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Full Length Research Paper

Evaluation of medium components using Plackett-Burman design for phenol degradation by marine degrading *Fusarium oxysporum*

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A new phenol-degrading marine fungal strain *Fusarium oxysporum* with high biodegradation activity and high tolerance of phenol was isolated from marine effluent of El-Max bay Alexandria, Egypt. Morphological, biochemical and 18S rRNA sequencing analysis identified the strain as *F. oxysporum RA*. This strain was capable of removing 12 and 8 mM of phenol and p-cresol in liquid minimal medium by 86 and 78% within 168 h, respectively. The fungus showed maximum activities of phenol hydroxylase (652 U/min/l at 8 mM of phenol) and aerobic p-cresol hydroxylase (377 U/min/l at 6 mM of p-cresol) at 72 h of incubation at 30°C. The Placket-Burman design was applied for medium optimization with respect to phenol degradation and phenol hydroxylase activity by *F. oxysporum RA*. The increase of KH_2PO_4 concentration and incubation temperature is significant (90%) for phenol degradation, while the increase of yeast concentration is significant (95%) for phenol hydroxylase activity production. These data demonstrated the prospect in protecting the environment from phenol and p-cresol pollution after the application of filamentous fungal strain of *F. oxysporum RA*.

Key words: Phenol and p-cresol biodegradation, marine *Fusarium oxysporum RA*, Placket Burman design, phenol and p-cresol hydroxylase assay.

INTRODUCTION

Phenols and other phenolic compounds are well known components in a wide variety of waste water including those from coal conversion processes, cooking plants, petroleum refineries, several chemical industries, pharmacy, resins and dye manufacturing companies (Afzal et al., 2007). Phenol and its derivatives are among the most frequently found pollutants in rivers, industrial effluents and landfill run off waters (Wang et al., 2006).

Due to the toxic properties of these compounds, the efficiency of their biodegradation is of great importance (Fialová et al., 2003). Diverse microorganisms, including bacteria (Prieto et al., 2002), yeast (Tsai et al., 2005), algae (Semple and Cain, 1996) and filamentous fungi (Santos et al., 2003; Mendonca et al., 2004) have evolved the metabolic capacities to degrade phenol of various concentrations.

Fungi are famous for their wide incidence and the outstanding capacity of degrading complex and inert natural product like lignin, chitin and cellulose. They adapt more easily than bacteria and are capable of growing in extreme conditions like nutrient deficiency, low pH and limited water supply (Atagana, 2004; Stoilova et al., 2008). Literature describes a number of individual

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representatives of the genera *Candida*, *Rhodoturola* and *Trichosporon*, which are capable of metabolizing aromatic compounds (Katayama-Hirayama et al., 1994). The specific enzymes responsible for the biodegradation occupy an important place in these investigations. There are certain studies attesting the ability of strains from *Penicillium, Aspergillus, Fusarium, Graphium* and *Phanerocheste* genera to disintegrate aromatic compounds (Jones et al., 1995). Santos and Linardi (2004) reported on the isolation and examination of thirteen fungal strains with respect to their phenol tolerance and the presence of phenol hydroxylase has been confirmed. *Fusarium flocciferum* and *Aspergillus fumigates* have been cited for their potential for phenol degradation (Anselmo and Novais, 1992; Jones et al., 1994). A phenol-degrading fungus, *Fusarium oxysporum* GJ4, was isolated from contaminated soil and was able to use phenol as a sole carbon and energy source (Park et al., 2009).

Hydroxylation of aromatic compounds is the initial step in their degradation by fungi (Nakagawa et al., 2006) Hydroxylase are monooxygenase or mixed function oxidases. All these enzymes are flavoproteins requiring a reduced nicotinamide adenine dinucleotide (NADH) as cofactor. In addition, these enzymes share the properties of incorporating one atom of molecular oxygen into their aromatic substrate while the second oxygen atom is reduced to H_2O by an appropriate hydrogen donor different from other enzymes (Neujahr and Gaal, 1973).

The purpose of this study was to investigate the ability of the fungus *F. oxysporum* to degrade high concentrations of some phenolic compounds as phenol and p-cresol usually presented in agro-industrial effluent, and extracellular phenol hydroxylase production ability of an isolate, elucidation of medium components affects phenol degradation and phenol hydroxylase production ability by the concerned strain to degrade and detoxify this wastes.

MATERIALS AND METHODS

Isolation and cultivation

F. oxysporum was isolated from marine water sample collected from El-Max bay that received many industrial waste effluents containing phenolic compounds, especially those discharged from refining factories like Alexandria Petroleum Company (APC) This organism was selected according to its ability to use these phenolic compounds as a sole carbon sources. Stock culture was maintained at 4°C on Sabouraud agar (0.5% yeast extract, 1% peptone, 2% glucose and 2% agar). Liquid minimal medium for the biodegradation contained in g/l: 3.4 K_2HPO_4, 4.3 KH_2PO_4, 0.3 $MgSO_4.7H_2O$, 1 $(NH_4)_2 SO_4$, 0.05 yeast extract, plus 5 ml of trace element solution (mg/l: 0.6 $FeSO_4.7H_2O$, 2.6 $CaCl_2.H_2O$) adjusted to a pH 6.5.

Phenol and p-cresol were obtained from the Microbiological Department, Faculty of Science, Alexandria University. These phenols were added from sterile stock solution to the autoclaved medium prior to inoculation.

F. oxysporum was identified by Assiut University Mycological Center, Faculty of Science and confirmed by 18S rRNA gene sequencing analysis using universal primers (18S-forward primer 5V TCCGCAGGTTCACCTACGGA -3V and 18S-reverse primer 5V-AACTTAAGGAAATTGACGGA -3V) by using chain termination reaction as described by Heinfling et al. (1997). The nucleotide sequences were analyzed with the BLAST database (Lipman, 1997).

Biodegradation experiment

Phenol degradation experiment was performed in 250 ml Erlenmeyer flasks containing 50 ml of the basal liquid minimal medium plus phenol or p-cresol (1-16 mM) as single carbon and energy sources. The flasks were aseptically inoculated with 5 disks of 5 mm for each of *F. oxysporum* RA from Sabouraud agar plate of an actively growing culture and incubated on a rotary shaker (130 rpm) at 30°C for 168 h. Non inoculated culture medium was used as control. Samples were drawn at regular interval of 24, 48, 72, 96, 120, 144 and 168 h. Mycelia were collected by filtration and the supernatant was analyzed for phenol or p-cresol degradation. Quantitative estimation of phenol and other phenolic compounds was carried out according to the method described by Martin (1949). This method is based on rapid condensation with 2.5% solution of NH_3 and 0.6% 4-aminoantipyrene, followed by oxidation with 2.5% solution of NH_3 and 2% potassium ferricyanide under alkaline conditions to give a red-coloured product which is measured immediately at 492 nm.

Degradation efficiency (DE)

The degradation efficiency (DE) expressed in percentage (%) of the initial phenol concentration was calculated as follows:

$$DE = 100 (A_o-A_t)/A_o$$

Where A_o is the absorbance value of the initial phenolic compound concentration and A_t is the absorbance value of the final phenolic compound concentration at time (t).

Biomass production

Biomass production was evaluated by determining the dry mass of mycelia (mg/10 ml). Mycelia were harvested from the cultivation liquid medium by filtration using a weighted filter paper, then dried at 50°C overnight and weighted.

Enzyme assay

Phenol hydroxylase and aerobic p-cresol hydroxylase activities were usually measured by following the disappearance of the enzyme's specific co-substrates NADPH (decrease of absorbance at 340 nm) due to the oxidation of NADPH in a reaction mixture. Standard assay mixture in 1-cm quartz cuvettes contained: 100 µmol potassium phosphate, pH 7.6, 0.5 µmol phenolic substrate in 0.1 ml distilled water and completed to 3 ml. One enzyme unit is defined as the amount of enzyme which in the presence of phenol causes oxidation of 1 µmol NADPH per min (Neujahr and Gaal, 1973).

Thin layer chromatography (TLC)

TLC of the hydroxylation products of phenol and p-cresol was performed on 0.20-mm-thick precoated plastic-backed silica gel

Table 1. Plackett-Burman design for seven variables.

Trials(n)	Independent variables						
	1	2	3	4	5	6	7
1	+	+	+	-	+	-	-
2	+	+	-	+	-	-	+
3	+	-	+	-	-	+	+
4	-	+	-	-	+	+	+
5	+	-	-	+	+	+	-
6	-	-	+	+	+	-	+
7	-	+	+	+	-	+	-
8	-	-	-	-	-	-	-

F_{254}. Chromatograms were developed in one or two solvent systems: (I) *n*-butanol-acetic acid- H_2O (12:3:5, v/v/v), (II) 5% $Na_2HPO_4.12\ H_2O$ in H_2O (Fazekas and Kokai, 1971).

Statistical design (Plackett-Burman design)

The effect of medium components on the phenol degradation and phenol hydroxylase production abilities by *F. oxysporum* were studied by applying the Plackett Burman Experimental Design (Plackett and Burman, 1946; Greasham and Inamine, 1986). In this experiment, seven factors (medium components and culturing conditions) were screened in eight combinations organized according to the Plackett Burman matrix shown in the materials and methods section (Table 1). For each variable, a high level (+1) and a low level (-1) were tested (Rajendran et al., 2007). The factors tested were: K_2HPO_4, KH_2PO_4, $MgSO_4.7H_2O$, $(NH_4)_2SO_4$ and yeast extract concentrations. Also, the pH and the degradation temperature of the tested fungus were also tested. The assays were performed in duplicate.

The main effect of each factor was determined using the following equation:

$$E_{xi} = (\sum M_{i+} - \sum M_{i-})/N)$$

Where E_{xi} is the variable main effect, M_{i+}, M_{i-} are the calculated results of the phenol degradation rate also for the phenol hydroxylase activities recorded by trial which contains positive and negative levels of independent variables (xi), respectively and N is the number of trials divided by 2. A main effect figure with a positive sign indicates that the positive level of this variable is nearer to optimum percentage of phenol degradation or enzyme activity, while a negative sign indicates that the negative level of this variable is closer to optimum percentage of phenol degradation or enzyme activity. Using Microsoft Excel, statistical t-values for equal unpaired samples were calculated for the determination of variable significance (Al-Sarrani and El-Naggar, 2006).

RESULTS AND DISCUSSION

Isolation and characterization of *F. oxysporum* strain using liquid minimal medium. The strain was identified as a member of genus *F. oxysporum* by 18S rRNA gene analysis. We compared on molecular basis, the phylogenetic relationship between the gene we analyzed and some other strains of *F. oxysporum* to find the molecular relationship as shown in Figure 1.

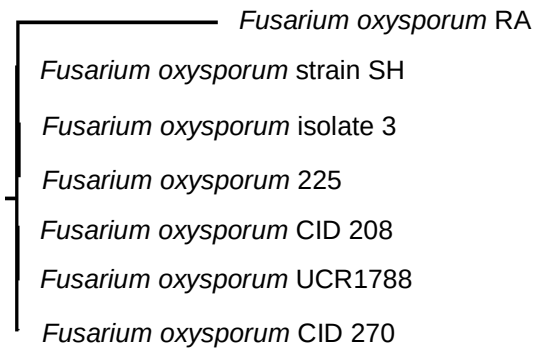

Figure 1. Comparative phylogenetic analysis of *F. oxysporum* RA with other identified *F. oxysporum* strains.

The fungal isolate is capable of growth on phenol or p-cresol as a sole carbon and energy source. The strain was selected for detailed studies because of its high phenol or p-cresol degrading rate. This strain was capable of removing 12 mM of phenol in liquid minimal medium by 86% in 7 days and 8 mM of p-cresol in liquid minimal medium by 78% in 7 days and metabolizing phenol and P-cresol at concentrations up to 16 mM.

Santos and Linardi (2004) studied the ability of phenol degradation by FIB4, LEA5 and AE2 strains of *Graphium* sp. and FE11 of *Fusarium* sp., the highest percentage of phenol degradation, with 75% degradation of (10 mM) phenol in 168 h for FIB4 strain of *Graphium* sp.

Biodegradation of phenolic compounds

The ability of *F. oxysporum RA* to degrade phenol or p-cresol separately in liquid minimal medium was compared (Figure 2A and B), the extent of phenolic compounds degradation and the time required for phenol degradation varied as a function of the initial phenol or p-cresol concentrations in the medium. As increased biodegradation time was observed, the initial phenol or p-cresol concentrations were increased. Phenolic compounds were almost completely removed in assays containing phenol concentration of 12 and 8 mM in the case of p-cresol.

For higher concentrations, considerable reductions in phenol and p-cresol degradation rates were observed and phenolic compounds were not completely removed. This behavior is probably due to the toxic effect of phenols on the cell viability at these concentrations. At high concentrations, phenols inhibits microbial growth and results in a lower removal efficiency of phenol (Israelachvili and Wennerstrom, 1996; Santos et al., 2009) At which time, the cells were began to grow vigorously (Figure 3A and B) and reached its maximum biomass. In addition, there was increase in phenol degradation rate of 12 mM from 78% by the second day

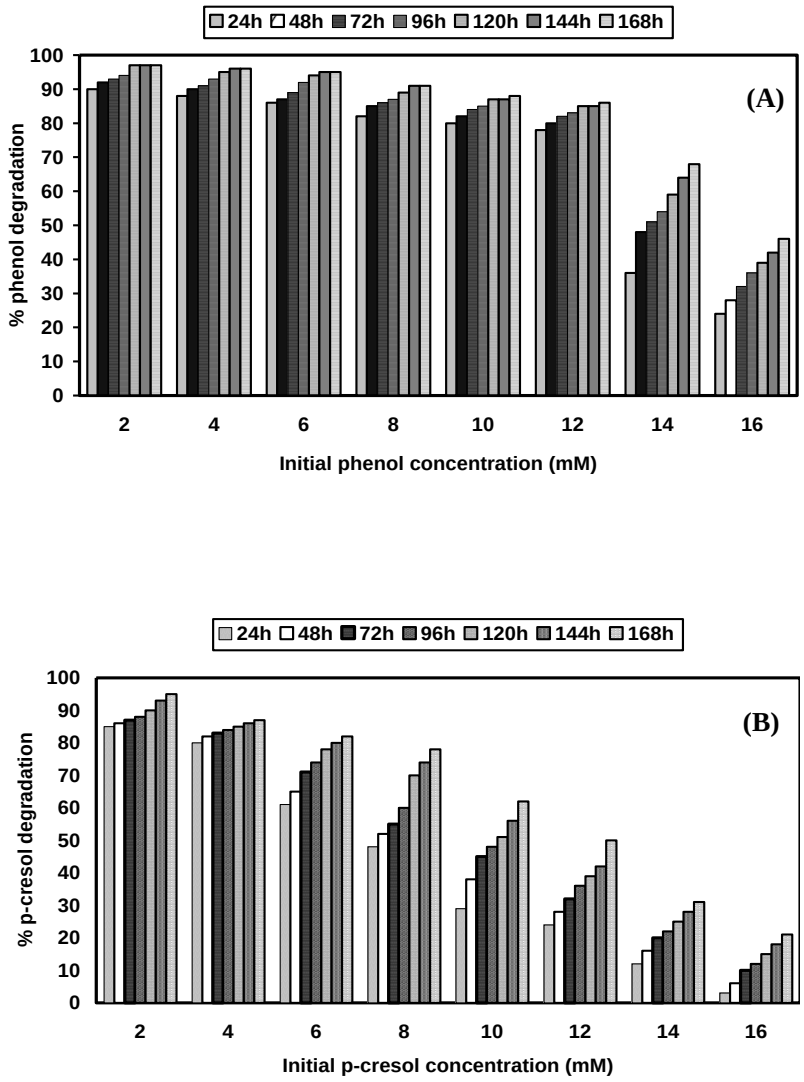

Figure 2. Effect of initial concentration (mM) of (A) phenol and (B) *p*-cresol on the rate of degradation by *F. oxysporum RA*.

up to 86% by the 7th day with the increase in the production of biomass from 41 to 120 mg/10 ml (4100 to 12000 mg/l). While there was increase in degradation rate of p-cresol (8 mM) from 48% by the 2nd day up to 78% by the 7th day with the increase in the production of a biomass from 52 to 218 mg/10 ml (5200 to 21800 mg/l). It has been observed that an increase in phenol or p-cresol concentration from 2 to 12 mM was caused a decline in fungal biomass from 450 to 120 mg/10 ml in the case of phenol by the 7th day and from 613 to 62 mg/10 ml for p-cresol. In this context, phenols can undergo cellular lyses (Ordaz et al., 1998). The decline in viability of microbial population and decrease in the growth rate with an increase in phenol concentration in the medium were determined (Hobson and Millis, 1990; Santos et al., 2001).

The maintenance of enzyme activity in the phenol degradation was studied in *F. oxysporum RA* mycelium incubated at different phenol concentration (Figure 4A and B). Activity was dependant on the phenol concentration and on the time of incubation used, levels of activity of phenol hydroxylase were higher in the case of phenol degradation than aerobic p-cresol hydroxylase in p-cresol degradation. The values of phenol and p-cresol hydroxylase activities increased as concentrations increased up to 6 and 8 mM in the assays of phenol and p-cresol, respectively; and above these values, the enzyme activities decreased. The enzyme activities were observed after 24 h of the process when the phenol and p-cresol hydroxylase were secreted in the media. Enzyme activity increased in the first 72 h in the assays involving phenolic compounds up to 2 mM concentration and it was followed by a decrease in activity with incubation time.

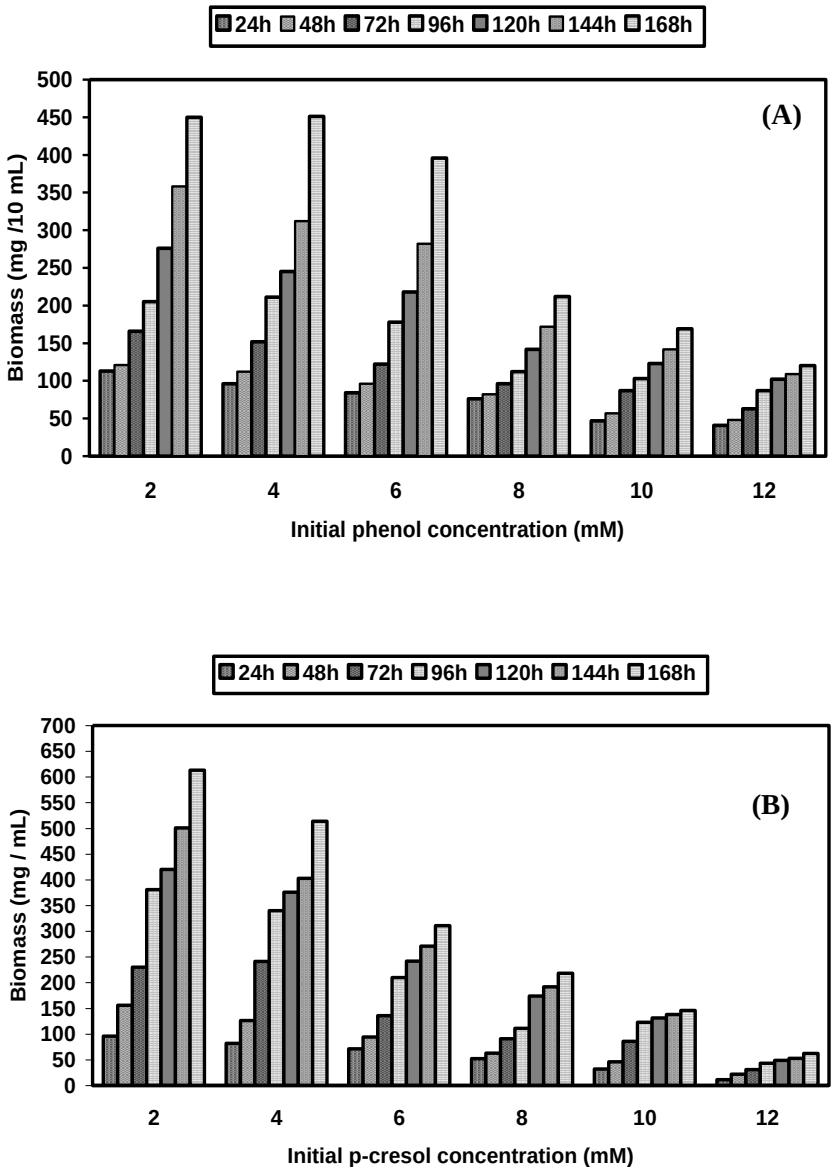

Figure 3. Effect of initial concentration (mM) of (A) phenol and (B) *p*-cresol on the production of biomass by *F. oxysporum RA*.

Mycelia growth by 72 h on 8 mM phenol exhibited extracellular phenol hydroxylase (0.652 U/min/ml) activity and at 72 h on 6 mM p-cresol exhibited extracellular aerobic p-cresol hydroxylase (0.377 U/min/ml) activity.

The results of the metabolic studies suggested that those methyl group hydroxylase and ring hydroxylations are involved in the catabolic pathways. Hydroxylation of phenol and p-cresol by phenol hydroxylase and p-cresol hydroxylase is the initial step in their degradation by the isolated fungus (Figure 5).

The identification of 4-hydroxymethyl benzyl alcohol by thin layer chromatography (TLC) as an initial attack on the methyl group of p-cresol by aerobic p-cresol hydroxylase was observed. Also, phenol hydroxylase which catalyzes a similar ring hydroxylation in the conversion of phenol into catechol was carried out (Fazekas and Kokai, 1971; Jones et al., 1994; Neujahr and Gaal, 1973).

The requirement for NADHP and oxygen for the phenol hydroxylase and p-cresol methyl hydroxylase activities in cell extracts is typical of a monooxygenase type of enzyme (Anderson and Dagley, 1980).

It seems that this mechanism of phenol and p-cresol degradation is preferable for the mass of the fungi investigated (Alexievaa et al., 2004; Jones et al., 1995; Santos et al., 2003). This mechanism may contribute to metabolic adaptation of ubiquitous fungus found in nature, such as *Graphium*, *Aspergillus* and *Penicillium*,

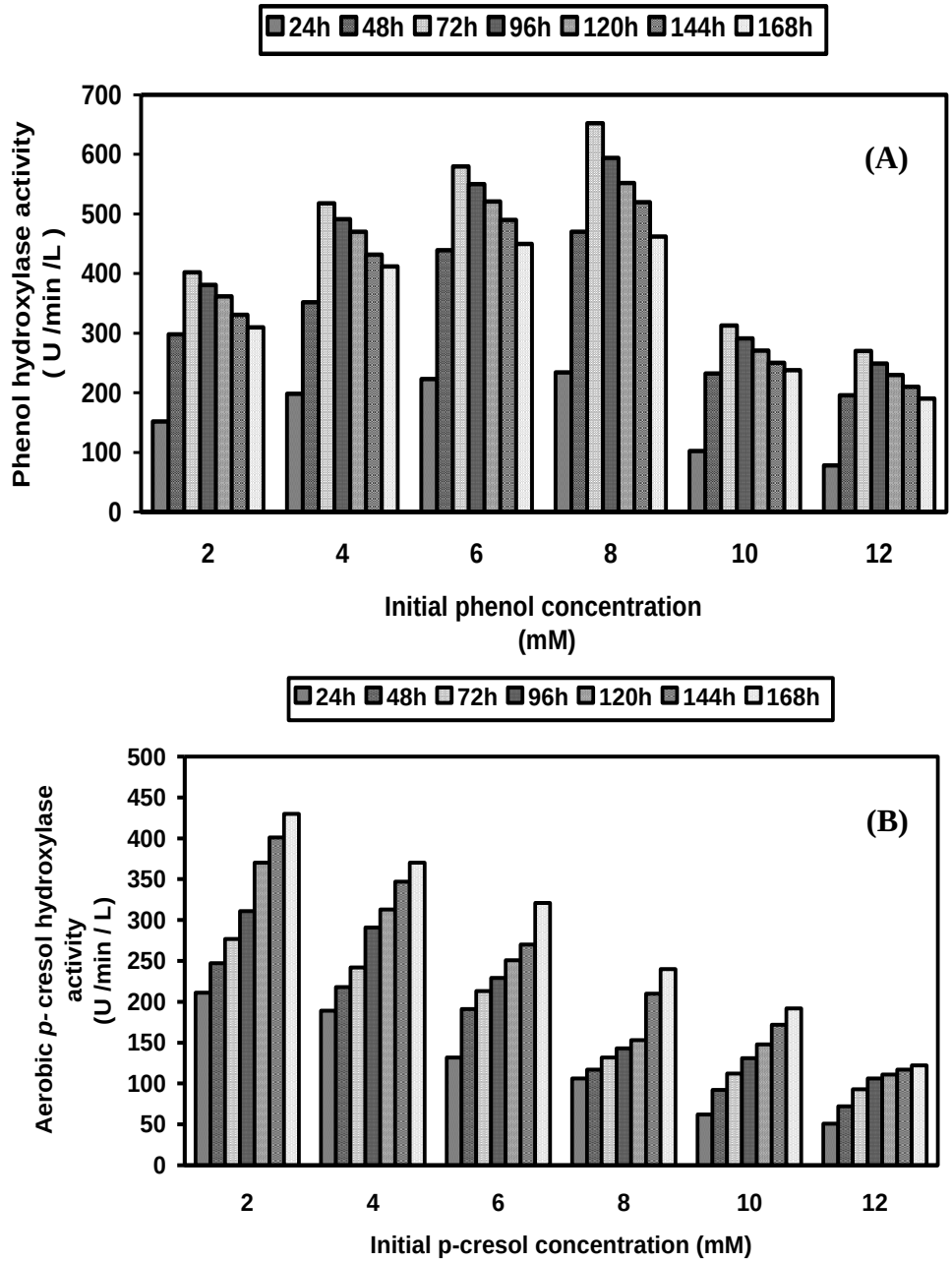

Figure 4. Effect of initial concentration (mM) of (A) phenol and (B) *p*-cresol on the production of hydroxylase activity by *F. oxysporum RA*.

exposed to xenobiotic and aromatic compounds (Stoilova et al., 2006).

Elucidation of medium components controlling phenol degradation

Optimization of the components of the liquid minimal medium for the biodegradation used for the growth of *F. oxysporum RA* and its rate of phenol degradation and phenol hydroxylase production was determined. The nutrient medium factors and cultivation conditions were screened by applying the Plackett-Burmann design as described in the materials and methods section. Eight combinations were performed and percentage of phenol degradation and unit activities of phenol hydroxylase records and calculations are shown in Tables 2 and 3.

Table 2 shows that trial 3 yielded the highest percentage of phenol degradation where 97% was obtained on incubating 5 discs (5 mm) containing the fungus for 7 days in 100 ml medium composed in g/l:

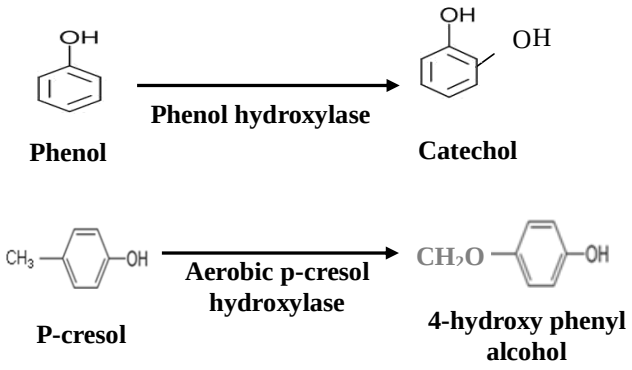

Figure 5. Ring hydroxylation of phenol and p-cresol by phenol and p-cresol hydroxylases.

Table 2. Degree of positive or negative effect of independent variables on the rate of phenol degradation by *Fusarium oxysporum* RA according to levels in the Plackett Burman experiments

Variables	Phenol degradation (%)													
	K_2HPO_4		KH_2PO_4		$MgSO_4.7H_2O$		$(NH_4)_2SO_4$		Yeast extract		pH		Temperature (°C)	
	+	-	+	-	+	-	+	-	+	-	+	-	+	-
	89	95	89	97	89	92	92	89	89	92	97	89	92	89
	92	88	92	75	97	95	75	97	95	97	95	92	97	75
	97	94	95	88	88	75	88	95	75	94	75	88	95	94
	75	67	94	67	94	67	94	67	88	67	94	67	88	67
Mean	88.25	86.00	92.50	81.75	92.00	82.25	87.25	87.00	86.75	87.50	90.25	84.00	93.00	81.25
Main effect	2.25		10.75		9.75		0.25		-0.75		56.25		11.75	
T-value	0.279		0.813		1.382		0.031		-0.090		1.579		1.801	
Deg.of sign.	N.S		90% (+)		N.S		N.S		N.S		N.S		90% (+)	

$t_{\alpha 0.05}$=1.943, $t_{\alpha 0.1}$=1.43, Deg.of sign. : Degree of significance.

K_2HPO_4 5.1, KH_2PO_4 2.15, $MgSO_4.7H_2O$ 0.45, $(NH_4)_2SO_4$ 0.5, yeast extract 0.025 and phenol (8 mM) at incubation temperature of 35°C. These results revealed that the degree of significance of KH_2PO_4 and incubating temperature was highest (90%). It showed also that, the increase of KH_2PO_4 and the increase of incubating temperature caused an increase in percentage of phenol degradation by *F. oxysporum RA* (at the 10% level of significance).

Trial 5 that yielded the highest phenol hydroxylase activity (714 U/min/ml) was obtained on incubating 5 discs (5mm) containing the fungus for 3 days in 100 ml medium composed in g/l: K_2HPO_4 5.1, KH_2PO_4 2.15, $MgSO_4.7H_2O$ 0.15, $(NH_4)_2SO_4$ 1.5, yeast extract 0.075 and phenol (8 mM) at pH 8 and incubation temperature of 25°C (Table 3).

These results showed that degree of significance of yeast extract was highest (95%). The increase of yeast extracts concentration cause an increase in phenol hydroxylase activity (at the 5% level of significance).

It appears that biodegradation of phenol could occur at room temperature with 35°C being the optimum temperature for *F. oxysporum RA* at pH 8 with phenol concentration (8 mM). Temperature apparently had a strong impact on the rate of aromatic compounds degradation, as the mesophilic temperature produced the best conditions for their degradation, or this could be solely the consequence of the temperature effect on enzyme activity (Khleifat, 2007; Leven and Schürer, 2005). It has been reported that the temperature could play an equivalent or larger role than nutrient availability in the degradation of phenol (Margesin et al., 2005).

The optimum pH for phenol degradation by *F. oxysporum RA* was 8. It is possible that the enzyme for phenol degradation have their optimum enzymatic activities at pH 8. When pH fell below 8, the degradation rate was lower, where at pH 4, phenol degrading activity of *F. oxysporum RA* was inhibited.

Similarly, pH ranges between 8 and 11 were found to be optimum for the bacterium *Halomonas campisalis*

Table 3. Degree of positive or negative effect of independent variables on the production of phenol hydroxylase activity by *Fusarium oxysporum RA* according to levels in the Plackett Burman Experiments

Variables	Phenol hydroxylase activity (U/min/ml)													
	K_2HPO_4		KH_2PO_4		$MgSO_4.7H_2O$		$(NH_4)_2SO_4$		Yeast extract		pH		Temperature (ºC)	
	+	-	+	-	+	-	+	-	+	-	+	-	+	-
	701	670	701	654	701	432	432	701	701	432	654	701	432	701
	432	510	432	714	654	670	714	654	670	654	670	432	654	714
	654	440	670	510	510	714	510	670	714	440	714	510	670	440
	714	410	440	410	440	410	440	410	510	410	440	410	510	410
Mean	625	507	560	572	576	556	524	608	649	484	619	513	566	566
Main effect	118		-12		20		-84		165		106		0	
T-value	0.164		-0.113		0.198		-0.903		2.226		1.179		0.002	
Deg.of sign.	N.S		N.S		N.S		N.S		95 (%)		N.S		N.S	

$t_{\alpha 0.05}$=1.943, $t_{\alpha 0.1}$=1.43, Deg. of sign. : Degree of significance.

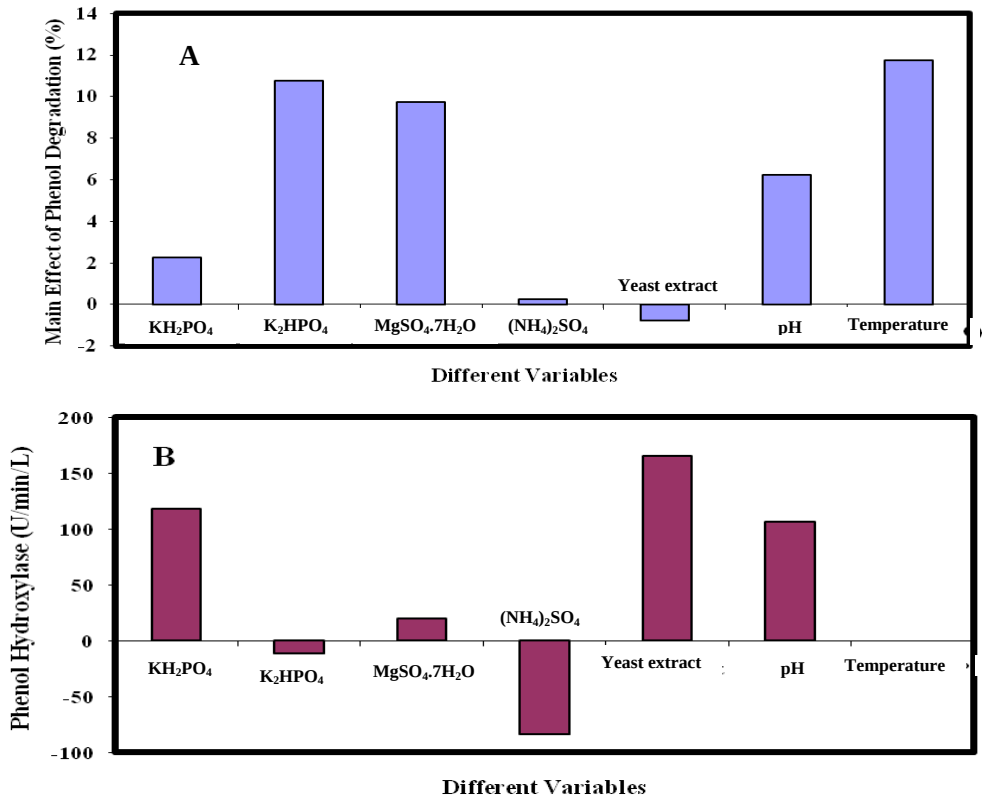

Figure 6. Main effect of (A) Phenol degradation (%) and (B) Phenol hydroxylase activity by *Fusarium oxysporum* RA according to Plackett Burman design.

required for the biodegradation of phenol (Alva and Peyton, 2003). The optimum pH and temperature were 6.8 and 37°C for phenol degradation by *Klebsiella oxytoca* (Shawabkeh et al., 2007). Also, when pH fell below 7.2, the degradation rates were lower, especially, when pH was at 5.5, phenol- degradation activity of *Acinetobacter* sp. strain PD12 was completely inhibited (Wang et al., 2007).

KH_2PO_4 as shown in Table 2 at a concentration of 2.15 g/l was significant and led to the increase of phenol degradation rate by *F. oxysporum RA*. At the same time, yeast extract as shown in Table 3 supplied at concentration of 0.075 g/l was significant and led to the increase of phenol hydroxylase activity production by the concerned fungus *F. oxysporum RA*. It was deduced from Figure 6A and B that KH_2PO_4 was the most significant

variable for phenol degradation and yeast extract concentration was the most significant variable for phenol hydroxylase activity.

Santos et al. (2003), showed that liquid minimal medium used for biodegradation of phenol by filamentous fungi isolated from industrial effluents contained in g/l: 4.3 KH_2PO_4 and yeast extract indicating that their low concentrations led to enhancement of phenol degradation and phenol hydroxylase activity.

Conclusion

Phenol and p-cresol were screened for degradation by *F. oxysporum RA* and the highest rate of degradation of phenol and p-cresol were 86 and 78% within 168 h, respectively. The maximum activity of phenol hydroxylase (652 U/min/l at 8 mM of phenol) and aerobic p-cresol hydroxylase (377 U/min/l at 6 mM of p-cresol) were attained at 72 h of incubation at 30°C. The Placket-Burman design was applied to determine the factors affecting phenol and phenol hydroxylase efficiency. The results represented by the main effect values of each variable revealed that KH_2PO_4 concentration and incubation temperature were significant for phenol degradation, while yeast extract concentration was significant for phenol hydroxylase activity. The marine *F. oxysporum* RA has a potential application in the biodegradation of phenol and p-cresol in order to insure the development of an eco-friendly technology.

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