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# Biodegradation of bisphenol A by the newly-isolated *Enterobacter gergoviae* strain BYK-7 enhanced using genetic manipulation

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## Abstract

Endogenous bacterial strains possessing a high bisphenol A (BPA)-tolerance/degradation activity were isolated from different outlets of petrochemical wastewater in Iran using the enrichment cultivation approach. Two bacterial isolates with high efficiency for BPA degradation in basal medium and petrochemical wastewater were identified as *Enterobacter gergoviae* strain BYK-7 and *Klebsiella pneumoniae* strain BYK-9 using morphology, 16s rDNA analysis and MALDI-TOF mass spectrometry systems. Due to the pathogenicity of *K. pneumoniae*, the *E. gergoviae* strain was selected for further studies. This strain with very high BPA tolerance (up to 2000 mg/L) degraded 23.10±0.126 mg/L BPA in basal medium, 31.35±4.05 mg/L BPA in petrochemical wastewater and 53.50±0.153 mg/L BPA in nutritious medium within 8, 72 and 48h, respectively. Biostimulation by mineral salts and ethanol was effective on the BPA-degradation activity of the *E. gergoviae*. In addition, recombinant *E. gergoviae* [pBR*bisd*] was able to degrade 45.02±0.334 mg/L BPA in basal medium within 48 h. These results point out this strain as a very promising organism for BPA removal in industrial wastewater.

**Keywords:** Bisphenol A, Petrochemical wastewater, Bioremediation, Biostimulation

## 1 Introduction

Bisphenol A [BPA; 2,2-bis(4-hydroxyphenyl) propane] is a synthetic aromatic compound with two phenol rings that is abundantly used as a key material for the production of epoxy resins and polycarbonate plastics, with a wide variety of applications.[1] BPA has acute toxicity towards algae, invertebrates and fish as well as mutagenic and estrogenic effects on the human health.[2, 3] In addition, it has been classified as an endocrine-disrupting compound (EDC) by the US Environmental Protection Agency (USEPA) and the World Wide Fund for Nature (WWF).[4, 5]

The important properties of BPA include low vapor pressure, moderate water solubility (120-300 mg/L) and low volatility.[1, 6] Due to these properties, BPA can be found in the wastewater of BPA-manufacturing and BPA-using industries and, thereby, released into the environment. Therefore, the development of efficient systems for its removal is necessary. Bioremediation using BPA-degrading microorganisms may be useful for BPA removal from exposed environments. In this sense, several BPA-degrading bacteria have been isolated, including the unidentified Gram-negative MV1 and WH1 strains, *Sphingomonas bisphenolicum* strain AO1, *Achromobacter xylosoxidans* strain B-16, *Pseudomonas paucimobilis* strain FJ-4, *Pseudomonas* sp. strains, *Streptomyces* sp. strain, *Sphingomonas* sp.strains, *Novosphingobium* sp. strain TYA-1, *Bacillus* sp. strains and *Cupriavidus basilensis* JF1.[3, 7, 11-21] Previous studies have shown that BPA-degrading bacteria can mineralize 60% of the total carbon of BPA to $CO_2$, assimilate 20% into bacterial cells and convert 20% into soluble organic compounds.[3] Lobos et al.[3] and Spivack et al.[22] proposed a major and a minor pathway for the BPA-biodegradation by a Gram-negative bacterium strain MV1. The major pathway produces 4-hydroxyacetophenone and 4-hydroxybenzoic acid as intermediates, whereas the minor one produces 2,2-bis (4-hydroxyphenyl)-1-propanol and 2,3-bis (4-hydroxyphenyl)1,2-propanediol as primary metabolites.[14] Further, these two metabolic pathways and metabolites were confirmed in other bacterial strains.[12, 16, 17, 18, 19]

BPA removal by several native bacterial strains has been reported but usually the BPA degradation ability of these native strains is not high enough to use them as practical systems for removing this compound. The success of bioremediation depends mainly on the ability of the selected bacterium to thrive in the polluted environment. Thus, the use of bacterial strains isolated from polluted sites for the treatment of hazardous pollutants is promising. [25] Therefore, in this study the BPA degradation potential of a bacterial strain isolated from the outlet of a petrochemical wastewater was investigated. In addition, genetic modification of the BPA

biodegradation pathway in this strain for development of a strain with high potential for BPA-biodegradation was investigated.

## 2 Experimental methods

### 2.1 Study site and sampling

Five samples from different outlets of Khuzestan petrochemical wastewater (named 1 to 5) and six samples from different outlets of Fajr wastewater treatment plant at the Bandar Imam Khomeini petrochemical complex (named 6 to 11) in Iran were collected for the isolation of BPA degrading bacterial strains during February 2011. Samples were collected and transferred to the laboratory under standard conditions.

### 2.2 Strains and chemicals

*Escherichia coli* (DH5α) was used as a host strain for gene cloning (Novagen, Germany). BPA, with purity of 99%, was purchased from Merck (Germany). The pUCH37 (pUC19 with the 3.7-kb *Hind* III fragment carrying *tnpA1*, *bisdA*, *bisdB*, and *tnpA2*) was received from Dr Y. Matsumura (Kansai University, Japan). All chemicals, enzymes, plasmids and kits were purchased from specific manufacturers. Solvents for HPLC were of HPLC grade.

### 2.3 Media and growth conditions

Basal salt medium (BSM, consisting of 1.0 g $K_2HPO_4$, 1.0 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4$. $7H_2O$, 0.01 g $FeCl_3$, 0.05 g NaCl and 0.05 g $CaCl_2$ per liter, pH 7) and L-medium (10 g Bacto Tryptone, 5 g Bacto Yeast extract and 5 g NaCl per liter, pH 7) were used for isolation and cultivation of BPA degrading isolates. BPA was added to BSM and L-medium before autoclaving at a final concentration of 200 mg/L (BSMB (200) and L-BPA (200), respectively), unless other concentrations stated. In some experiments, BPA (1 g/L) was dissolved in distilled water

containing 5% (v/v) ethanol and 20% (v/v) of this BPA solution was added to BSM (BSMBE (200); final concentrations of BPA and ethanol were 200 mg/L and 1% (v/v), respectively). To confirm growth and BPA-degradation activity of the selected bacterial isolates in the original petrochemical wastewater, the selected strains were cultivated in 250-mL Erlenmeyer flasks containing the Khuzestan petrochemical wastewater (PWW) and the same medium supplemented with concentrated solutions of BSM (PWW-BSM). Cultures were incubated at 30ºC in the dark on a rotary shaker incubator (150 rpm) for suitable incubation times. Growth was monitored by measuring the turbidity (optical density) at 600 nm ($OD_{600}$) spectrophotometrically (BECKMAN, USA) during different cultivation times. Agar plates were prepared by addition of 1.5% (w/v) pure agar to the liquid BSMBE (200). The solid media were used for purifying the individual members of the bacterial consortium and growth of the individual isolates (from 2 to 4 days of incubation at 30ºC). Recombinant *Escherichia coli* (DH5α) and the selected bacterial isolates were cultivated on Luria-Bertani (LB) agar plates containing appropriate concentrations of antibiotics.

**2.4 Isolation of BPA-tolerant/degrading bacteria**

One mL of each petrochemical wastewater sample was inoculated in 250-mL Erlenmeyer flasks containing 50 mL of BSMBE(20). The cultures were incubated in the dark on a rotary shaker incubator at 150 rpm and 30ºC for 12 days. During the incubation period, 1 ml of grown culture media was transferred to 250-mL Erlenmeyer flasks containing 50 mL BSMBE(100) or 50 mL BSMBE(200), according to the experiment. The grown bacterial cells in BSMBE(200) were isolated by spreading and cultivating them on BSMBE(200) agar plates. The distinct colonies on the plates were stored in 30% (v/v) glycerol and 1% (v/v) tryptone solution at -70ºC.

**2.5 Determination of BPA degradation**

Bacterial isolates were pre-cultured in L-medium and grown aerobically under shaking (180 rpm) for 16 h at 37ºC. The cells were centrifugally separated (5000 rpm, 20 min) and washed twice with 5 mL of fresh culture medium in which they will be cultured and inoculated to BSMBE(200), PWW and PWW-BSM media at an $OD_{600}$ of 0.2 and incubated under shaking (150 rpm) at 30ºC for 72 h. After incubation time, the residual BPA content of the cultures were determined using the 4- aminoantipyrine colorimetric method. Cultures were centrifuged (13000 rpm, 5 minutes) and the phenol content of the supernatants was extracted by a steam-distillation system (UDK 129). The concentration (mg/L) of the extracted phenol (*C*p) was determined colorimetrically as described by Ghioureliotis et al. [26] Then, the concentrations (mg/L) of total phenol and bisphenol (*C*pb) of the supernatants were calculated by the same method and finally, the concentration (mg/L) of the bisphenol (*C*b) was obtained using the formula: *C*b = (*C*pb-*C*p)×2.4.

For the determination of the BPA-degradation activity, the recombinant and native strains were cultivated in BSMB(200) and L-BPA(200) media at 30ºC and 150 rpm. Samples (1 mL) were collected from the cultures at certain time points and centrifuged at 13000 rpm for 5 min. The resultant supernatants were filtrated through a 0.2 µm membrane filter (Millipore, USA). The amount of BPA in the filtrates was determined by a high performance liquid chromatography (HPLC) system equipped with a UV detector (Agilent 1260, USA) and a reverse-phase C18 column (4.6×250 mm, 5 µm, Zorbax RX-C18). The samples were eluted with a linear gradient (10-90 % acetonitrile/water) at 1 mL/min for 40 min. The injection volume was 25 µl and the absorbance was monitored at 280 nm.

Non-inoculated media were used as controls. The decrease of BPA concentration in the samples was calculated from the remained BPA in the samples, compared with the BPA content in the

controls. All BPA-degradation experiments were carried out in duplicates and the data presented are the average of duplicate experiments.

**2.6 Identification of bacterial strains**

The selected isolates were identified using morphology (Gram staining), 16s rDNA analysis and MALDI-TOF mass spectrometry systems. For the 16s rDNA analysis, the isolates were cultivated in liquid LB medium for 18 h under shaking (180 rpm) at 37ºC and then collected by centrifugation (13000 rpm, 5 min). The genomic DNA was extracted using a conventional method.[27] The 16S rDNA fragment was amplified by PCR using *Taq* DNA polymerase and 3 universal primer pairs of fD1 and rP1; fD1 and 1387R; fD1 and rD1. The PCR conditions were as follows: 5 min at 95ºC; followed by 30 cycles of 95°C for 1 min, 50ºC for 1 min, and 72°C for 1.5 min; and a final extension step of 10 min at 72°C[28]. The amplicons were inserted into the T/A cloning vectors. Then *E. coli* (DH5α) cells were transformed with the recombinant vectors and the recombinant colonies were selected by plating on the LB medium supplemented with 100 μg/mL ampicillin, 0.2 mM IPTG and 40 μg/mL X-gal.[27] Finally, the vectors carrying amplicons were extracted using High Pure Plasmid Isolation Kit (Roche, Germany) and the 16S rDNA fragment was sequenced with an ABI Prism 377 automatic sequencer (Applied Biosystems, CA, USA) using V6 primers. The similarity of the 16S rDNA sequences of the selected isolates with the sequences in the GenBank database (National Center for Biotechnology Information, NCBI) was evaluated using the BLAST algorithm.[29] Related sequences were obtained from GenBank database, and a phylogenetic tree was constructed using the neighbour-joining method of MEGA (Version 5.05) software based on the 16s rDNA sequences.

The identification of the selected isolates (i.e. 7A and 9C) by MALDI-TOF MS was performed on a Bruker Microflex system (Bruker, Germany) instrument equipped with a nitrogen laser with

an output wavelength of 337 nm ~~is used~~ at a repetition rate of 60 Hz. All spectra were acquired in the linear positive mode within a range of 2-20 kDa.

A rapid, on-plate method was used for sample preparation. This method requires a small amount of bacteria which was picked up with a sterile toothpick from a colony and hand spoted onto a 96-spot polished stainless steel MALDI target plate. The spots were allowed to dry at room temperature and overlaid with 1 µL of absolute formic acid. Once dried, the samples were overlaid with 1 µL of MALDI matrix α-cyano-4-hydroxycinnamic acid (CHCA). CHCA was dissolved in a solvent (acetonitrile 50%, water 47.5% and trifluoroacetic acid 2.5%) to a final concentration of 2.5 mg/mL. When the matrix was air dried, the MALDI sample plate was inserted into the spectrometer and spectra were acquired under high vacuum conditions. MALDI-Biotyper 3.1 software library versión V4.0.0.1 (5.627 MSPs) (Bruker Daltonik GmbH, Germany) was used for the identification of each bacteria.

**2.7 Cloning of *bisdAB* operon**

The pUCH37 (pUC19 harbouring 3.7-kb *Hind* III fragment including *tnpA1*, *bisdA*, *bisd*B, and *tnpA2*) (Fig. 1)[31] was digested with *Pst*I (Roche, Mannheim, Germany) in order to obtain one 2339-bp *Pst*I fragment including *bisdAB* operon. Restriction enzyme digestion was performed according to the manufacturer's instruction. The digestion product was subjected to electrophoresis in agarose gel (1% w/v) and the *Pst*I fragment was purified from the gel using High Pure PCR Product Purification kit (Roche, Germany). The purified fragment was then inserted into *Pst*I site of pBR322 vector. Ligation of the *Pst*I fragment and vector was done using the T4 DNA ligase (Roche), according to the manufacturer's instruction. The resulting construct was transferred to *E. coli* DH5α (Novagen).[27] The transformants were cultivated on the LB agar plate containing tetracycline (25 µg/mL). Among the clones grew on this medium, recombinant

clones were selected by colony PCR using specific primers for *Pst*I fragment (forward primer: 5GGAAGCTTGGCCTCCGCACAGC3; reverse primer: 5AGCTGCAGGCCTACCTCTGACTGC3). PCR amplification was performed as following: After an initial 5 min denaturation step at 94℃, two loops including 20 and 10 cycles of programmed amplifications were carried out (loop 1: denaturation at 94°C for 1 min, annealing at 56℃ for 1 min, extension at 72°C for 2 min; loop 2: denaturation at 94°C for 1 min, annealing at 61℃ for 1 min, extension at 72°C for 2 min, and final incubation for 10 min at 72℃). The pBR322 carrying *bisdAB* operon was extracted from 3 mL liquid overnight culture of a recombinant clone using High Pure Plasmid Isolation Kit (Roche-Germany) and transferred to the competent selected strain using the $CaCl_2$ method.[27] The transformants were identified on the LB agar plates containing 25 µg/mL tetracycline by colony PCR using specific primers for *Pst*I fragment and confirmed by specific restriction digestion analysis.

Plasmid stability was tested using serial cultures in non-selective and selective media.[30]

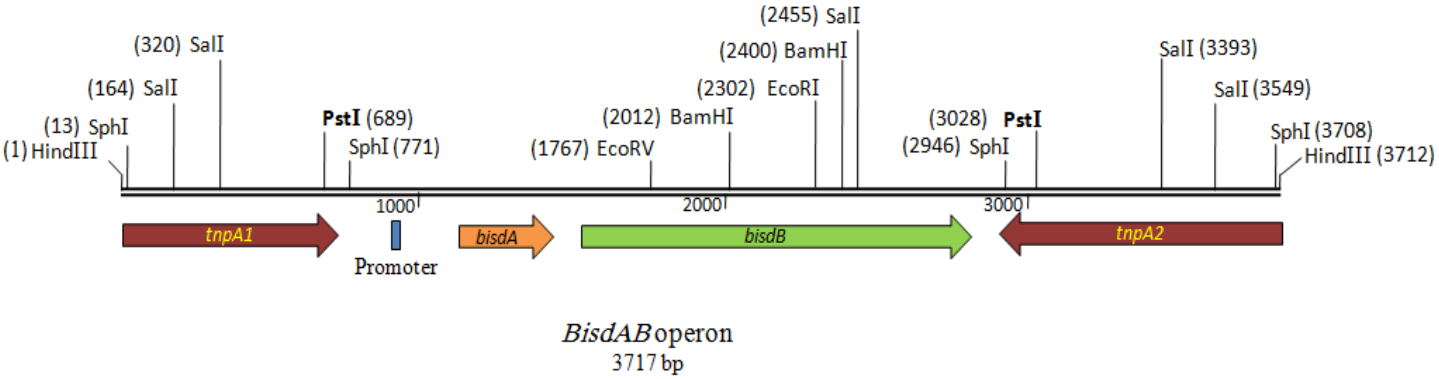


**Fig. 1** 3.7-kb *Hind* III fragment carrying *tnpA1*, *bisdA*, *bisdB*, and *tnpA2*.[31]

# 3 Results

## 3.1 Isolation of BPA-tolerant/degrading bacteria

Collected samples (named 1 to 11) were cultured in BSMBE containing 20, 100 and 200 mg/L BPA using stepwise enrichment manner. BSMBE(200) cultures showed considerable turbidity ($OD_{600}$=0.6-1.1) during 3 to 7 days of incubation at 30ºC; suggesting the existence of BPA-tolerant/degrading bacteria in samples. Finally, 25 microorganisms from BSMBE(200) liquid cultures were isolated on BSMBE(200) agar plates and designated as 1A, 2A, 3A, 4A, 5A, 6A to 6J, 7A to 7C, 8A, 9A to 9C, 10A and 11A. The isolates 6A, 6B, 6E, 6H, 6J, 7A, 9A, 9B and 9C were able to grow on BSMBE(200) agar plates containing 30 g/L NaCL, at pH 7-10 and temperatures of 25-37ºC. The suitable pH and temperature for growth of these isolates were 7 and 30ºC, respectively. Therefore they will be presumably able to grow in the conditions existing in the petrochemical wastewater.

## 3.2 BPA-degradation activity

The BPA-degrading activity of nine selected isolates including 6A, 6B, 6E, 6J, 6H, 7A, 9A, 9B and 9C was examined in both liquid BSMBE and petrochemical wastewater (PWW) by the 4-aminoantipyrine colorimetric method. All isolates were able to grow in BSMBE(200) and degraded 19.52±1.3 to 52.83±4.8 mg/L of the BPA in the culture media within 72 h (Fig. 2). BPA-degradation activity of 7A (52.83±4.80 mg/L) and 9C (47.10±1.20 mg/L) isolates were higher than the others. Furthermore, comparison of growth and BPA-removal of 6E, 6J and 6H isolates indicated that the growth of 6J ($OD_{600}$=1.039±0.05) was higher than the two other isolates, while BPA-removal activity of these three isolates was similar. So it was concluded that the higher growth of 6J in comparison to 6E ($OD_{600}$=0.895±0.006) and 6H ($OD_{600}$=0.749±0.009) was due to the utilization of ethanol as a carbon source. Since less bacterial counts of 6H isolate

were able to degrade similar amounts of BPA (45.17±1.30 mg/L) as 6E (44.96±4.10 mg/L) and 6J (44.26±2.82 mg/L) isolates, it was suggested that BPA-degradation ability of this isolate was higher than that of the other two. Therefore the three isolates 6H, 7A and 9C showed higher ability for BPA removal than the other ones.

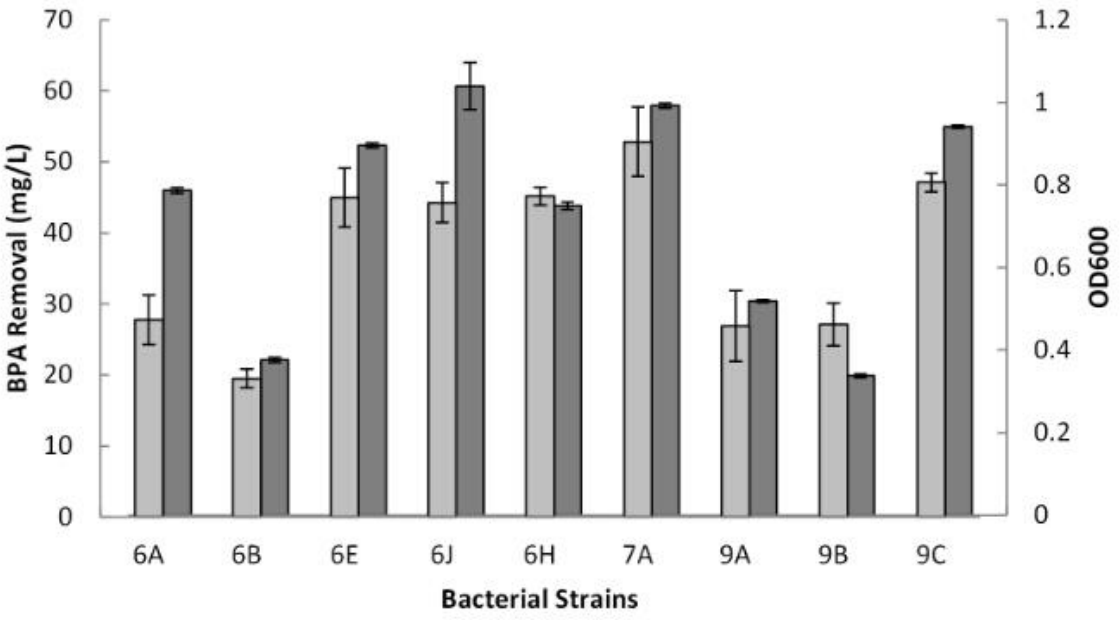


**Fig. 2** Growth ($OD_{600}$) and BPA degradation efficiency of bacterial isolates in BSMBE(200) within 72 h of cultivation. ■, BPA removal (mg/L); ■, $OD_{600}$.

Subsequently, the growth and BPA-degradation activity of the three selected isolates (i.e. 6H, 7A and 9C) were confirmed in PWW and PWW-BSM. The BPA concentration of PWW was determined to be 451.8±4.2 mg/L by the 4-aminoantipyrine colorimetric method. $OD_{600}$ and BPA-degradation of all the cultures after 72 h of cultivation (Table 1) showed that the three isolates were able to grow in both PWW and PWW-BSM media and degrade BPA. The growth

and BPA-degradation activity of the three isolates in the PWW-BSM were higher than those in the PWW. Therefore, the addition of mineral salts to the petrochemical wastewater was effective on both the growth and the BPA-degradation activity of these isolates.

**Table 1** Growth and BPA degradation of the three selected isolates within 72 h of cultivation.

| Isolate code | PWW medium | | PWW-BSM medium | |
|---|---|---|---|---|
| | $OD_{600}$ | BPA degradation (mg/L) | $OD_{600}$ | BPA degradation (mg/L) |
| 6H | 0.365±0.018 | 10.51±3.73 | 0.841±0.010 | 62.74±7.11 |
| 7A | 0.447±0.020 | 31.35±4.05 | 0.902±0.014 | 78.33±5.26 |
| 9C | 0.552±0.010 | 36.68±3.61 | 0.830±0.016 | 65.47±5.43 |

The data in the table 1 show that the two isolates 7A and 9C were able to remove 31.35±4.05 and 36.68±3.61 mg/L of BPA in PWW as well as 78.33±5.26 and 65.47±5.43 mg/L of BPA in PWW-BSM, respectively. Consequently, these two isolates were selected for further studies.

### 3.3 Bacterial identification

The two selected isolates (i.e. 7A and 9C) were rod-shaped, Gram-negative, non-spore forming bacteria. After 24 h of cultivation on LB agar plates, the 7A isolate formed milk-white, non transparent and circular colonies with a smooth surface. The 9C isolate colonies were milk-white, non transparent, slightly gummy/wet looking, circular and convex under the same conditions.

To identify the 7A and 9C isolates, their partial 16s rDNA fragments were amplified using PCR by primer pairs of fD1 and rP1. The amplicons were sequenced and aligned using the BLAST algorithm of the NCBI (National Center for Biotechnology Information, US). The analysis showed that 16s rRNA genes of 7A (accession no. KP255918) and 9C (accession no. KP255917)

were similar to those of *Enterobacter gergoviae* (99% homology, AB682278.1) and *Klebsiella pneumoniae* (98% homology, KC524425.1), respectively.

Aliquots of each bacterial colony were transferred to individual spots on a 96-spot polished stainless steel target plate (Bruker) and allowed to dry. Each spot was overlaid first with formic acid and after drying, with α-cyano-4-hydroxycinnamic acid (CHCA) matrix. When dried, the target plate was inserted into the Bruker Microflex LT MALDI-TOF MS system for analysis. Spectra acquisition was completed after a few minutes and data evaluation was directly linked to the measurement. MALDI-Biotyper 3.1 software (Bruker Daltonik GmbH, Germany) was used for the identification of bacteria by comparing their spectral fingerprints with those existing in the database (composed for 5627 entries). A matching score based on identified masses and their intensity correlation was generated and used for ranking of the results.

Biotyper 3.1 software (Bruker), returned the top 10 identification matches along with confidence scores ranging from 0.0 to 3.0. Score values of 2.3 or higher were considered high-confidence and indicate that the identifications of genus and species are highly probable (secure species), score values between 2.0 and 2.29 show that the genus is reliable and the species is probable. Score values between 1.7 and 1.99 were considered intermediate confidence and indicate that the identification of the genus is probable. Score values lower than 1.7 were considered “not reliable” evincing that spectra acquisition was insufficient or no peak protein was detected, and further analyses are required for the sample.

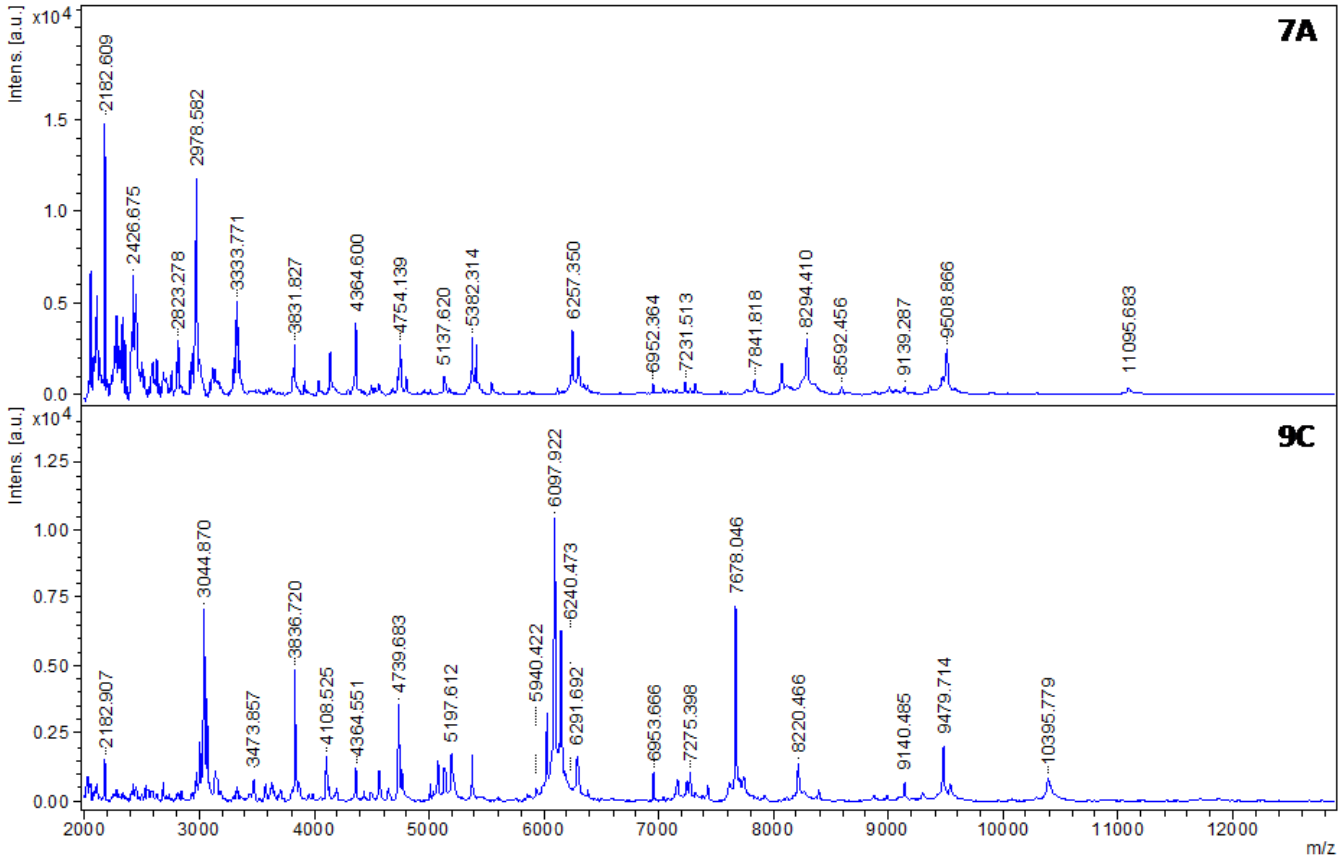


**Fig. 3** MALDI-TOF mass spectra of 7A and 9C samples in the 2-13 kDa mass range.

The isolates 7A and 9A were identified by MALDI-TOF MS as *Enterobacter gergoviae* (NCBI code 61647, score 2.001) and *Klebsiella pneumoniae ssp. pneumoniae* (NCBI code 72407; score 2.049), respectively (fig 3) which are in agreement with the molecular identification based on 16s rDNA sequence. According to MALDI-TOF scores, the species *gergoviae* and *pneumoniae* are reliable. Therefore, the 7A and 9C isolates were named as *Enterobacter gergoviae* strain BYK-7 and *Klebsiella pneumoniae* strain BYK-9.

Because of the pathogenicity of *K. pneumoniae* (Deutsche Sammlung von Mikroorganism-DSM) and 98% similarity of the *K. pneumoniae* strain BYK-9 to this strain, *E. gergoviae* strain BYK-7 was selected for further studies. Figure 4 is phylogenetic tree of the *E. gergoviae* strain BYK-7 based on 16s rDNA, showing its position among the representative species of the genus *Enterobacter*.

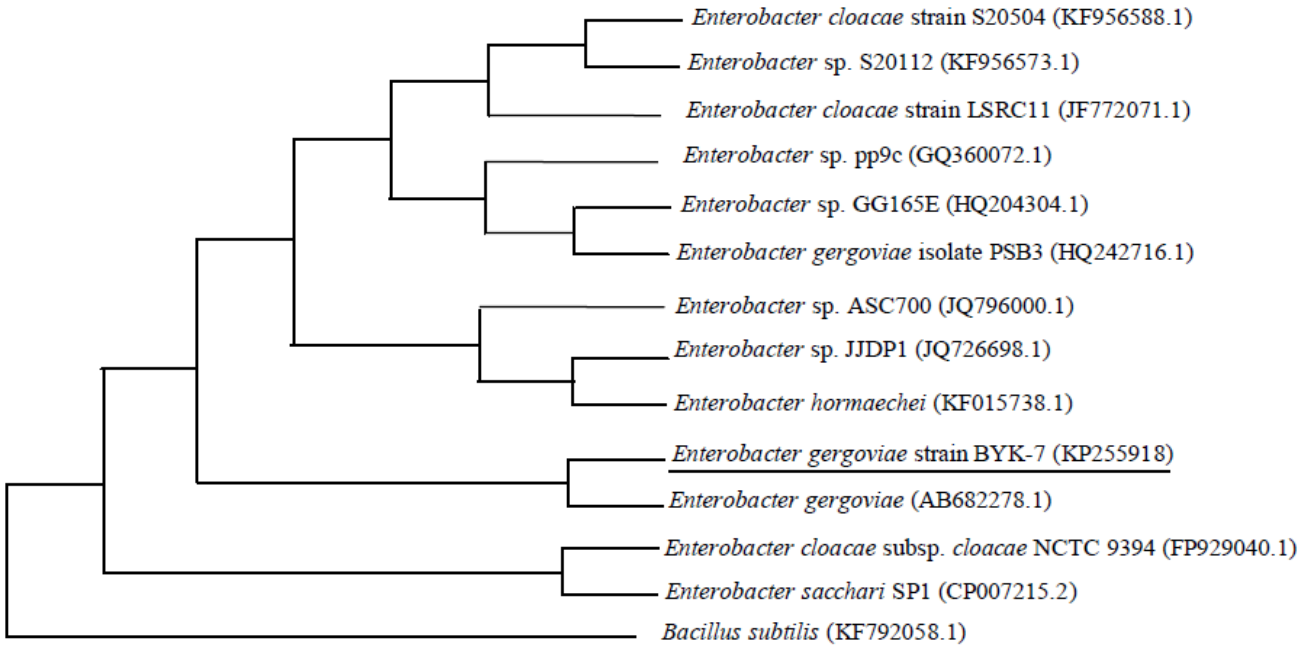


**Fig. 4** Neighbour-joining trees based on 16s rDNA sequences of *Enterobacter gergoviae* strain BYK-7 and related bacteria. Bootstrap values at 100 replications are indicated. The numbers in parentheses are GenBank accession numbers. The tree is rooted with a sequence from *Bacillus subtilis*.

### 3.4 BPA tolerance of *Enterobacter gergoviae* strain BYK-7

The effect of BPA toxicity on the growth of *E. gergoviae* BYK-7 was examined in liquid L-medium containing BPA (0 to 2000 mg/L) and compared to the *Escherichia coli* (DH5α) under the same conditions. Both strains were cultivated in a liquid medium at 37ºC, pH 7.0 under shaking at 180 rpm for 7 h. The growth of *E. coli* (DH5α) was inhibited at a BPA concentration of 200 mg/L, while *E. gergoviae* BYK-7 was able to grow at a BPA concentration higher than 1000 mg/L (Fig. 5). Therefore, *E. gergoviae* BYK-7 showed good tolerance to high concentrations of BPA which can be useful for its application in wastewater treatment.

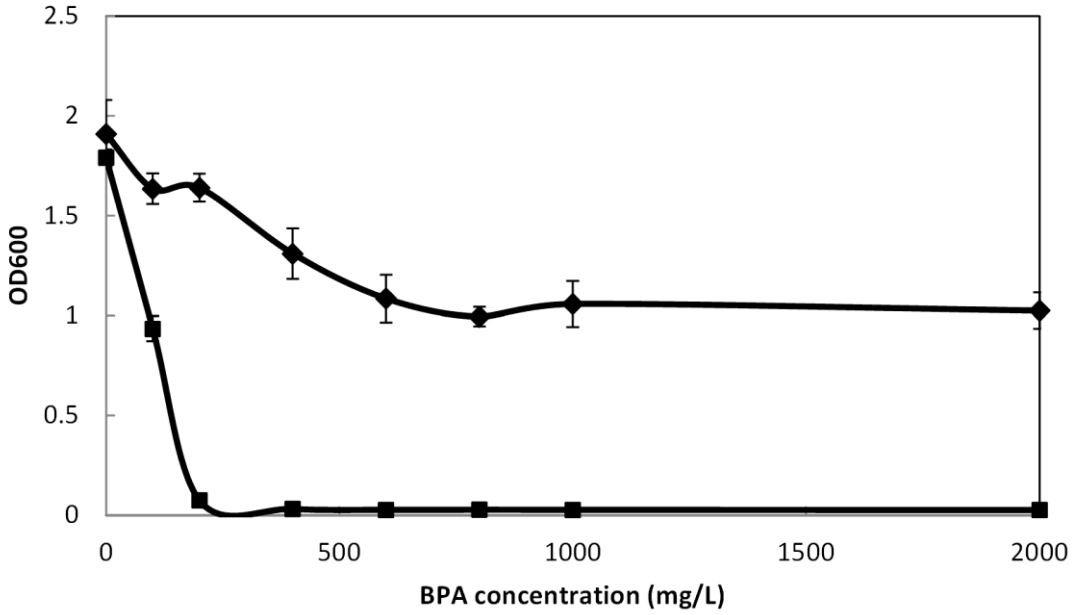


**Fig. 5** Effect of BPA concentration on the growth of *E. gergoviae* BYK-7 and *E. coli* (DH5α). The measurements were performed after 7 h of incubation. Error bars indicate the standard deviation obtained in two independent experiments. ▲ *E. gergoviae* BYK-7; ■ *E. coli* (DH5α)

### 3.5 BPA-degradation activity assay

*Enterobacter gergoviae* BYK-7 was cultivated in BSMB containing 200 mg/L BPA as a sole carbon source (30°C, 150 rpm). After 8, 24 and 48 h, the residual concentration of BPA in the cultures was determined by HPLC analysis. The retention time of BPA was 23.9 min (Fig. 6). The calibration curve equation for detection of the BPA concentration was as follows: peak area = 21.12 $C_{BPA}$ – 9.742 ($R^2$=0.999), where $C_{BPA}$ was the BPA concentration (within the range of 1-300.0 mg/L). The growth was also monitored by measuring the $OD_{600}$ at 8, 24 and 48 h. The results (Table 2) showed that *E. gergoviae* BYK-7 could grow up to an $OD_{600}$ of 0.401±0.035 and degrade 23.10±0.126 mg/L of BPA in BSMB(200) without detectable new peaks in HPLC analysis within 8 h (Fig. 6). Therefore, this strain is able to utilize BPA as a carbon source and may metabolize it to $CO_2$, $H_2O$, and cell components, similar to the strain MV1, as reported by Lobos et al.[3] After 24 and 48 h, growth decreased and the concentration of BPA in the cultures remained constant. Subsequently, the growth and BPA-degradation activity of *E. gergoviae* BYK-7 were determined in L-medium containing 200 mg/L BPA (L-BPA (200) medium) (Table 2). Growth in the L-medium increased until 24 h ($OD_{600}$ 2.725±0.03) and thereafter till 48 h it did not increase. In this nutritious medium, *E. gergoviae* BYK-7 was able to degrade 53.50±0.153 mg/L BPA within 48 h which was higher than that in the BSM medium. Since the L-medium provides more available carbon sources, the increase in the BPA-degradation activity was due to growth stimulation.[11]

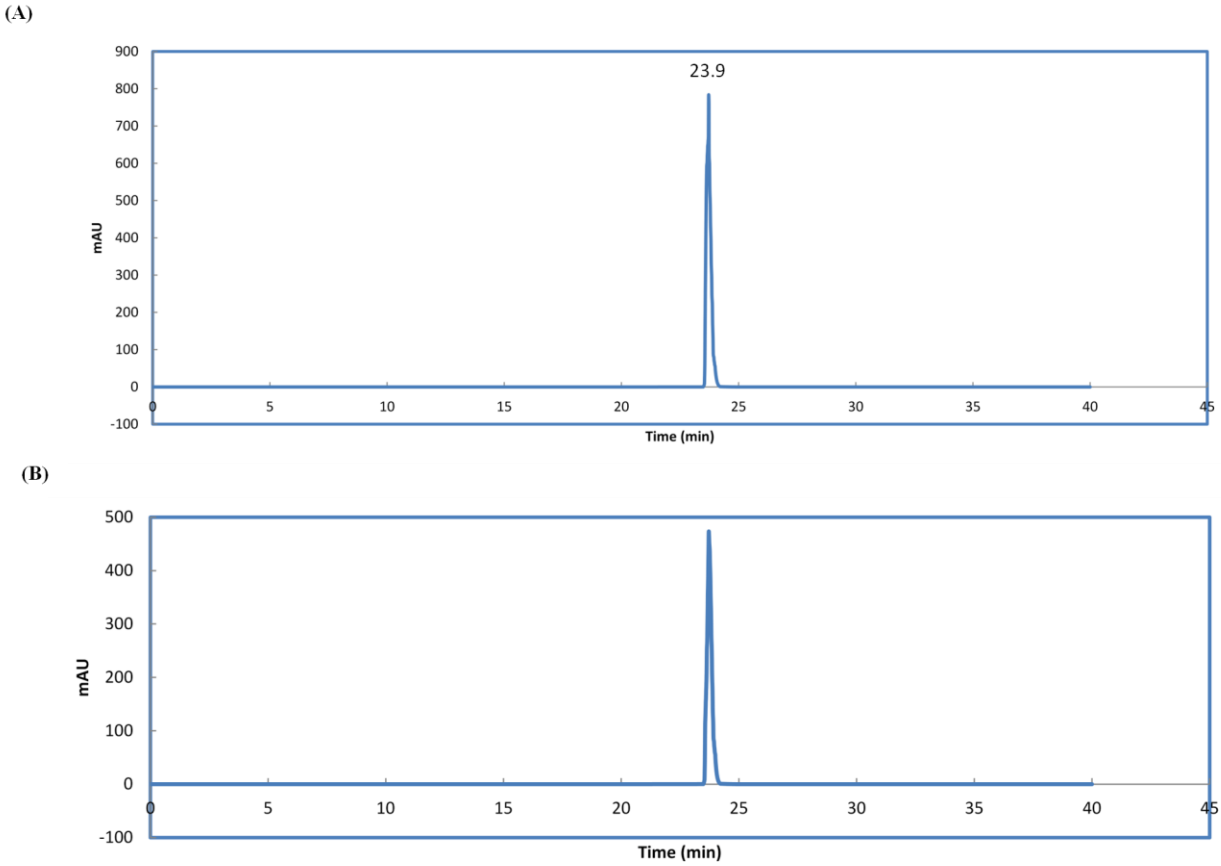


**Fig. 6** (A) The chromatogram of the HPLC system for the measurement of 300 mg/L bisphenol A in BSM medium. (B) HPLC analysis of BPA degradation by *E. gergoviae* BYK-7 in BSMB(200). (linear gradient 10-90% acetonitrile/water; flow rate: 1mL/min.)

**Table 2** Growth and BPA degradation of *E. gergoviae* BYK-7 in BSM and L medium.

| Time (h) | BSMB(200) | | L-BPA(200) | |
|---|---|---|---|---|
| | $OD_{600}$ | BPA degradation (mg/L) | $OD_{600}$ | BPA degradation (mg/L) |
| 0 | 0.270±0.001 | 0 | 0.272±0.019 | 0 |
| 8 | 0.401±0.035 | 23.10±0.126 | 2.105±0.021 | 36.77±0.061 |
| 24 | 0.375±0.049 | 23.13±0.152 | 2.725±0.035 | 47.27±0.126 |
| 48 | 0.349±0.015 | 23.03±0.160 | 2.690±0.014 | 53.50±0.153 |

The growth of *E. gergoviae* BYK-7 in the BSMBE(200) medium increased up to an $OD_{600}$ of 0.992±0.006 and its BPA-degradation activity was 52.83±4.8 mg/L (Fig. 2) which were higher than the growth ($OD_{600}$= 0.401±0.035) and BPA-degradation (23.05±0.059) in the BSMB(200) medium. Thus, 1% (v/v) ethanol could stimulate the growth and BPA degradation activity of *E. gergoviae* BYK-7 in the basal salt medium. In addition, the growth and BPA-degradation activity in PWW ($OD_{600}$=0.447±0.020; BPA degradation= 31.35±4.05 mg/L) and PWW-BSM ($OD_{600}$=0.902±0.014; BPA degradation= 78.33±5.26 mg/L) were higher than those in the BSMB(200) medium. It was probable that other organic compounds (phenol, Isopropyl alcohol, methyl ethyl ketone, according to the Khuzestan petrochemical wastewater treatment plant analysis) existing in the petrochemical wastewater could stimulate the growth and degradation activity of *E. gergoviae* BYK-7. Furthermore, other living microorganisms existing in this environment (i.e. petrochemical wastewater) might synergistically enhance the growth and BPA degradation of this strain. These results suggest that this strain is promising for the bioremediation and bioaugmentation of wastewater containing BPA.

### 3.6 Genetic manipulation of *Enterobacter gergoviae* strain BYK-7

The *bisdAB* operon encoding cytochrome P450 ($P450_{bisd}$) and ferredoxin ($Fd_{bisd}$) of the cytochrome P450 monooxygenase system was found to be responsible for BPA degradation in the *S. bisphenolicum* strain AO1.[31] Consequently, the *bisdAB* operon of the *S. bisphenolicum* strain AO1 was cloned into *E. gergoviae* BYK-7 and the effect of its expression on the BPA-degradation activity was studied.

The pBR322 carrying *bisdAB* operon (designated as pBR *bisd*) was introduced into *E. gergoviae* BYK-7. Transformants were successfully obtained on LB agar plates containing 25 µg/mL tetracycline (originally sensitive to tetracycline) and confirmed using the colony PCR approach. Introduction and maintenance of the pBR*bisd* plasmid into *E. gergoviae* BYK-7 was confirmed by plasmid DNA extraction and agarose gel electrophoresis analysis. Afterwards, the correct

structure of the recombinant plasmids and the presence of the *bisdAB* operon were verified by restriction mapping and PCR methods (Fig. 7). The resulting strain, *E. gergoviae* BYK-7 [pBR*bisd*], was used for BPA-degradation activity analysis.

The pBR*bisd* plasmid stability examination showed that *E. gergoviae* BYK-7 was able to maintain this construct after 6 sub-cultures in the antibiotic-free liquid LB medium.

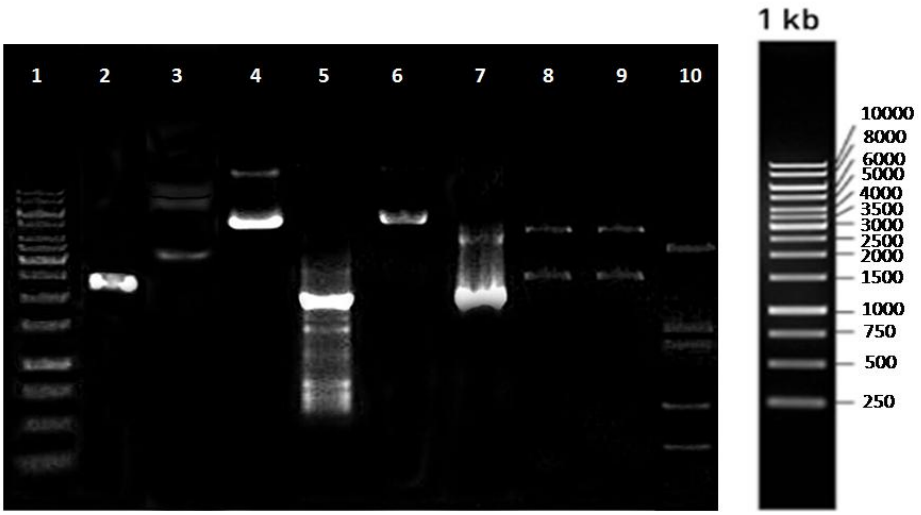


**Fig. 7** Agarose gel electrophoresis analysis. 1: 1 kb ladder (SM 1163). 2: Digested and gel purified *Pst*I fragment including *bisdAB* operon. 3: Non-recombinant pBR322 plasmid. 4: pBR*bisd* extracted from *Eschrichia coli* ($DH_{5\alpha}$). 5: Colony PCR of recombinant *E. gergoviae* BYK-7 using specific primers for *Pst*I fragment. 6: pBR*bisd* extracted from *E. gergoviae* BYK-7. 7: Amplified *Pst*I fragment by PCR using specific primers and pBR*bisd* extracted from *E. gergoviae* BYK-7 as template. 8: Digestion products of pBR*bisd* using *Pst*I. 9: Digestion products of pBR*bisd* using *Eco*RI. 10: Digestion products of pBR*bisd* using *Pst*I and *Bam*HI.

### 3.7 BPA-degrading activity of *Enterobacter gergoviae* strain BYK-7 [pBR*bisd*]

*Enterobacter gergoviae* BYK-7 [pBR*bisd*] was cultivated in BSMB(200). The HPLC analysis of the samples collected at 8, 24 and 48 h showed that this strain was able to degrade 5.77±0.385, 31.08±0.402 and 45.02±0.334 mg/L BPA (Fig. 8), respectively; while the non-recombinant *E. gergoviae* BYK-7 degraded 23.10±0.126 mg/L BPA after 8 h under the same conditions, and its degradation activity did not increase after 24 and 48 h (Table 2). Furthermore, the growth ($OD_{600}$) of *E. gergoviae* BYK-7 [pBR*bisd*] in BSMB(200) was higher than that of *E. gergoviae* BYK-7 (Fig. 9). Therefore, the growth and BPA-degradation activity of native *E. gergoviae* was improved by the expression of *bisd*AB operon from the high potential *S. bisphenolicum* strain AO1 in this strain for the first time.

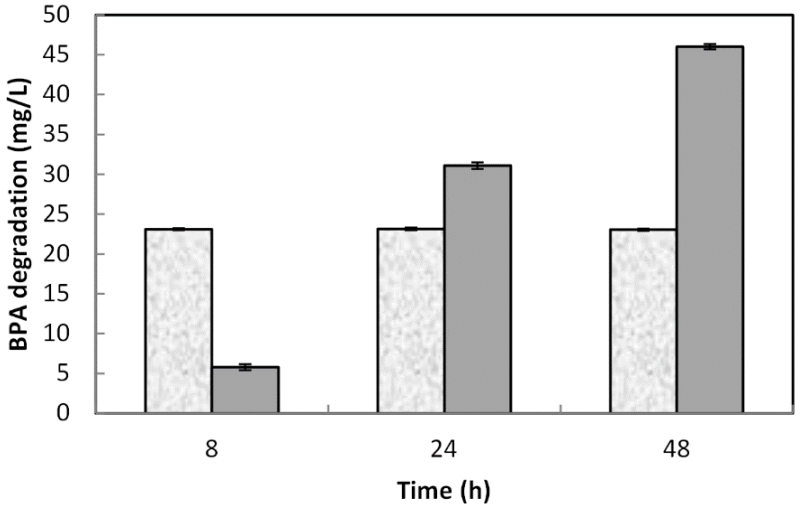


**Fig. 8** Comparison of the BPA-degradation efficiency of *E. gergoviae* BYK-7 (■) and *E. gergoviae* BYK-7 [pBR*bisd*] in BSMB(200) (■).

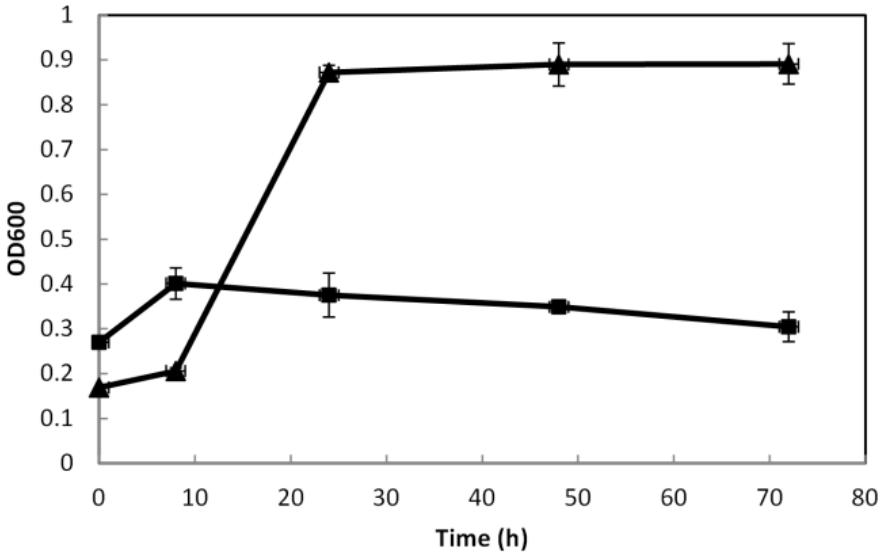


**Fig. 9** Comparison of the growth values of *E. gergoviae* BYK-7 (■) and *E. gergoviae* BYK-7 [pBR*bisd*] in BSMB(200) (▲) within 8, 24, 48 and 72h of cultivation.

## 4 Discussion

Since 1992, there have been several reports about isolation of bacterial strains with different BPA-degradation abilities.[3, 7, 8, 11-21, 32] Among them, a few strains such as *Pseudomonas* sp. strain KU1, KU2 and *Bacillus* sp. strain KU3[13] were able to degrade 780, 810 and 740 mg/L of BPA in a basal medium within 7 to 12 days, respectively. Other strains such as *S. bisphenolicum* strain AO1[17] could degrade high concentrations (up to 110 mg/L) of BPA in the nutritious MYPG-medium in a short cultivation time (6 h), but BPA-degradation activity in the basal medium was very low (about 23 mg/L after 72 h). Furthermore, some of strains including *Bacillus pumilus*,[21] *Sphingomonas* sp. strain BP-7[19] and *Cuprividus basilensis* JF1[7] were only able to degrade 10, 98 and 45 mg/L of BPA in the nutritious medium after 24 h, 4 days and 225 days and were not able to grow in the basal medium containing BPA as a sole carbon source. In

the current study, we have succeeded in isolating *E. gergoviae* BYK-7 which is able to degrade 23 mg/L of BPA in the basal medium within 8 h without production of detectable metabolites based on HPLC analysis. This is a great advantage, because it has been indicated that some BPA metabolites exhibited obvious estrogenic activities and may create new environmental pollution concerns.[2, 17] There are several reports on the degradation of BPA in the basal and nutritious media, river water and pond sediments, while degradation of this compound in the petrochemical wastewater has not been reported previously. Our results for the first time showed that *E. gergoviae* BYK-7 can degrade 31.35±4.05 mg/L BPA in PWW and 78.33±5.26 mg/L BPA in PWW-BSM in 72 h, respectively. In comparison to the previous reported strains, this strain has a relatively high BPA-degradation ability, which is promising for application in practical BPA removal systems. It is probable that the optimization of its environmental conditions and modification of the BPA-degradation pathways using genetic engineering techniques increase the potential of this strain for BPA-degradation and its application in the treatment of wastewater containing BPA.

Sasaki et al.[31] reported that the expression of *bisd B* and *bisdAB* genes of *S. bisphenolicum* strain AO1 in *E. coli* BL21 (DE3) using the L-BPA medium, increased the BPA-degradation activity in 18 h from 10 mg/L to 30 and 90 mg/L, respectively. These recombinant *E. coli* strains converted BPA to only 1,2-bis(4-hydroxyphenyl)-2-propanol (metabolite 1) that is the product of cytochrome P450 monooxygenase in strain AO1. The other detected product of BPA-degradation by these recombinant cells was metabolite II that was not detectable in the BPA-degradation pathway of the strain AO1. Therefore, it was suggested that an alternative BPA metabolic pathway may exist in *E. coli*. But, those experiments were performed in the nutritious L-medium and they did not report whether these recombinant *E. coli* cells were able to grow in

the basal medium containing BPA as a sole carbon source. In the current study, *E. gergoviae* BYK-7 [pBR*bisd*] could grow in the BSM containing BPA as a sole carbon source and degrade 45.02±0.334 mg/L of BPA without producing detectable metabolites. From these results, it can be suggested that this recombinant *Enterobacter* strain is able to metabolize this amount of BPA to $CO_2$, $H_2O$ and cell components. These findings are in agreement with those reported by Hashizume et al.[33] and Kang and Kondo[32] who found that bacteria isolated from rivers and soils were able to degrade BPA without producing detectable metabolites.

In comparison to non-recombinant *Enterobacter*, remarkable delay was observed in the growth and BPA-degradation activity of recombinant *Enterobacter* (Figs. 8 and 9), which could be due to the cell metabolic burden. Consequently, expression of cytochrome P450 and ferredoxin encoded by *bisdAB* operon caused higher BPA degradation and growth.

Most bacterial cytochrome P450 monooxygenase systems need to have a flavin adenine dinucleotide-containing NADH-dependent reductase (ferredoxin reductase) and an iron-sulfur redoxin (ferredoxin).[34-36] There are two reports about the existence of cytochrome P450-like proteins in *Enterobacter cloacae*. subsp. dissolvens SDM[37] and *Enterobacter cloacae*. subsp. cloacae ENHKU01[38], but the gene encoding cytochrome P450 has not been found in the sequenced genome of the other *Enterobacter* species including *Enterobacter cloacae*. cloacae ATCC13047 (CP001918.1), *Enterobacter cloacae* ECWSU1 (CP002886.1), *Enterobacter cloacae*. subsp. cloacae NCTC 9394 (FP929040.1), *Enterobacter aerogenes* KCTC 2190 (CP002824.1), *Enterobacter aerogenes* EA1509E (NC-020180.1), *Enterobacter asburiae* LF7a (CP003026.1), and *Enterobacter asburiae* L1 (CP007546.1). All the reported genome sequences for the *Enterobacter* species contain the gene that encodes ferredoxin, but only some of them have the ferredoxin reductase gene. In the current study, the increase of BPA-degradation

activity of *E. gergoviae* BYK-7 [pBR*bisd*] in comparison to that of the native *Enterobacter* showed that cytochrome P450 and ferredoxin produced by *E. gergoviae* BYK-7 [pBR*bisd*] were functional. So, this suggests that *E. gergoviae* BYK-7 produces ferredoxin reductase that contributed to BPA-degradation by $P450_{bisd}$ and $Fd_{bisd}$.

Earlier studies showed that under environmental conditions and with strains cultivated in mineral medium, BPA was found not to be easily degradable.[39] Biostimulation by simply providing the cultures with easier degradable compounds can accelerate the biological degradation of more persistent compounds.[7] Our results clearly demonstrated that the addition of simple compounds including mineral salts to PWW and ethanol to basal salt medium enhanced the BPA-degradation activity of the *E. gergoviae* BYK-7. Therefore, it seems that in addition to bioaugmentation, biostimulation by primary substrates is a very promising strategy for bioremediation of petrochemical wastewater containing BPA.

## 5 Conclusion

According to the risks of BPA as an environmental endocrine disruptor, elimination of this compound from environment is necessary. This study demonstrated the efficiency of BPA removal using microorganisms. We initially succeeded to isolate one BPA-tolerant/degrading *E. gergoviae* BYK-7 from petrochemical wastewater. Thereafter, BPA-degradation activity of this strain enhanced using genetic manipulation. Since the expression of *bisdAB* operon in *Enterobacter* led to an increase in the BPA-degradation activity, it is probable that the identification of other key genes in the metabolic pathway of BPA-degradation and further genetic manipulation of *E. gergoviae* BYK-7 [pBR*bisd*] using these genes may be effective in increasing the degradation activity of the *E. gergoviae* BYK-7.

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