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Biodegradation and Detoxification of Bisphenol-A by Filamentous Fungi Screened from Nature

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Authors' contributions

This work was carried out in collaboration between all authors. Author AF designed the study and wrote the protocol. Author AMAK performed writing and editing of the manuscript with managing the analyses of the study and the literature searches. Authors HHE-S and EMA-R review the first draft of the manuscript. Author AHH performed the most of experimental work. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This study demonstrated obtaining fungal isolates able to degrade and reduce the toxicity of Bisphenol-A (BPA).
Study Design: Soils enclosed by; gas stations, paint industries, and pesticides wastes, Cairo, Egypt, will be used for fungal isolation. BPA will be utilized as a sole carbon and energy source for fungal selectivity. Selected fungal strains will be optimized for BPA degradation. The residues of BPA in cultures will be determined. BPA degradation products will be identified. The toxicity of BPA degradation products on in-vitro cell viability of mammalian cell line will be investigated.
Place and Duration of Study: The study was performed in Mycological lab in botany & microbiology department in faculty of science, Al-azhar university and the Regional Center for Mycology and Biotechnology from October 2012 until May 2014.
Methodology: Different types of media shall be used for isolation, identification and purification processes. Determination of BPA concentrations will be assayed by High performance liquid chromatography (HPLC). BPA degradation products will be identified by Gas chromatography–mass spectrometry (GC-MS). Cytotoxicity test will be measured by Mammalian cell line: Vero cells

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(derived from the kidney of a normal African green monkey).
Results: Six soil samples were collected from different localities contaminated with petroleum and industrial wastes then 52 fungal isolates were purified before screened for BPA degradation. 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. The effect of different conditions on BPA degradation by (C10) and (G1) including nitrogen sources, incubation temperatures, pH and incubation periods were studied. The highest degradation amount of BPA was obtained from isolates (C10) and (G1) using medium containing sodium nitrate at pH 5 and Yeast extract at pH 7 respectively and incubation temp at 30ºC after incubation at 6 days at shaking state. According to GC-mass the BPA degradation products were identified as following compounds; Thiopropionamide, Methanone, (3-amino-2- benzofuryle) (4- chlorophenyle), 1H-pyrazole, 4,5-dihydro-5,5-dimrthyle-4-isoprpylidene, Phenol, 2,4-isopropylidenedi, Phenol, 2,6-bis(1,1-dimethylethyle)-4- (1-methyle-1-phenylethyle), Bis (2-ethylehexyle) phthalate. The toxicity of BPA was reduced after metabolized by selected fungal strains. Toxicity reduction was measured on cell viability of mammalian cell line.
Conclusion: Our results showed that *Aspergillus terreus* and *A. flavus*, have ability to degrade BPA and alter it to less toxic products. These products were tested for their toxicity by cytotoxicity test; the test showed hopeful results while compared it with the toxicity of the original compound.

Keywords: Bisphenol A; bioremediation; Aspergillus sp; cytotoxicity.

ABBREVIATIONS

BPA: Bisphenol-A, DMSO: dimethyl sulfoxide, MEA: Malt Extract Agar, CzDA: Czapek's Dox Agar, HPLC: High performance liquid chromatography, GC-MS: Gas chromatography-Mass spectroscopy, HEPES buffer: (4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid).

1. INTRODUCTION

Bisphenol-A (BPA) is an important monomer in the manufacture of polycarbonate plastics, food cans, and other daily used chemicals. Regularly and universal usage of BPA and BPA-contained products led to its ubiquitous spreading in water, sediment/soil, and atmosphere. Moreover, BPA has been identified as an environmental endocrine disruptor for its estrogenic and genotoxic activity [1]. Endocrine disruptors may be responsible for decline in sperm counts, abnormalities in the female reproductive tract, slow development in infants, increases in the incidence of testicular and breast cancer, and other medical disorders [2]. One of the cheapest possible solutions to resolve phenols contamination problem is by bioremediation using microbial cells. Many studies on biodegradation of phenol using pure and mixed cultures have been reported [3-6]. Up until now, most BPA degradation studies were mainly focused on the oxidation reaction including photo-degradation [7] and biodegradation [8,9]. Organisms such as fungi [10], bacteria [9] can all be used as biocatalysts for BPA biodegradation. Using fungi as a method of bioremediation provides an option to clean up environmental pollutants. Bioremediation using fungi has drawn little attention in the past two decades since most bioremediation research has focused mainly on the use of bacteria. Nevertheless, recently fungi have received considerable attention for their bioremediation potential which is attributed to the enzymes they produce. In addition, fungi have advantages over bacteria such as fungal hyphae that can penetrate contaminated soil to reach the pollutants [11]. Filamentous fungi are highly adaptable organisms that can grow in extreme environmental conditions, such as in contaminated areas with potentially toxic chemical compounds and elements [12]. *Aspergillus sp* was used in biodegradation of BPA [13,14] such as *Aspergillus fumigatus* [15], *Aspergillus terreus* [16] and *Aspergillus awamori* [14]. Assessing the toxicological effects of BPA, as opposed to its oestrogenic effects, has not been the main focus of the literature published on BPA. Cytotoxic effects in addition to oestrogenic effects may well be extremely important in understanding of the broader range of effects that BPA has on organisms [17]. A mammalian cell line was chosen here to present our results in a manner that is as relevant to humans as possible. The current study was designed to study the ability of different fungal isolates to degrade and detoxify the BPA. This study includes isolation of fungi from different

contaminated soils, screening as well as the optimization process to get the optimum conditions to produce the maximum activity of biodegradation. Cytotoxicity was used to evaluate toxicity of biodegradable products.

2. MATERIALS AND METHODS

2.1 Isolation of Fungal Strains

Fungi were isolated from surface and mid-depth layers of six soil samples independently collected from gas stations, paint industries, and pesticides wastes in Egypt. Around 1.0 g of sample was diluted in sterile distilled water and plated onto MEA and CzDA plates and incubated at 30ºC for 3-4 days. Various colonies of different morphologies were individually picked off and replicated on MEA plates and then kept at 4ºC for further use.

2.2 Chemicals

BPA (GC grade ≥ 99%) was purchased from sigma Aldrich (Germany). Malt Extract Agar (MEA) and Czapek Dox Agar (CzDA) were readymade (Oxoid). Tap water agar medium [18], Ingredients (g/l) consist of: (g/l) Agar 15; Tap water 1l. Mineral salt medium [19] Ingredients (g/l) consist of: Ammonium sulphate 1.0; Potassium chloride 0.2; Magnesium sulphate hepta hydrate 0.2; Calcium chloride 0.04; Ferric chloride 0.005; dipotassium hydrogen phosphate 1.0; Potassium di hydrogen phosphate 0.2; Distilled water 1l.

2.3 Screening

Tab water agar medium was supplemented with BPA (10 ppm); fresh isolated fungal disk (9mm) was placed on medium surface under a sterile conditions; Incubated at 30ºC for 21 days; concentration of BPA increased up gradually to distinguish the best tolerant fungi. Different concentrations were applied as 20, 50, 100, 150, 200, 400, 600, 800, 900, and 1000 mg/l Table 1.

2.4 Identification of Fungal Strains

Identification of fungal genera and species were carried out according to (Raper et al.; McClenny N.; Diba K et al.; Domsch KH et al. and Samson & Pitt [20-24], and confirmed by Mycology Research Laboratory, Botany & Microbiology department, Faculty of science, Al-Azhar University.

2.5 Factors affecting BPA Degradation

Different parameters (pH values, temperatures, nitrogen sources, and incubation periods and conditions) that controlling BPA biodegradation were performed. Each parameter was performed by using mineral salt medium supplemented with 200 ppm of BPA.

2.6 Assay of BPA

2.6.1 Analysis of BPA degradation by Spectrophotometric method

BPA cconcentration after degraded by selected fungal isolates was determined using Folin–Ciocalteu reagent. The residual concentration of BPA in the samples was determined after centrifugation the crude extract of culture medium for 10 min at 3500 rpm. One milliliter of the supernatant was added to 10 mL of distilled water and 1 mL of Folin–Ciocalteu reagents. The mixture was allowed to stay for 5 min and 2 mL of 20% Na_2CO_3 (w/v) was added to the mixture. The solution was left in a dark place for 60 min and the absorbance at 750 nm was measured [14].

2.6.2 Analysis of BPA degradation by High Performance Liquid Chromatography (HPLC)

A. terreus and *A. flavus* were cultured in the mineral salt medium for 6 days at 30 ^{o}C containing 200 ppm of BPA as a sole carbon source under shaking conditions. Samples were withdrawn after 6 days of culture and centrifuged at 9200 rpm for 10 minutes at 4^{o}C. The resulting supernatant was filtered through 0.22-µm Millex filter paper for removal of insoluble. The filtrate was used for the quantification of BPA and its degradation products by HPLC. BPA concentration in the filtrate was assayed by HPLC which provided with GBC U.V/visible Detector, GBC LC 1110 Pump, Winchrome chromatography Version. 1.3 Kromasil (250x4.6 mm) column. The mobile phase was a mixture of water and acetonitrile at a ratio of 1:1 (V/V). The flow rate was 1 ml/min and elution was monitored at 280nm. Detection limit by the HPLC was 0.05 ppm [25].

2.6.3 Gas chromatography- Mass spectroscopy (GC-MS) analysis

Selected fungal isolates were grown in mineral salt medium containing BPA as a sole carbon

source under all optimum environmental and nutritional culture condition. Liquid cultures were extracted for GC-MS analysis to determine the biodegradation products of BPA.

2.7 Cytotoxicity Test

2.7.1 Cell culture

Mammalian cell line: Vero cells (derived from the kidney of a normal African green monkey) were obtained from the American Type Culture Collection (ATTC cci - 81). The Vero cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactive fetal bovine serum, 1% L-glutamine, HEPES buffer, and 0.05 mg/ml gentamycin. All cells were maintained at 37ºC in humidified atmosphere with 5% CO_2 and were sub cultured two times a week.

2.7.2 Cell viability

Vero cell lines were seeded into 96-well plate at a density of $10x10^4$ cells per well in 100µl of growth medium. Three treatments were performed for this experiment, (1) Standard BPA; (2) Fungal metabolites; and (3) Fungal metabolites included degradation products of BPA. Serial two-fold dilutions of BPA were added to confluent cell monolayers dispensed into 96-well, flat-bottomed microliter plates (Falcon, NJ, USA) using a multichannel pipette. The microliter plates were incubated at 37ºC in 5% CO_2 humidified incubator for 48 h. Three individual wells were measured per treatment point. Untreated control cells were incubated with or without DMSO. Slight ratio of DMSO (maximal 0.1%) had no effect on the experiment. After the end of incubation period, the viable cells yield was examined by a colorimetric method. All experiments were carried out in triplicate. The cell cytotoxic effect was analysed by MTT assay according to the method of Mosmann and Wilson [26,27].The 50% cell cytotoxic concentration (CC_{50}), the concentration required to kill or cause visible change in 50% of intact mammalian cells, was estimated from graphic plots. STATA statistical analysis package was used for the dose response curve drawing in order to calculate CC_{50}.

3. RESULTS AND DISCUSSION

3.1 Isolation, Purification and Screening of Fungal Strains for BPA Biodegradation

Fifty-two fungal species were isolated from different soils contaminated with phenolic compounds. All isolates were purified and cultured onto MEA and CzDA media. All fungal isolates were screened for BPA biodegradation process. Fifty isolates failed to grow in the medium containing 500 ppm of BPA. Only two isolates *A. terreus* and *A. flavus* were succeeded to grow and tolerate the high concentration of BPA.

3.2 Identification of the Most Potent Fungal Isolates

Fungal isolates were identified depending on their morphological characters as a following C10 was *Aspergillus terreus*, and G2 was *Aspergillus flavus* Table 2, Fig. 1 and Fig. 2. *Aspergillus* species have been used in previous studies successfully to degrade BPA [28,29]. Many other fungi can degrade BPA but fungi with high BPA degradability are also limited [30,15]. For example, Chai et al. found that among 26 fungi strains, 11 strains could biodegrade BPA at >50% and four strains (*Fusarium sporotrichioides*, *Fusarium moniliforme*, *Aspergillus terreus* and *Emericella nidulans*) were more effective for BPA biodegradation. In this study, two *Aspergillus* species (*A. terreus* and *A. flavus*) out of fifty two fungal species screened from nature were able to degrade BPA and reduce its toxicity.

3.3 Factors Affecting BPA Degradation

Spectrophotometric method was used for measuring the quantity of BPA biodegradation process. As a result of the present investigation, the incubation temperature, pH and nitrogen sources were listed as one of the most factors affecting BPA biodegradation process [31,29]. Effect of incubation periods and incubation conditions on BPA biodegradation by both *A. terreus* and *A. flavus* was illustrated in Figs. 3A and B. It was clear from figures that, the best incubation period for biodegradation of BPA by both *A. terreus* and *A. flavus* was after incubation at 6 days under shaking condition. However, the best pH value was differing between the two *Aspergillus* species. The highest BPA

biodegradation by *A. terreus* was obtained at pH 5 while *A. flavus* was obtained at pH 7 (Figs. 3C and D). A variety of nitrogen sources were studied for BPA biodegradation process. Sodium nitrate was the best nitrogen source for BPA biodegradation process by *A. terreus* while yeast extract was the best for *A. flavus* (Figs. 3E and F). To end with temperature, 30ºC was the best temperature for both *Aspergillus* species intended for breaking down BPA (Fig. 3 G and H).

3.4 HPLC Analysis of BPA Obtained after Degradation by Selected Fungi

Concentration 200ppm of BPA standard was analyzed by HPLC and showed peak at retention time 5.02 minutes. However, when the medium supplemented with the same concentration of BPA and incubated to degrade by *A. terreus* and *A. flavus* for 6 days at optimum conditions; it showed peaks at retention times 4.87 and 5.3 minutes respectively. As shown in Table 3, 50% and 40.2% of 200 ppm (200mg/l), BPA were degraded by *A. terreus* and *A. flavus*, and each metabolite was a single peak. These results clearly indicated that BPA, at concentrations of up to 200mg/l could be degraded to simpler compounds by *Aspergillus* species. It has been reported that *Aspergillus orayzae* degrades 79% of BPA in minimal salt medium supplemented with 20mg/l [28]. Two fungi namely *Heterobasidium insulare* and *Stereum hirsutum*, cultivated in shallow stationary culture (SSC) in YMPG containing 100 ppm of bisphenol A, were shown to remove 68 to 77% of BPA. By HPLC analysis after incubation for seven days only a trace amount of BPA was found [32]. It has also been reported that degradation of 115 mg/l of BPA in LB medium was by *Sphinogomonas* sp. within six hours [33]. Further, it has been reported that the white rot basidiomycetous fungus, *Plerutous ostreatus*, degrades 0.4 mg/l of BPA, with the appearance of several intermediates, as evidenced by HPLC analysis [34]. The results of the present study clearly demonstrated that 200µg/l of BPA could be degraded more effectively by the isolated *A. terreus*.

Table 1. Screening test of C10 and G2 at different concentrations of BPA

Fungal isolate	Growth at different BPA concentrations (ppm)										
	10	20	50	100	150	200	400	600	800	900	1000
C10	+++	+++	+++	+++	++	++	++	+	+	+	-
G2	+++	+++	+++	++	++	+	+	+	+	+	-

(+++), (++), (+) and (-) means highly, moderate, poor growth and can't grow, respectively

Table 2. Showing culture characteristics of *Aspergillus* species growing on Malt Extract Agar medium (MEA)

Character	Examination (*A. terreus*)	Character	Examination (*A. flavus*)
Growth characteristics	Colonies restricted reaching 2-4 cm diameter in 4 days at 28°C, on Malt Extract Agar medium (MEA), cinnamon to light brown, buff colonies, reverse light brown.	**Growth characteristics**	Colonies grow rapidly reaching 3-5 cm diameter in 4 days at 28°C, on Malt Extract Agar medium (MEA), dark yellowish green colonies (powdery or wooly) Reverse sometimes creamy, pale brown.
Conidial heads	Columnar.	**Conidial heads**	Radiate, loosely columnar.
Conidiophore	7-10µm in diameter. (200-250) µm in length.	**Conidiophore**	10-20µm in diameter. (400-800) µm in length.
Vesicle	Sub-globose, elongated12-20 µm.	**Vesicle**	Globose to sub-globose shape.20-42 µm.
Sterigmata	In two series (biseriate), 8 µm length.	**Sterigmata**	In single series (uniseriate) 8-11µm
Conidia	Conidia, sub-spherical or oval, rough walled conidia with2.0-5.0 µm in size	**Conidia**	Conidia, spherical, smooth walled 3-7 µm.

Table 3. HPLC analysis of BPA Standard and BPA obtained after degradation by *A. terreus* and *A. flavus*

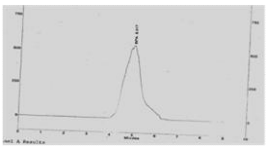
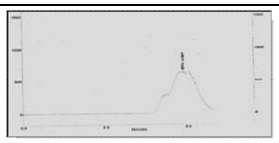
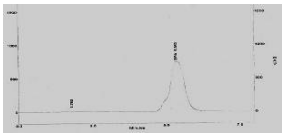
Factors	Conc. of BPA	RT (min)	Absorbance Spectrum (HPLC)
BPA Standard	200ppm	5.02	
A. terreus	100ppm	4.87	
A. flavus	119.58ppm	5.3	

Fig. 1. *Aspergillus terreus*

Fig. 2. Aspergillus flavus

Analysis of BPA metabolites using GC –MS

To investigate the degradation pathway of BPA; *A. terreus* and *A. flavus* were inoculated into mineral salt medium for 6 days at 30 °C containing BPA as a sole carbon source under shaking conditions. Many different metabolites were recognized in the GC-MS spectrum which could be identified by comparisons with the known authentic compounds at corresponding retention times. Many reports are available stating that BPA underwent a major and minor metabolic pathway of degradation pattern [35], [36]. Further it has also been reported that BPA is cleaved to 4, 2-propanol phenol and p hydroquinone. The splitting of 4-(2-proapanol) - phenol leads to 4-isopropenylphenol and 4 hydroxyacetophenone. This has been converted to p-hydroxybenzoic acid which was then mineralized. The major intermediates were 4 – hydroxyacetophenone and p-hydroxybenzoic acid. BPA was metabolized to form three major intermediates which were p-hydroxyacetophenone, p-hydroxybenzaldehyde and p-isopropenylphenol [37]. In the present study, the *Aspergillus sp*. could degrade BPA and split it to most of the previous products (Tables 4 & 5).

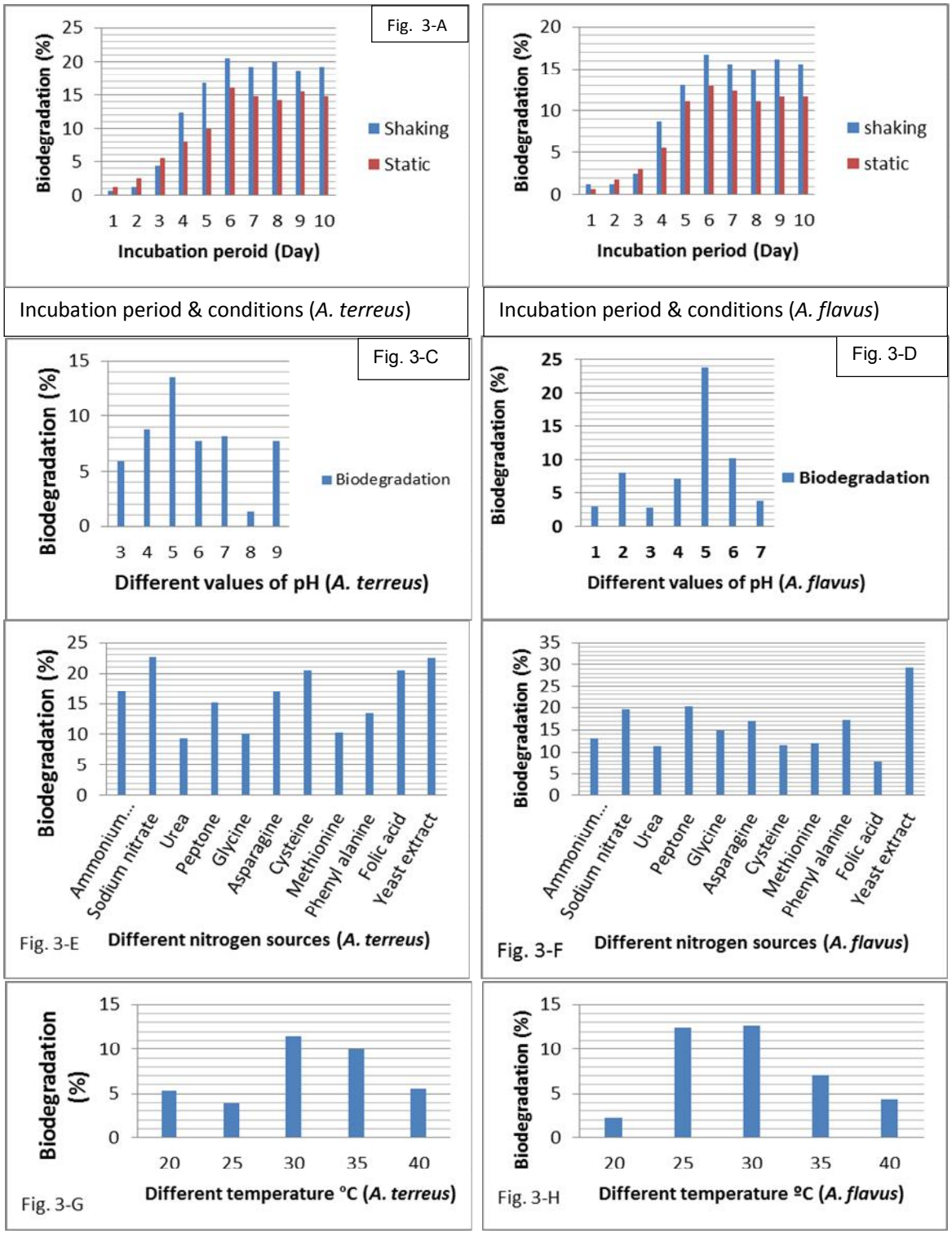


Fig. 3. Effect of different environmental parameters on BPA biodegradation by *A. terreus* and *A. flavus*

3.5 BPA Cytotoxicity

Most of BPA studies were achieved on its effects concentrate on oestrogenic property and not on direct cytotoxicity. An in-vitro approach was taken here, showing that cytotoxicity effect of BPA on the viability of kidney (Vero) cells. It has been investigated that BPA has cytotoxic effects in-vitro at low concentration [17]. Therefore, using cytotoxicity for measuring the reduction of BPA toxicity is a sensitive and an efficient test. In the present study, the cytotoxicity of BPA and its metabolites against Vero cells is shown in Fig. 4.

The 50% cytotoxic concentration (CC_{50}) of BPA for each cell line was calculated. The CC_{50} for BPA against Vero cells was approximately 0.589µg/ml. To calculate the CC_{50} of BPA after degraded by fungal cells; the metabolic activity of *A. terreus* and *A. flavus* against Vero cells with and without BPA was measured. When the metabolic activity of *A. terreus* excluded BPA the CC_{50} was 41.1µg/ml and when included BPA CC_{50} was 16.0µg/ml. Regarding *A. flavus* the CC_{50} for the metabolic activity without BPA was 17.2µg/ml while with BPA was 1.7µg/ml.

Table 4. Mass spectrum analysis of BPA degradation products of by *A. terreus*

Peak no.	RT(min)	Conc. %	Compound name
1	1.57	34.87	Thiopropionamide
2	32.01	0.61	2-(1,1-dimethylethyl)-4-(1-methyl-1-phenylethyl)phenol
3	32.10	4.5	Phenol, 2,4-isopropylidenedi
4	32.39	1.43	N-(2,4-dinitrophenyle)-2-naphthyle amine
5	33.22	27	BPA
6	35.77	1.39	Phenol, 2,4-bis(1-methyle-1-phenyle ethyle)
7	35.84	1.82	Bis (2-ethylehexyle) phthalate
8	35.88	2.33	Phenol, 2,6-ditert-butyle-4-2,3,4,5,6-pentaflurobenzyle

Table 5. Mass spectrum analysis of BPA degradation products of by *A. flavus*

Peak no.	RT(min)	Conc. %	Compound name
1	0.31	16.61	Thiopropionamide
2	13.35	0.49	1H-pyrazole, 4,5-dihydro-5,5-dimrthyle-4-isoprpylidene
3	32.25	0.90	Benzene, 3chloro -1- propenyl
4	32.41	0.81	Phenol, 2,6-bis(1,1-dimethylethyle)-4- (1-methyle-1-phenyleethyle)
6	32.6	3.09	P-chloroaniline
7	…32.82	…2.04.	1.6-di(trimethylsilyl)hexan
8	33.25	51	BPA
9	33.82	8.61	Bis (2-ethylehexyle) phthalate

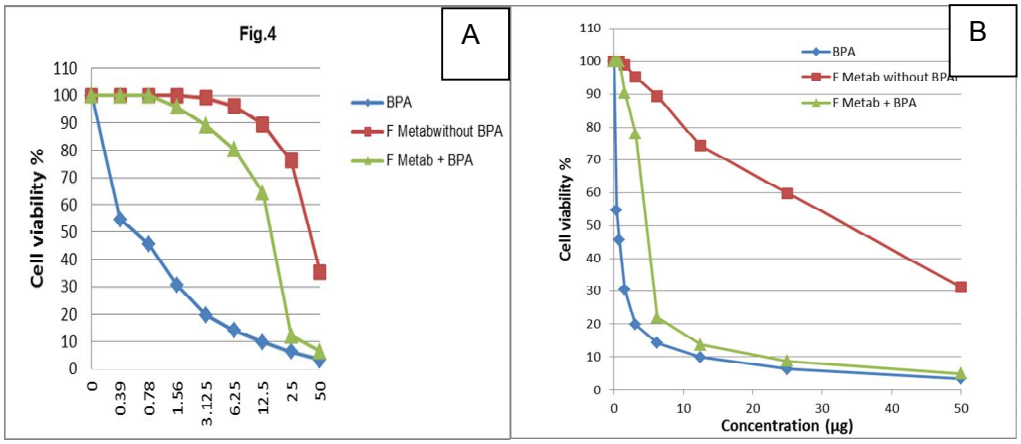


Fig. 4. Cytotoxic of BPA and its degradation products obtained in the culture of (A) *A. terreus* and (B) *A. flavus* against Vero cells

4. CONCLUSION

Aspergillus species namely *A. terreus* and *A. flavus* showed resistance to high concentration of BPA in medium, thereby utilizing it as a sole carbon source in mineral salt medium. Furthermore, it had excellent potential for degrading and detoxing BPA against Mammalian cell line in vitro. Moreover, the results obtained by HPLC, GC-MS analysis, and cytotoxicity test suggested that environmental pollution and toxicity by BPA could be eliminated by biological treatment (filamentous fungi).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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