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Research Article

Biodegradation of Bisphenol A by some bacterial species and significance role of plasmids

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Abstract

Bacterial strains having high Bisphenol A (BPA) tolerance were isolated from contaminated soil samples at different manufactures companies from Helwan governorate, Egypt. Two different bacteria, designated as J.2 and L.4 showed the best degradation efficiency at 150rpm in mineral medium supplemented with 400ppm BPA. The best degradation was obtained at 24h for two isolates but the BPA degrading by isolate J.2 was better than that of isolates L.4. The optimum conditions for BPA biodegradation exhibited at 35°C to 40°C and pH 7 for two isolates, and it subsequently decreased with alteration of temperature and pH. The best inoculum size was 0.1% and 0.3% for isolates L.4 and J.2, respectively. The initial reading of BPA residual was 400ppm and decreased to 157.69ppm, 285.45ppm and 132.89ppm for isolate J.2, L.4 and consortium, respectively by using HPLC. Biodegradation product from BPA degradation was determined by GC-MS. These strain were identified as *Klebsiella pneumoniae* J2 *and Enterobacter asburiae* L4 by morphological, physiological, biochemical characters and 16s rDNA analysis. The role of plasmid-borne gene in BPA degradation by two isolates was determined. Plasmid extraction and curing were carried out using standard procedures. Plasmid extraction studies showed that, *Klebsiella pneumoniae* J2 have two plasmid (small, 300bp and large, ›1500bp size), as well as, *Enterobacter asburiae* L4 have one plasmid (1500bp size). The result also showed that, the isolates were successfully cured of plasmid using ethidium bromide. From data analysis of curing of plasmid, we concluded that, loss of plasmid by *Klebsiella pneumoniae* J2 *and Enterobacter asburiae* L4 lead to complete loss of their degradative abilities.

Keywords: Bisphenol A biodegradation, BPA tolerance bacteria, HPLC, GC-MS, Plasmid DNA, Plasmid curing.

1. Introduction

Environmental pollutants such as alkylphenolic compounds, polychlorinated biphenyls, pesticides, dioxins and textile dyes have been released into the environment. Most of these chemicals and/or their degradation products are toxic, mutagenic, carcinogen and endocrine disrupting for aquatic and terrestrial life. Bisphenols (BPA: 2,2-Bis(4-hydroxyphenyl) propane) are a group of chemical compounds that consist of two phenolic rings joined together through abridging carbon or other chemical structure. BPA and some of its derivatives, such as bisphenol-B (BPB), bisphenol-F (BPF), and bisphenol-S, are used as materials for epoxyresins and polycarbonates lining large food containers and water pipes (**Nicolucci, *et al*., 2011**). Tetrabromobisphenol-A (TBBPA), a halogenated derivative of BPA, is used as a flame retardant (**Sjodin, *et al*., 2001**). Tetrachlorobisphenol-A (TCBPA) has been found in the effluent from waste-paper recycling plants (**Kuruto-Niwa, *et al*., 2002**).All these BPAs have also been classified as endocrine-disrupting chemicals **(Kitamura, *et al*., 2005**). Bisphenol A is an industrially important monomer used in many chemical manufacturing plants throughout the world for the synthesis of polycarbonates, epoxy resins, phenol resins, polyesters, polyacrylates and lacquer coatings on food cans as well as storage vessels (**Ike, *et al*., 2000: Snyder, *et al*., 2000**).

As a frequently detected environment pollutant, BPA was not only found in the environmental samples, such as water, sludge and air (**Zafra *et al*., 2003)**, but also in the biological samples as well as human tissues (**Vandenberg *et al*., 2007**).

Most BPA degradation studies were mainly focused on the oxidation reaction including photodegradation (**Nomiyama *et al.*, 2007**) and biodegradation (**Kolvenbach *et al.*, 2007; Yamanaka *et al.*, 2007; Yim *et al.*, 2003)**. The organisms such as fungi (**Kabiersch*et al.*, 2011**), plant-cultured cells (**Saiyood *et al.*, 2010**) as well as bacteria(**Yamanaka *et al.*, 2007**) can all be used as biocatalysts for BPA biodegradation. (**Fouda, *et al.*, 2015)** reported that, fifty two (52) purified fungal strain screening for BPA degradation and select two promising fungal isolates *Aspergillus terreus* C10 and *A. flavus* G1 were selected based on their ability to degrade BPA with percentage 50% and 40% respectively.

Environmental factors that may influence the biodegradation of toxic chemicals in the environment include pH, salinity, temperature, oxygen availability, and nutrients (**Fan, *et al.*, 2004**). The size of the sediment particles is also of fundamental importance; if the particles are large, the specific surface area is small, and the number of potential sites for microbial activity is affected (**Delhomenie, *et al.*, 2002)**.

Plants can rapidly absorb BPA through their roots from water and metabolize it to several glycosidic compounds. The glycosylation of BPA by plants leads to estrogenicity of the parent compound. Two oxidative enzymes, peroxidase and polyphenol oxidase, are closely associated with BPA metabolism (**Kang, *et al.*, 2006)**.

Genetic information in bacteria, as in all organisms, is stored in the form of DNA. The information is physically present in bacterial cells in two forms – the chromosome and the plasmids. The bacterial chromosome is a single circular, highly folded double-strand of DNA. In addition to chromosomal DNA, a larger number of bacteria also have extra-chromosomal DNA in the form of plasmids (**Zylstra and Gibson, 1991**).

Many plasmids contain genes which code for the enzymes necessary for the derivative pathways important to bioremediation. Enzymes involved in the degradation of toluene, naphthalene, salicylate, octane etc. have been shown to be plasmid encoded (**Nelson, 1990**).

In this study, examine the ability of different bacterial isolates in biodegradation of BPA and study the suitable environmental and nutritional condition for BPA biodegradation.in addition, determine the biodegradation products by HPLC and GC-MS spectrum. Study the role of plasmid-borne gene in degrading BPA in bacterial isolates.

2. Materials and Methods

2-1- Samples collection

Five different hazardous soil samples were collected from different manufactures, companies from Helwan governorates, Egypt.

2-2- Chemicals and media used

2-2-1- BPA (99%) analytical standard grade, are purchased from the Sigma chemicals company (*Sigma-Aldrich*) Germany.

2-2-2-Nutrient agar media (Shirling and Gottlieb, 1966).

The nutrient agar medium is widely used as a routine medium for comparative study for isolation of different species of microorganisms & consist of (g/L) peptone, 5.0; Beef extract, 3.0; Nacl, 5.0; 1000 ml with distilled water.

2-2-3- Mineral Salt medium (MS)

According to **Mariusz *et al.*, (2009),**the medium was composed of (g/L): $(NH_4)2So_4$,1.0; KCl, 0.2; $MgSo_4.7H_2o$, 0.2; $CaCl_2$, 0.04; $Fecl_3.6H_2o$, 0.005; $K_2Hpo_4.3H_2o$, 1.0; kH_2Po_4, 0.2; was prepared and distilled water up to 1000ml/L , Agar, 15.0g/L for solid media.

MS was used for study the BPA biodegradation. After sterilized, BPA was added separately under sterile conditions.

2-3- Isolation and Screening.

2-3-1- Isolation.

The bacterial population occurred in collected soil samples were suggested to be induced and enhanced to promote its initiation of growth. This carried out by preparation the previous MSM and supplemented with 1% yeast extract, 50 ppm of BPA and inoculated with 1 g soil, incubating under shaking condition for 2 days. After this, new MSM containing 50 ppm of BPA and 0.5% yeast extract was prepared and inoculated with 2 ml of the previous MSM. This procedures were repeated till removing yeast extract completely, then,

MSM containing only 50 ppm of BPA as a sole carbon and energy source. After this MS agar media were prepared and inoculated from last previous MS for further purification.

2-3-2- Screening.

The purified bacterial isolates were subjected to growing on MS agar media containing different concentration of BPA (200, 400, 500,600 and 700 ppm) as a sole source for carbon and energy to select the most potent bacterial isolates according to growing on high concentration of BPA.

2-4- Identification of the most potent bacterial isolates.

The most potent bacterial isolates were identified using **Morphological, Physiological and Biochemical test** according to ***Bergy's manual of systematic bacteriology (2005),*** and 16s ribosomal DNA sequence (16s rDNA) analysis for most potent bacterial isolates were amplified by polymerase chain reaction (PCR) using the universal primers. The forward and reverse primers used for PCR amplification were 27^{f} (5`-AGAGTTTGATCCTGGCTCAG -3`) and 1592^{r} (5` GGTTACCTTGTTACGACTT -3`) (16S r DNA universal primer).The sequencing for the PCR product was occurred in **GATC BiotechCompany by use ABI 3730xl DNA sequencer** by using forward and reverses primers.

2-5- Analysis of BPA biodegradation.

2-5-1- Spectrophotometric methods.

The residue of BPA in culture after growth was determined by Folin-Ciocalteu reagent according to **Yordanova, *et al.*, (2013).** After centrifugation of the culture medium for 10min at 3500rpm. One milliliter of the supernatant was added to 10 mL of dis.H_2O and 1 mL of Folin-Ciocalteu reagent. The mixture was then allowed to stay for 5min and 2 mL of 20% Na_2CO_3 (W/V) was added to the mixture. The solution was put in a dark place for 60min and then the absorbance at 750nm was measured.

2-5-2. High performance Liquid Chromatography (HPLC) method.

Two volume of methanol was added to cultured medium and filtered to remove insoluble materials and cell. BPA concentration in the filtrate was assayed by HPLC which provide with GBC U.V vis detector, GBC LC 1110 Pump.winchrome chromatography Ver I 3, Kromasil (250x4.6 mm 5u) column. The solvent system was 50% acetonitrile. The flow rate was I mL/min and elution was monitored at 280 nm Detection limit by the HPLC was 0.05 ppm (**Takita *et al*., 2005**).

2-5-3.Gas-Chromatography-Mass-Spectrum (GC-MS) Method.

To determine the biodegradation products result from BPA biodegradation, GC-MS was done. Selected bacterial isolates were grown in mineral salt medium containing BPA as a sole carbon source under all optimum environmental and nutritional culture condition. Cultures were extracted for GC-MS analysis to determine the biodegradation products of BPA.

2-6- Factors affecting on BPA biodegradation ratio.

2-6-1. Incubation periods and incubation conditions.

Three replicate of the most potent bacterial isolates were allowed to grown on MSM containing BPA as a sole carbon and energy source for 8, 10, 12, 14, 16, 18, 20, 22, 24 and 32hours under static and shaking conditions. At the end of each incubation period, Folin-Ciocalteu reagent was used to determine the residue of BPA comparable with control.

2-6-2. Effect of different temperatures.

To determine the effect of different temperatures on BPA degradation, the two most potent bacterial isolates were allowed to grow in different temperatures (25^{o}C, 30^{o}C, 35^{o}C, 40^{o}C, 45^{o}C and 50^{o}C) at appropriate time and incubation condition for each bacterial isolates.

2-6-3.Effect of different pH values.

To determine the effect of different pH on BPA degradation, Three replicate for each one of the most potent bacterial isolates were allowed to grown on MSM at different pH values (3,4,5,6,7,8,9,10) under optimal time, incubation condition and optimal temperature for each bacterial isolates.

2-6-4. Effect of different inoclum sizes.

The inoculum size was adjusted by means of cell forming units(CFU) to obtain the definite number of

bacterial cell in 1 mL of culture. The following concentration was applied, 0.1%, 0.3%, 0.5%, 1%, 1.5%, 2%, 2.5%.

2-7. Plasmid DNA isolation and detection procedure.

Plasmid DNA was extracted from the isolates under study by alkaline lysis method according to **Birnboim and Doly (1979).**

2-8.Curing of plasmid.

Plasmid curing experiment was performed according to a procedure described by **Head *et al*. (1992).**

2-9. Statistical analysis.

Data were statistically analyzed by SPSS v17, one-way analysis of variance (ANOVA) test was used for multiple sample comparison, when normality and homogeneity of variance were satisfied, followed by multiple comparison Tukey test.

3. Results and Discussion

The contaminated soil samples (five samples) were selected for the isolation of BPA resistant bacteria. Out of 12 bacterial isolates, eight isolates were gram positive and four isolates were gram negative. All of the isolates were screened for their ability to resist BPA at 200, 400, 500, 600 and 700ppm. Two out of 12 isolates were found to resist 600ppm of BPA (Table 1).

Table. [1]. Effect of different concentrations of BPA on bacterial isolates

Isolate No.	200ppm	400ppm	500ppm	600ppm	700ppm
J.1	+	+	-	-	-
J.2	+	+	+	+	-
J.3	+	+	-	-	-
I.1	+	+	-	-	-
I.2	+	-	-	-	-
L.1	+	-	-	-	-
L.2	+	+	-	-	-
L.4	+	+	+	+	-
G.1	+	-	-	-	-
G.2	+	+	-	-	-
F.1	+	+	-	-	-
F.2	+	-	-	-	-

Two most potent bacterial isolates (J.2 and L.4) are identified based on morphological, physiological and biochemical characters as shown in table [2] and 16sr RNA sequence analysis. According to this results, two most potent isolates belonging to *Klebsiella pneumoniae* and *Enterobacter asburiae*. So that, given code *Klebsiella pneumoniae* J2 and *Enterobacter asburiae* L4. **Ajaz, *et al.*, (2004),** were isolate 30 soil isolate have the ability to degrade phenol. Out of 30 isolates, 23 isolates were gram positive and 7 isolates were gram negative and determined their resistant to phenol on solid media at different concentration of phenol.

Table. [2]. Biochemical and physiological properties of the two selected isolate strains.

Biochemical Characters	Bacterial isolate J.2	Bacterial isolate L.4
Gram reaction	-	-
KoH	+	+
Growth at 41^{o}C	+	+
Indole production	-	-
Methyle red test	-	+
Vogas-proskour	+	-
Esculine hydrolysis	+	+
Urea hydrolysis	+	-
Carbohydrate Fermentation		
L-Arabinose	-	+

Cellobiose	+	+
Glycerol	-	-
Lactose	+	-
Maltose	+	+
D-Mannitol	+	+
Melibiose	-	-
Raffinose	+	+
L-Rhamnose	+	+
D-Sorbitol	-	+
Sucrose	+	+
D-Xylose	+	+
Utilization of:		
D-Arabitol	-	+
Benzoate	+	-
Citrate	+	+
Histamine	-	+
Lactose	+	+
Lactulose	+	-
D-Malate	+	-
Malonate	-	-
D-Melibiose	-	+
L-Proline	+	+
Raffinose	+	-
L-Rhamnose	+	-
D-Sorbitol	+	+
Sucrose	+	+
L-Tyrosine	-	-

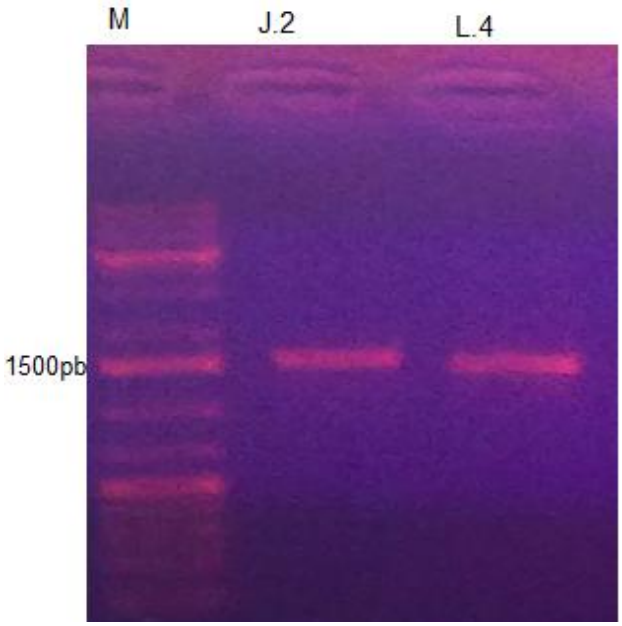

Plate (1): PCR product of 16S rDNA gene for the isolates J.2, lane (1) and L.4, lane (2), where (M) DNA ladder (marker).

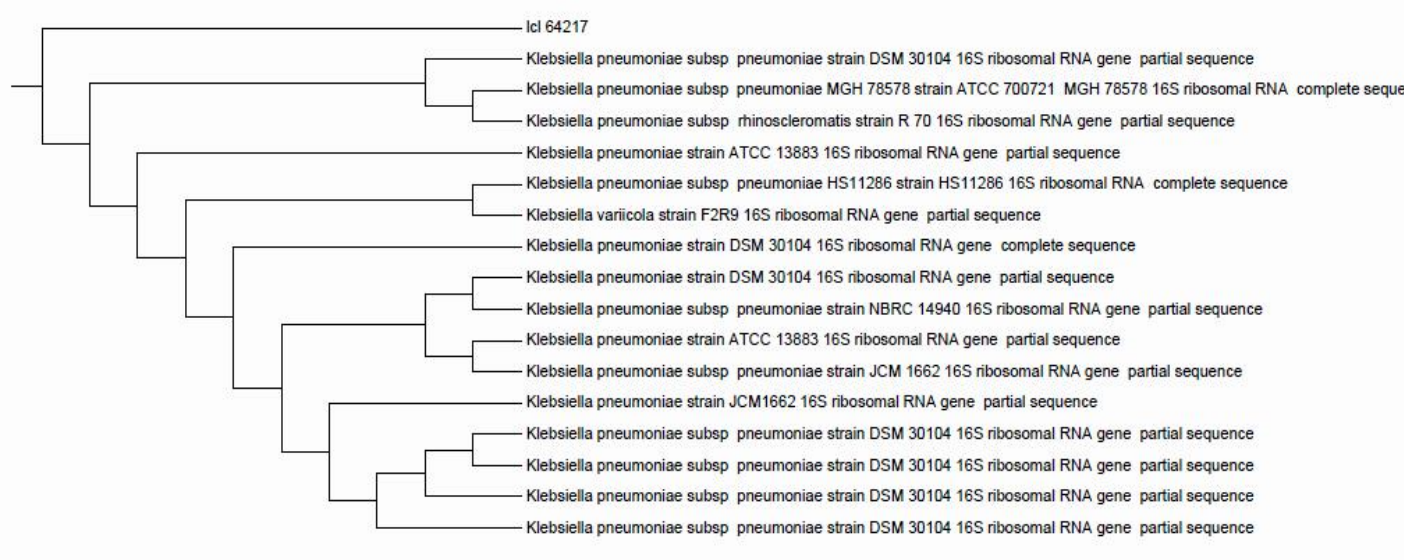

Fig. (1): Phylogenetic analysis of the bacterial isolate J.2.

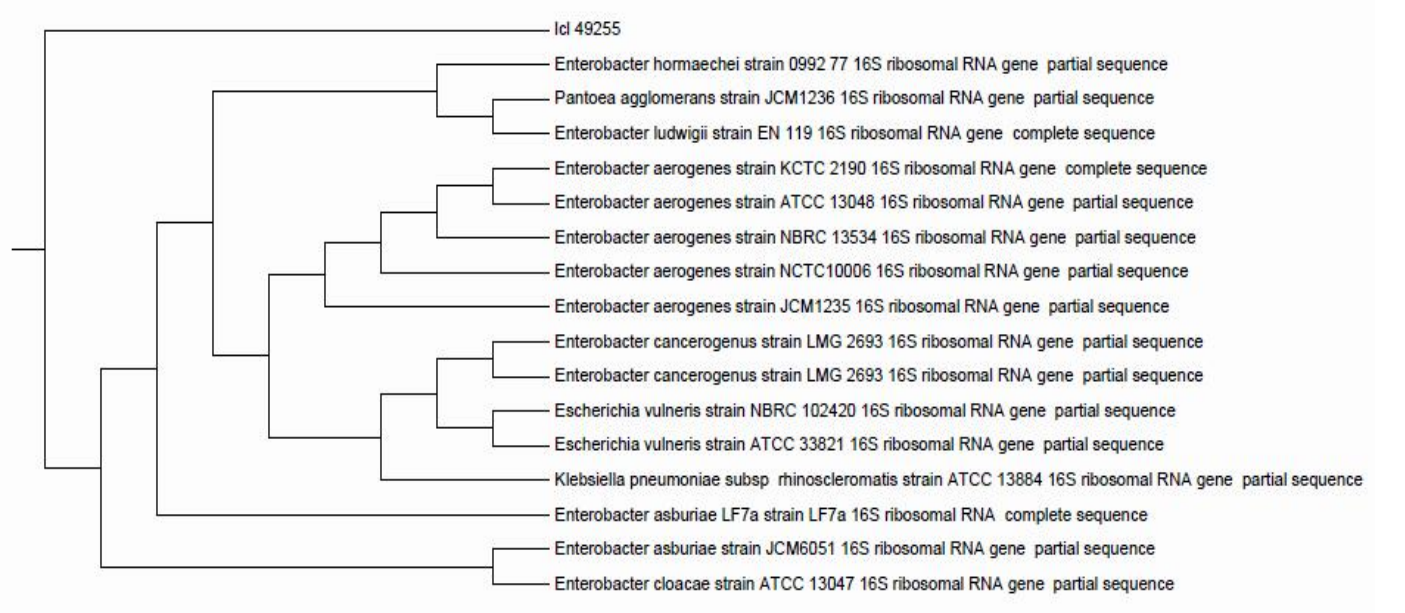

Fig. (2): Phylogenetic analysis of the bacterial isolate L.4.

3.1.Effect of different Incubation periods and incubation status.

The biodegradation of BPA under different static and shaking conditions was shown in (Table 3). In general, shaking status to degrade BPA was better than static conditions through 8 to 32 h. Under static condition, there was no significant variation between control and bacterial inoculation by *Klebsiella pneumoniae* J.2 or *EnterobacterasburiaeL.4* after 8 and 10h, but the significant variation in biodegradation of BPA was recorded between different treatments at time courses of 16-32h. Similar results were found under shaking condition except the significant difference was found through time of 18 to 32h. Under static or shaking conditions, inoculation with bacterial isolates *Enterobacter asburiae* L.4 or by *Klebsiella pneumoniae* J.2 was significantly increased the biodegrading of BPA as compared to control treatment at time courses ranged between 16 to 32h (Figs 3 and 4). The data analysis showed that, the degradation of BPA reached maximum level at 24h, and the BPA degrading by isolate *Klebsiella pneumoniae* J.2 was better than that of isolate *Enterobacter asburiae* L.4

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Table [3].Biodegradation of bisphenol A by two bacterial isolates under static and shaking conditions at different times.

Residue of bisphenol A (O.D) at static condition			Residue of bisphenol A (O.D) at shaking condition		
		Mean ± SE			**Mean ± SE**
8hs	Control	1.71±0.231	8hs	Control	1.82±0.032
	J.2	1.95±0.009		J.2	1.60±0.136
	L.4	1.66±0.20		L.4	1.80±0.046
10hs	Control	1.83±0.064	10hs	Control	1.80±0.039
	J.2	1.67±0.074		J.2	1.58±0.120
	L.4	1.81±0.063		L.4	1.68±0.054
12hs	Control	1.82±0.067	12hs	Control	1.63±0.106
	J.2	1.54±0.049		J.2	1.56±0.106
	L.4	1.74±0.034		L.4	1.60±0.058
14hs	Control	1.80±0.069	14hs	Control	1.60±0.136
	J.2	1.51±0.037		J.2	1.49±0.107
	L.4	1.73±0.045		L.4	1.48±0.113
16hs	Control	1.81±0.058	16hs	Control	1.69±0.045
	J.2	1.52±0.043		J.2	1.46±0.118
	L.4	1.59±0.047		L.4	1.49±0.069
18hs	Control	1.81±0.042	18hs	Control	1.59±0.034
	J.2	1.58±0.058		J.2	1.43±0.068
	L.4	1.64±0.113		L.4	1.51±0.043
20hs	Control	1.81±0.046	20hs	Control	1.68±0.025
	J.2	1.51±0.022		J.2	1.44±0.049
	L.4	1.59±0.020		L.4	1.52±0.033
22hs	Control	1.80±0.036	22hs	Control	1.64±0.013
	J.2	1.54±0.013		J.2	1.47±0.017
	L.4	1.58±0.009		L.4	1.39±0.025
24hs	Control	1.80±0.041	24hs	Control	1.63±0.015
	J.2	1.46±0.017		J.2	1.35±0.022
	L.4	1.51±0.022		L.4	1.40±0.042
32hs	Control	1.79±0.034	32hs	Control	1.61±0.087
	J.2	1.49±0.026		J.2	1.41±0.054
	L.4	1.51±0.043		L.4	1.500.026

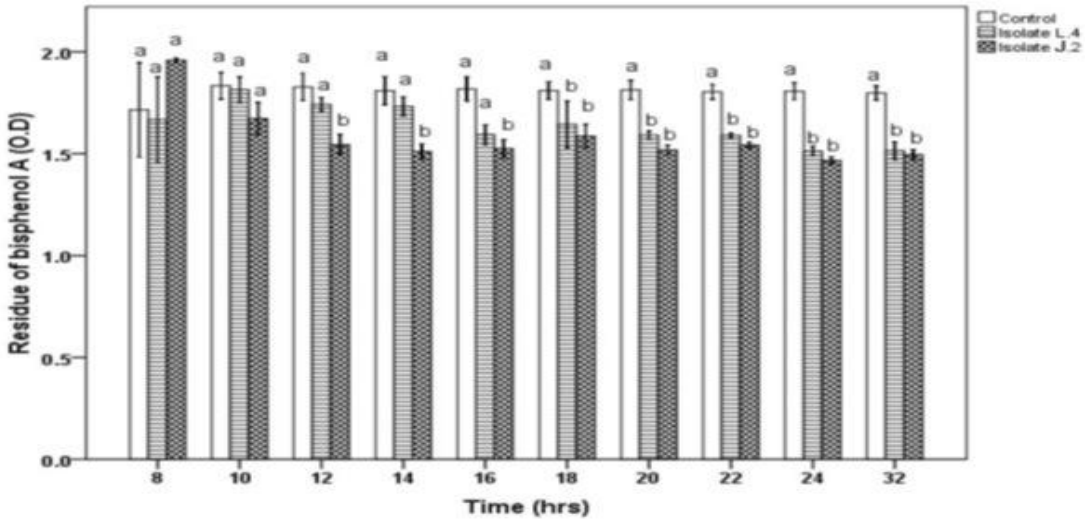


Fig. 3. Biodegradation of BPA by two bacterial isolates under static conditions at different times. Means with the same letter are not significantly different (P ≤0.05). Data are the mean ± SE of three replicates

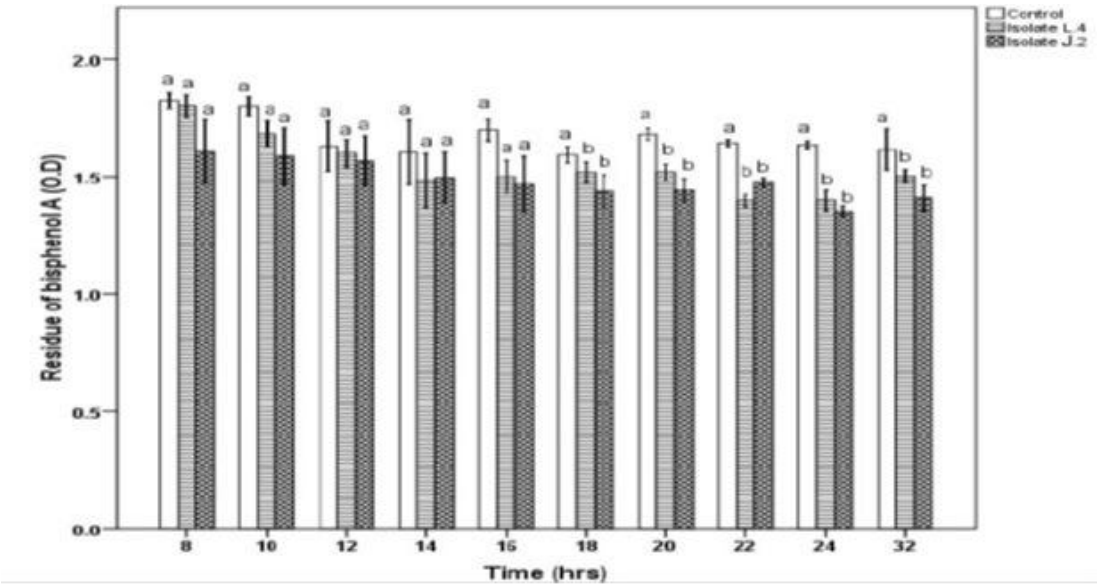


Fig. 4. Biodegradation of bisphenol A by bacterial isolates under shaking conditions at different times. Means with the same letter are not significantly different (P ≤0.05). Data are the mean ± SE of three replicates**.**

3.2.Effect of different temperatures on biodegradation of BPA.

The effect of different temperatures on biodegradation of BPA by bacterial inoculation was studied. The results showed that, the highest BPA degrading values were found at 35to 40^{o} C. Also, it was observed that, inoculation with isolate *Klebsiella pneumoniae* J.2 was significantly increased the BPA degrading as compared to inoculation with Isolate *Enterobacter asburiae* L.4 at 35 and 40^{o}C (Fig. 5). BPA degrading by isolate *Enterobacter asburiae* L.4 or control treatments did not significantly different at 25 to 45^{o}C. However at high temperature of 50^{o}C, inoculation by *Enterobacter asburiae* L.4 was significantly increased the BPA degrading compared to *Klebsiella pneumonia* J.2 isolate and control treatments (Fig. 5). Thus, our results point to the inoculation with *Klebsiella pneumonia* J.2 isolate is recommended under normal temperature degree of 35-40^{o}C, but inoculation with *Enterobacter asburiae* L.4 performed better in BPA degradation under high temperature of 50^{o}C.

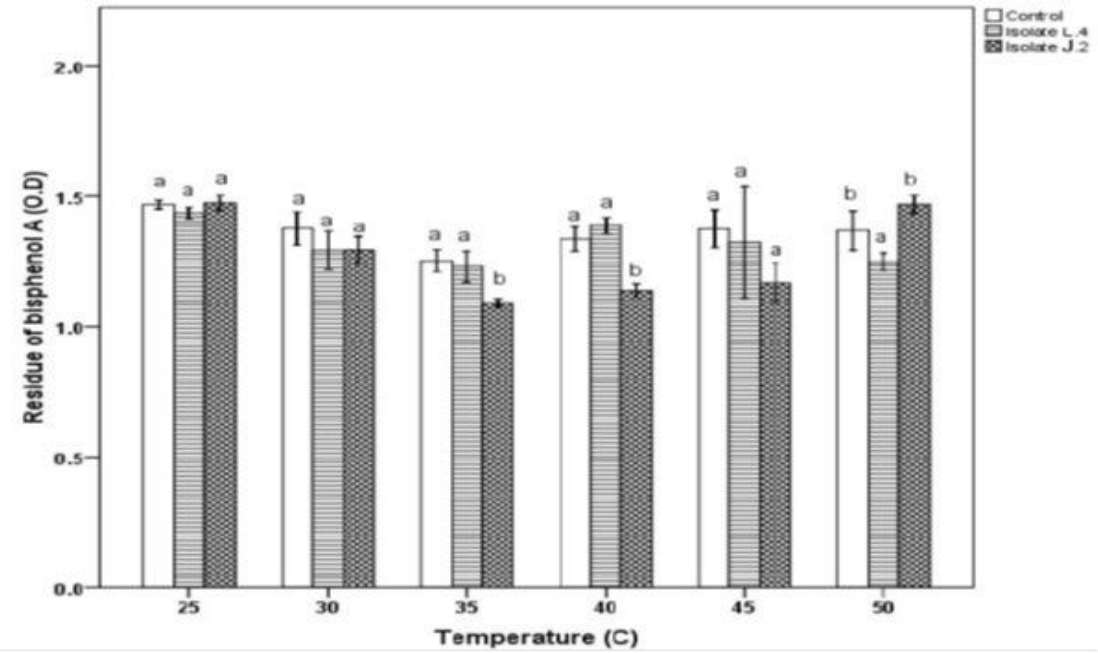


Fig. 5. Biodegradation of BPA by two bacterial isolates at different temperatures. Means with the same letter are not significantly different (P ≤0.05). Data are the mean ± SE of three replicates

3.3.Effect of different pH values on biodegradation of BPA.

The optimal pH value for BPA biodegradation by bacterial isolates was pH 7 as was shown in (Fig. 6). Analysis of variance in BPA degrading by bacterial isolates was found in (Fig. 6), where there was no significant difference in BPA degradation between bacterial isolates and control treatments at all pH values except at pH value of 7; in which, inoculation with bacterial isolates *Klebsiella pneumoniae* J.2 and *Enterobacter asburiae* L.4 were significantly increased the BPA degradation compared to non-inoculated control treatment.

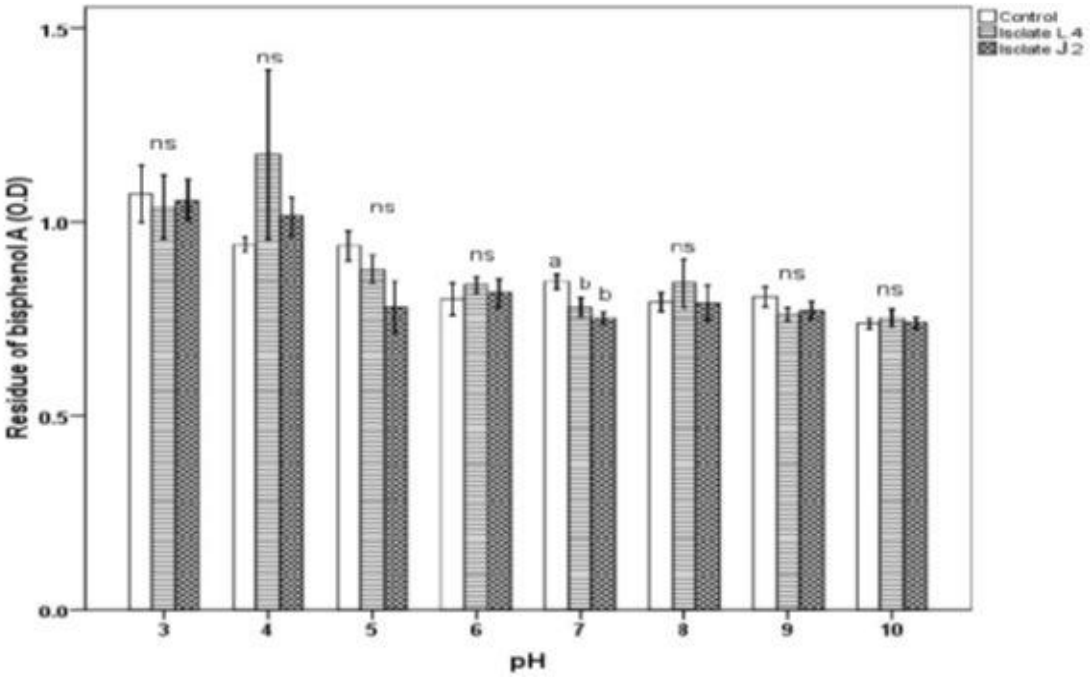


Fig. 6. Biodegradation of bisphenol A by bacterial isolates under different pH values. Means with the same letter are not significantly different (P ≤0.05). Data are the mean ± SE of three replicates

From the previous data analysis for the effect of static or shaking conditions, temperature, and pH; we concluded that the best optimal conditions in order to get the best BPA biodegradation by bacterial isolates were shaking condition at temperature of 35-40^{o}C and pH 7. So the effect of bacterial inoculation size on BPA degrading was conducted at these previous mentioned optimal conditions.

This result was matching with **Xia-ling *et al* (2010),** concluded that**,** The proper conditions for the BPA-degradation by *Klebsiella* ZY-B at pH 7.0 and temperature 35 °C and nearly complete degradation was achieved in 54 h.

3.4.Effect of different inoculum size.

One-way ANOVA analysis showed the significant effect of bacterial inoculation size on BPA degradation where *P*= 0.001 and ≤0.001 for *Enterobacter asburiae* L.4 and *Klebsiella pneumoniae* J.2 isolates, respectively (Table 4). It was observed that the inoculation sizes of 0.1% by *Enterobacter asburiae* L.4 and 0.3% by *Klebsiella pneumoniae* J.2 isolates produced the highest value of BPA degradation (Table 5). Generally, inoculation by 0.3% of *Klebsiella pneumoniae* J.2 was the best inoculation size to degrade BPA (Fig 7). Our data analysis showed that under minor inoculation size ranged between 0.3 to 0.5 ml, the BPA degradation by *Klebsiella pneumonia* J.2 isolate was significantly impacted than L.4 isolate treatment, while BPA degrading was significantly higher under major inoculation of 1% to 2% by *Enterobacter asburiae* L.4 isolate than was found by *Klebsiella pneumoniae* J.2 isolate (Fig. 7).

Table[4]. One-way analysis of variance (ANOVA) for the effect of bacterial inoculation size on BPA biodegradation

		Sum of Squares	df	Mean Square	F	Sig.
Effect of inoculation size (Isolate L.4)	Between Groups	1.690	7	.241	5.066	.001
	Within Groups	1.144	24	.048		
	Total	2.834	31			
Effect of inoculation size (Isolate J.2)	Between Groups	2.427	7	.347	7.250	.000
	Within Groups	1.148	24	.048		
	Total	3.575	31			

Table[5]. Effect of bacterial inoculation size on BPA biodegradation

Residue of bisphenol A (O.D) at different bacterial inoculation sizes					
		Mean± SE			**Mean± SE**
Inoculation size (ml) of (L.4)	.00	1.05±.529	Inoculation size (ml) of (J.2)	.00	1.05±.529
	.10	.356±.125		.10	.438±.216
	.30	.444±.094		.30	.237±.041
	.50	.465±.180		.50	.359±.041
	1.00	.425±.041		1.00	.823±.092
	1.50	.568±.108		1.50	.778±.137
	2.00	.412±.087		2.00	.581±.110
	2.50	.839±.151		2.50	.958±.113

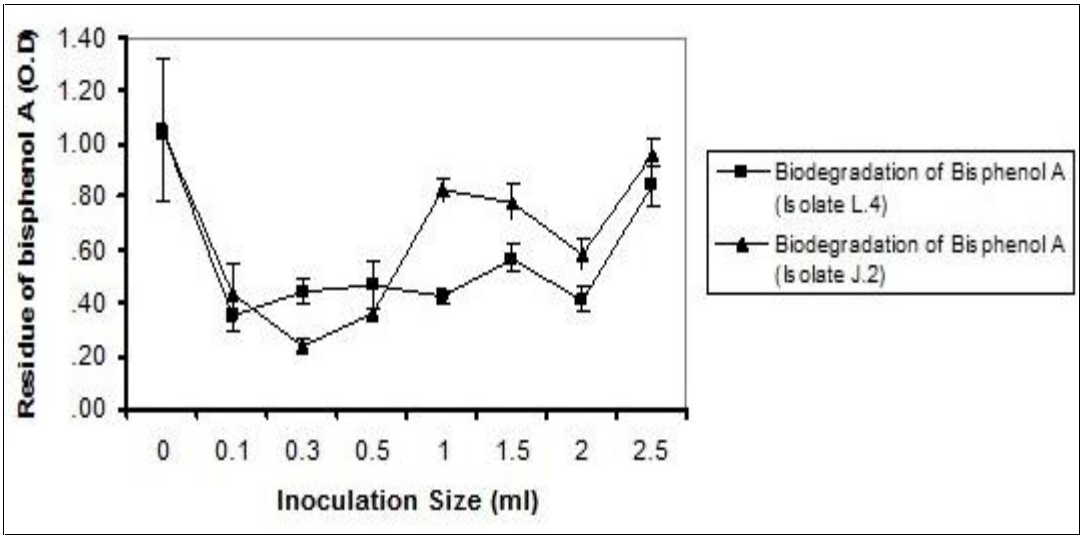
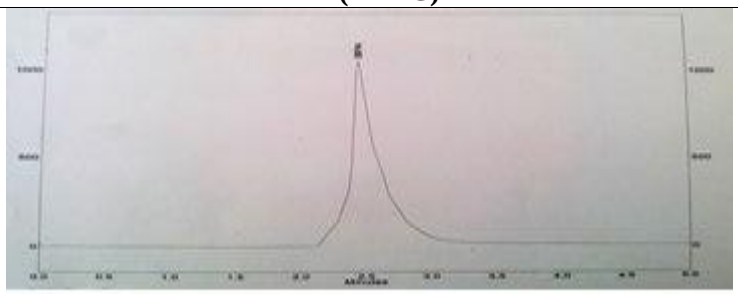
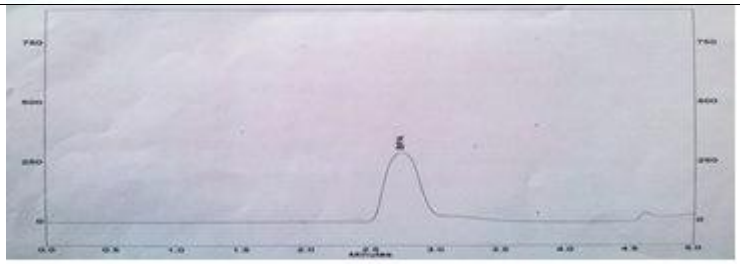
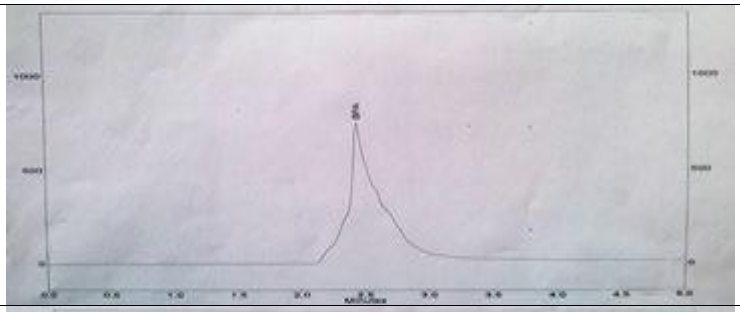
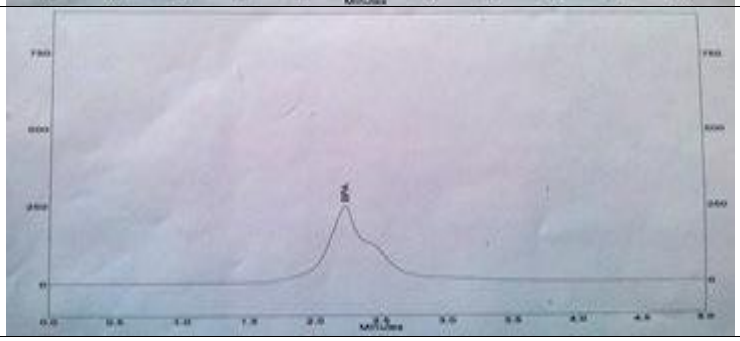


Fig. 7. Biodegradation of bisphenol A under different bacterial inoculation size. Data are the mean ± SE of three replicates

3.5.HPLC analysis for BPA biodegradation.

Mineral salt medium was supplemented with 400ppm of BPA to analyzed by HPLC for most potent bacterial isolates (*Klebsiella pneumoniae* J2 and *Enterobacter asburiae* L4) and consortium (*Klebsiella pneumoniae* J2 with *Enterobacter asburiae* L4 at the same flask). This result showed that, HPLC analysis for control (Mineral salt medium supplemented with 400ppm without inoculation) showed peak at retention time 2.43 minutes. While the mineral salt medium supplemented with the same concentration of BPA and inoculated by *Klebsiella pneumoniae* J2, *Enterobacter asburiae* L4 and consortium for 24 h. showed peaks at retention time 2.73, 2.43 and 2.23 minutes respectively. According to HPLC analysis, the concentration of BPA after degradation decreased from 400ppm to 157.69, 285.45 and 132.89 by *Klebsiella pneumoniae* J2, *Enterobacter asburiae* L4 and consortium respectively. According to HPLC data, concluded that, biodegradation of BPA was more effective by consortium followed by *Klebsiella pneumonia* J2 and finally *Enterobacter asburiae* L4.

Table [6]. HPLC analysis of BPA Standard and BPA obtained after degradation by *Klebsiella pneumoniae* J2 and *Enterobacter asburiae* L4 and consortium.

Factors	Conc. of BPA	RT (min)	Absorbance Spectrum (HPLC)
BPA Standard	400ppm	2.43	
***Klebsiella pneumoniae* J2**	157.69ppm	2.73	
***Enterobacter asburiae* L4.**	285.45ppm	2.43	
Consortium	132.89ppm	2.23	

3.6.Analysis of BPA Metabolites Using GC-MS.

To determine the biodegradation product result from BPA biodegradation, *Klebsiella pneumoniae* J2, *Enterobacter asburiae* L4 and the consortium were inoculated in to mineral medium containing BPA as a sole of carbon source under all optimum environmental and nutritional culture condition. According to data of GC-MS spectrum, BPA can be hydrolyzed and give the same product as flourene, Azobenzene, 4-Bromphenyl-phenyl ether and phenol-pentachloro at retention time 28.015, 29.04, 30.087 and 31.611 min, respectively from BPA biodegradation by two most potent isolate and consortium. Other biodegradation products as phenol 2, 4-dinitro and Diehtylphthalate at retention time 26.033 and 28.057 min respectively for isolate *Klebsiella pneumoniae* J2.

Other products as phenol 4-chloro-3-methyl, phenol-2,4,6-trichloro and Dimethy-phthalate produced at retention time 20.553, 22.926 and 24.704min respectively by *Enterobacter asburiae* L4. Phenol-4-chloro-3-methyl, Hexachlorocyclopentadine and Dimethyl phthalate are produced during biodegradation of BPA by consortium at retention time 20.553, 21.600 and 24.702min, respectively.

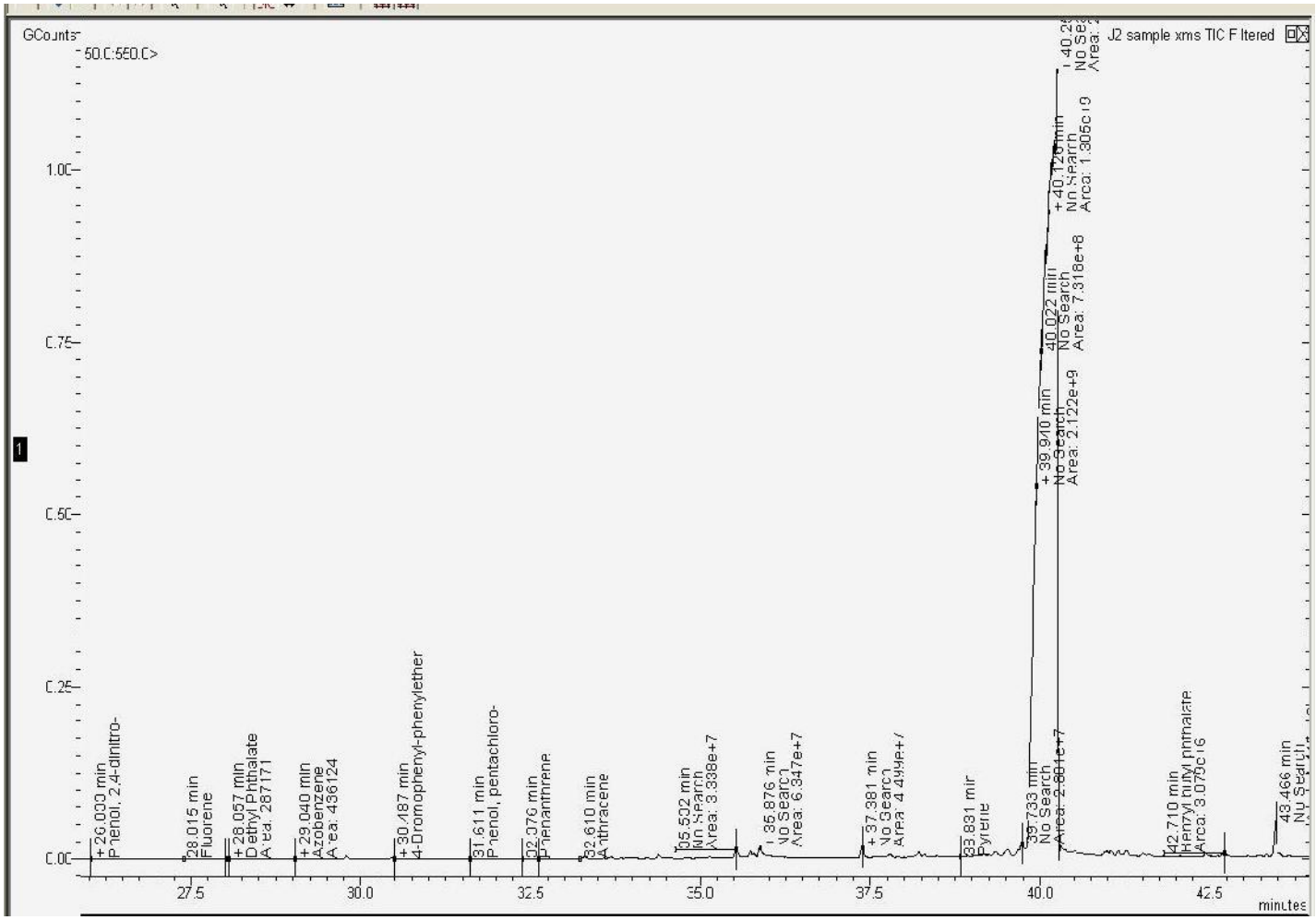


Fig. [8]. GC-MS analysis for BPA biodegradation by *Klebsiella pneumoniae* J2.

Lee, *et al*., (2005) used GC-MS to analysis of BPA metabolites produced by two species of white rot fungi and concluded that, The most abundant product among analyzed compounds was 2-hydroxy-3-phenyl propanoic acid, followed by 1-ethenyl-4-methoxybenzene, and then by phenylacetic acid and its hydroxylated compound at C2. These compounds were assumed to originate from the phenol of BPA through dehydroxylation, carboxylation, and hydroxylation on the side chain.

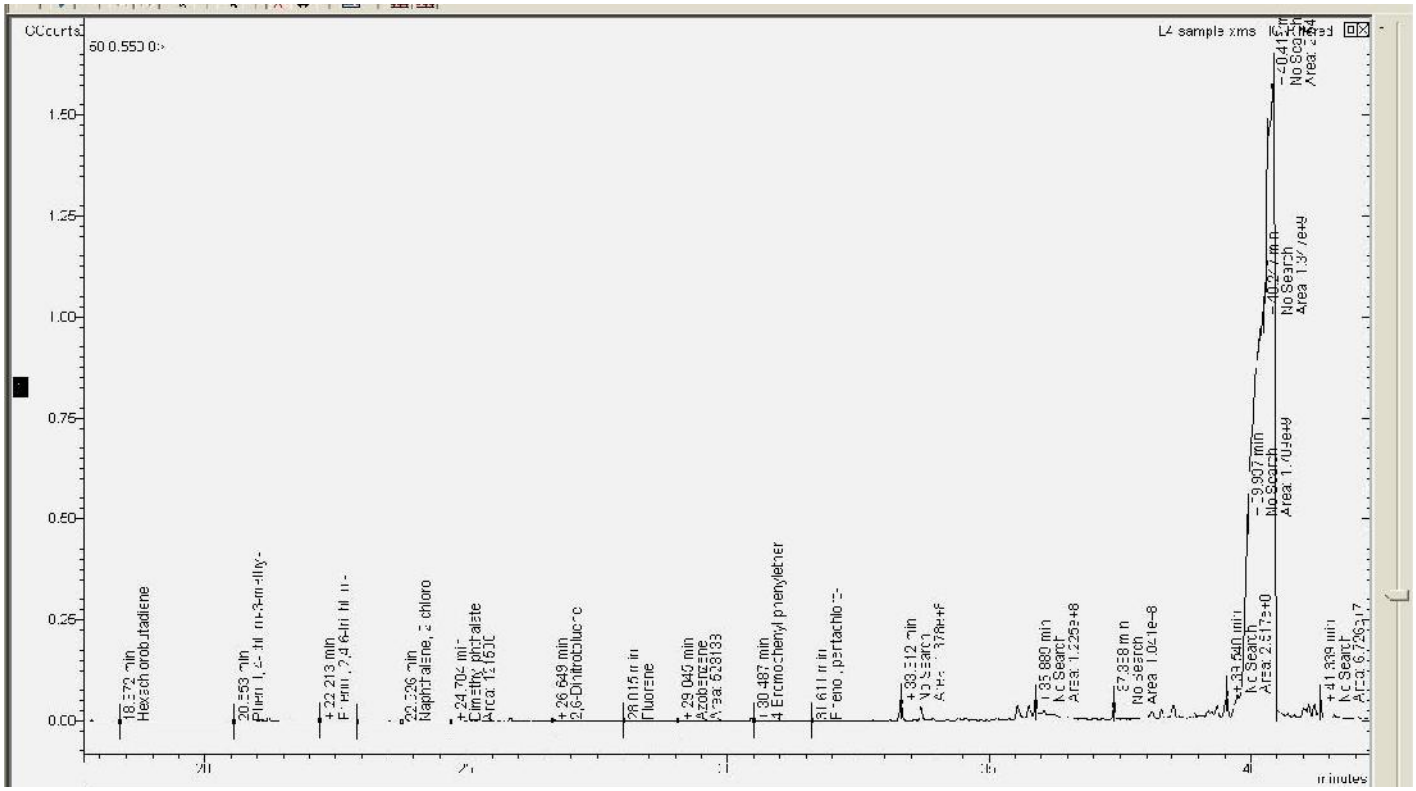


Fig. [9]. GC-MS analysis for BPA biodegradation by *Enterobacter asburiae* L4

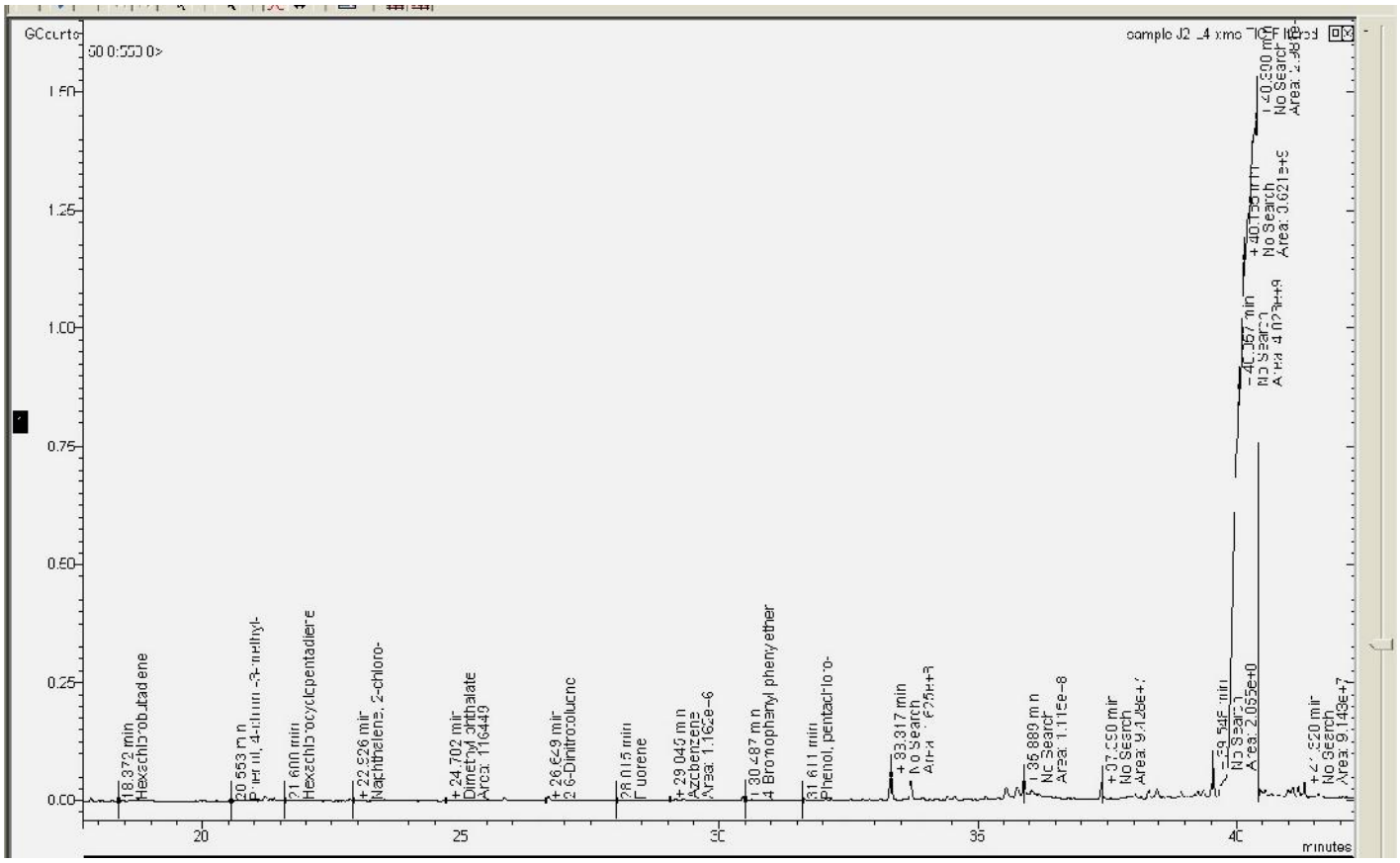


Fig. [10]. GC-MS analysis for BPA biodegradation by consortium (*Klebsiella pneumoniae* J2 with *Enterobacter asburiae* L4 at the same flask).

3.7.Isolation and curing of plasmids.

Results of plasmid extraction showed that, *Klebsiella pneumoniae* J2 containing two plasmid, their size, the smallest is 300pb while the largest was found to be above 1500pb. While *Enterobacter asburiae* L4 harboured one plasmid their size 1500pb as shown in lane 1,2 of plate (2).**Esumeh *et al.* (2009)** for instance isolated *Klebsiella pneumoniae, Enterobacter cloacae and Pseudomonas aeruginosa*, all these isolates had one plasmid of 23.1 kbp each except *Enterobacter cloacae*, which possessed an additional plasmid of 12.0 kbp. Also **Ling-zhi and Jun-wen (2006),** concluded that, the gene responsible for BPA biodegradation in *Pseudomonas* is located at a plasmid of 19kb. During current study of the plasmids were successfully cured hence the disappearance of the band post plasmid curing as shown in lane 3,4 of plate (2).

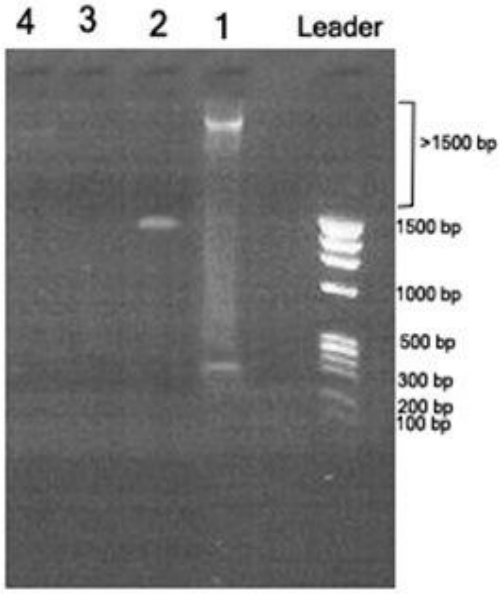


Plate [2].plasmid detection in non-cured and cured bacterial isolates

After curing of plasmid, two most potent bacterial isolates loss their ability to grow in mineral salt media containing BPA concentration which grown in it before curing. This indicated that, the loss of plasmids by *Klebsiella pneumoniae* J2 and *Enterobacter asburiae* L4, lead to loss of biodegradative ability. These observations suggest that, the gene responsible for BPA degradation was carried on plasmids. **Esumeh *et al.* (2009)** whose plasmid-cured isolates lost the ability to grow in crude oil medium while **Ajayi and Ebeigbe (2009)** whose *Staphylococcus aureus* isolate haboured plasmid gene that did not code for antibiotic resistance, these observations suggest that the chromosomal DNA of *Staphylococcus aureus* carry genes for antibiotic resistant.

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