results of strain J2

Substrate	DBP	DMP	DEP	DOP	DIOP	DINP
utilization	+	+	+	-	-	-
+ positive -negative						

Characteristics of DBP degradation

The effect of pH on DBP degradation by strain J2 was investigated at 35 °C. The degradation rate of DBP increased with the increase of pH from 5.0 to 8.0. Strain J2 had the highest degradation rate of DBP at pH 8.0. As pH exceeded 8.0, the degradation rate decreased sharply (Fig. 4A). In temperature experiment, when the temperature raised from 18 °C to 35 °C, the degradation rate of DBP increased rapidly (Fig. 4B). However, when the temperature reached 40 °C, the degradation rate decreases obviously. Strain J2 could not grow at 45 °C. Therefore, the optimal conditions for DBP degradation were 35 °C and at pH 8.0. Jin et al. reported that the efficiency of DBP degradation was the highest, when pH value reached 7.0 (Jin et al. 2015). *Achromobacter denitrificans* Sp1 could degrade DEHP efficiently at pH 8.0 and 35 °C. Xu et al. reported that *Pseudomonas fluorescens* B-1 could degrade DBP efficiently at pH 7.0 (Xu et al. 2015). Strain J2 and the reported bacteria had the same trend in the optimization of degradation conditions.

Under optimal conditions, strain J2 could degrade 97.6% of 1000 mg/L DBP within 5 days. Some bacteria played important roles in DBP degradation. For example, *Pseudomonas* sp. W1 could degrade effectively degrade DBP (Wang et al. 2020). *Rhodococcus ruber* CQ0301 was able to degrade DBP (Li et al. 2006). *Achromobacter* sp. W-1 identified from wastewater could utilize DBP (Jin et al. 2015). *Sphingobium* sp. TJ isolated from the Haihe estuary cloud grow on mixed PAEs (Jin et al. 2013). *Gordonia* sp. QH-11 identified from sludge was reported to use DBP (Jin et al. 2012). *Enterobacter* sp. DNB-S2 could degrade 91.08% DBP at 50 °C within 7 days (Sun et al. 2019). *Arthrobacter nicotianae* ZM05 isolated from soil was efficient at degrading DBP (Wang et al. 2021). Based on the analysis of previous reports, many genera could degrade DBP. This study was the first report that *Priestia* genus could degrade DBP.

Genome analysis and DBP-degrading genes analysis

The complete genome of strain J2 contains one complete circular chromosome of 5,067,299 bp, and four circular plasmids (147,924 bp, 75,940 bp, 11,604 bp, 11,333 bp, respectively) (Fig. 5). The genome sequences in this study have been submitted to GenBank under the accession number CP086208-CP086212. As shown in Table 3, GC content is 38.06% and the number of predicted coding sequences is 5,585, accounting for 83.55% of the whole genome. The chromosome of strain J2 contains 112 tRNA genes and 39 rRNA operons. The plasmid A has 1 tRNA genes and 0 rRNA operons. The plasmid B has 17 tRNA genes and 3 rRNA operons. The plasmid C and D have no tRNA and rRNA genes. Among the coding genes, 4210 (79.34%), 2553 (48.12%) and 3085 (58.14%) genes are categorized against the COG, KEGG and GO databases, respectively.

Table 3
General features of *Priestia* sp. J2

	1	2	3	4	5
Length (bp)	5067299	147924	75940	11604	11333
GC content (%)	38.25%	33.15%	36.31%	34.16%	32.86%
Total CDSs	5306	186	69	14	12
tRNA genes	112	1	17	0	0
rRNA genes (5S,16S, 23S)	39(13,13,13)	0(0,0,0)	3(1,1,1)	0(0,0,0)	0(0,0,0)
Genes assigned to COG	3977	113	37	4	6
Genes assigned to KEEG	2491	51	9	0	2
Genes assigned to GO	2986	70	21	6	2
1, 2, 3, 4, 5 were Chromosome, Plasmid A, Plasmid B, Plasmid C, Plasmid D, respectively.					

In general, biodegradation of PAEs is caused by esterases, which catalyze hydrolysis of ester bonds (Jin et al. 2013). However, esterase research is still lacking. In this study, based on six databases, there were a large number of genes encoding for putative esterase/carboxylesterase and hydrolase/ α/β hydrolase. According to sequence alignment, almost all these esterases showed no significant homology to those of three reported DBP-degrading enzymes, EstS1 (AEW03609), DphB (KC438416) and DBP hydrolase (AFK31309) (Ding et al. 2015; Zhang et al. 2014; Wu et al. 2013), implying that there may be new DBP-degrading genes. A gene from *Priestia* sp. J2 annotated as “carboxylesterase” was named Est-J2-1, whose amino acid sequence showed 36.66%, 31.08% and 25.34% identity with above three DBP-degrading esterase.

The sequence comparison revealed that the sequence of Est-J2-1 shared no intensive homology with the three enzymes. Thus, the results indicated that Est-J2-1 was a novel member of the DBP hydrolase family. The gene of Est-J2-1 was found to be located on the chromosome. Sequence analysis of Est-J2-1 gene indicated that the open reading frame consisted of 930 bp encoding 310 amino acids. The molecular weight of Est-J2-1 was 34700.14 Da and the isoelectric point was 5.04. No signal peptide

sequence was found. Est-J2-1 and the three DBP-degrading enzymes had highly conserved functional motifs. The catalytic triad (Ser-Asp-His) and consensus motif (Gly-X-Ser-X-Gly) around the active-site Ser154 are boxed in the black frame (Fig. 6).

Conclusion

In conclusion, the strain J2 isolated from peanut field soil could use DBP as its sole carbon source. Based on morphological, physiological characteristics and 16S rRNA gene sequence analysis, it was identified as *Priestia* sp. J2. This study was the first report that *Priestia* genus could degrade DBP.

The research results indicated that the optimal conditions for DBP degradation were 35 °C and at pH 8.0. Strain J2 could effectively degrade 97.6% DBP within 5 days. The genome of strain J2 contains one complete circular chromosome (5,067,299 bp) and four plasmids (147,924 bp, 75,940 bp, 11,604 bp, 11,333 bp, respectively). A novel DBP-degrading enzyme was identified, and contained 310 amino acids. This study provided strain resource for DBP removal from farmland and other environments.

Declarations

Acknowledgments

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Conflict of interest The authors declare no conflict of interests.

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Figures

Figure 1

The morphologic feature of strain J2

Figure 2

Electrophoretic profiles of 16S rRNA gene, Lane 1 was 16S rRNA gene of stain J2, lane 2 was DNA Marker

Figure 3

Phylogenetic tree based on 16S rRNA gene sequence. Bootstrap values based on 1000 replication are shown at branch node. *Halomonas elongata* ATCC 33173^T was used as an outgroup.

Figure 4

Effect of pH and temperature on biodegradation of DBP by *Priestia* sp. J2.

Figure 5

Circular map of the complete genome of *Priestia* sp. J2.

Est-J2-1		0
DphB		0
DBP_hydrolase	MNSAIKGTNVLTALPQKIQTILDKGQGPAAARALDKFPKIVQ	40
EstS1		0
Est-J2-1	MPLDPHIQIFLNQYNEMRP...SLEDVTP	27
DphB	...MNDGATRYTRPDVAFLAFLNAQEGP...KMEDMPP	33
DBP_hydrolase	ESLAKVLGYQYPDLDAFTKCLMAVQIKQGRIGFIGEDP	80
EstS1	MPLDPRVEQFLAQMPPLNRE...GLSLAEA	27
Est-J2-1	LQLREMEKMS..LTPSKE.AVKKVYNEEIQLNERTLTIRV	64
DphB	DGAREMFRVMGQLADVPRGDIKVEDRTIPGPAGDIAIRI	73
DBP_hydrolase	IESRRQFDAQMLAILNKATQIESVEDIRLPLQSGTVFARH	120
EstS1	RQQFKQGALL..LDQMVPVPVDTEDGTVVTTTHGPVRIIR	65
Est-J2-1	YEPEGTG.PFPALVYVHGGGWVLCGLDTHDSICRSYANET	103
DphB	YDNRPDREAGPVVVFYHGGGWVLCGLDTHOPYCAEAARQL	113
DBP_hydrolase	YHPAPNK.KLPMILFYHGGGFVVGGLDTHDEVCRLLAKYA	159
EstS1	YIPDLRL.FSHPLVFYHGGGFVFCGLDTHHGLVARLCQTV	104
Est-J2-1	NCIVVSVDYRLAPESEKFPAAVNDAYDALEKISAHAFLQNI	143
DphB	DMPVIAVDYRLAPEHPFPAAPDDCEATRIVADN....I	148
DBP_hydrolase	KVQVLSIDYPLAPEASPQLLIKSCEDALAVVYQNRRLKI	199
EstS1	GATVISVDYSLAPEAKFPVPVAECIDVARAAEAEPGWGL	144
Est-J2-1	DSNKIAVGGDSAGSNLAASVILAKQRQGPS.IVHQLLIY	182
DphB	ACTGLVLSGESAGSNLTIAATLTDRPASKPVLAMHPIY	188
DBP_hydrolase	YKNRIAVAGDSAGSNISTVVAQHTAGKSYAP..QAQLLIY	237
EstS1	KPS.IVVAGDSAGSNLAASVSQRAKDESLP..IAAQLLFY	181
Est-J2-1	PSVGFKNQHPASMKENAEGYLLSKDLMDFRQLYLNKKEE	222
DphB	PAVTTHNDWQS.YRDFGEGHLLTTGSMTWFGNHYAADPAD	227
DBP_hydrolase	PVVDGKSRHPS.FYAYGEGLVLTSKDQDVYVTQYYATQHNI	276
EstS1	PALDMVHETPS.KRDFARGYLLLEADAMQWFGEOYLRTD	220
Est-J2-1	EQHPYNAPVLLLEDLSSLPSATILTAQYDPLRDSGKDYADA	262
DphB	...YRASPLDFPAEDLPPTLLITAGLDPLRQGRAYAAK	263
DBP_hydrolase	ALDNPLISPTYGNLRKLAPAYVITAGHLLHDEGEIYSHK	316
EstS1	VSHPWASPALSPDLTGLPPALVITAEDPLRDEGEAYAEA	260
Est-J2-1	LKNHGVPTVYENYETMIHGFGLGFHEFVPLAQQAINKSAAQ	302
DphB	LVEAGVPTTYREAGNIHGYINLSQGIPSAKDDIRGALT	303
DBP_hydrolase	LRQSGMKVQYVDPDQTHGFINLTPISSKAKRNTIEIAKN	356
EstS1	LRAAGVPTQIRFDGMIHGFMTMPIF.PQMEAAIEAVARF	299
Est-J2-1	LRQVFDSI...	310
DphB	LKAIVAEANGA	314
DBP_hydrolase	FRKFWDKHS..	365
EstS1	LERID.....	304

Figure 6

Multiple-sequence alignment of esterases from *Priestia* sp. J2 (Est-J2-1, in this study), wastewater treatment plant (DphB) (KC438416), *Acinetobacter* ap. M673 (DBP hydrolase) (AFK31309), and *Sulfobacillus acidophilus* (EstS1) (AEW03609). The catalytic triad Ser154, Asp250, and His280 in the Est-J2-1 from *Priestia* sp. J2, is indicated by arrows. The consensus sequence Gly-X-Ser-X-Gly around the active-site Ser154 is boxed in the black frame.