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Evaluation of Nutritional and Environmental Conditions for Phenol Degradation by a Lebanese strain of *Candida tropicalis*

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A B S T R A C T

Phenol is a toxic hydrocarbon for nervous system, kidneys, liver, muscles and immune system. It is also mutagenic and possibly carcinogenic. Phenol containing- pollutants are dangerous and spread worldwide in the industrial effluents.The present study aimed to isolate a potent microorganism able to degrade phenol, as well as to evaluate the nutritional and environmental conditions that lead to its maximum degradation. A yeast isolated from petroleum-contaminated soil on MSM supplemented with phenol as sole carbon source, was identified by 28S rDNA sequencing. Residual phenol was estimated using 4-AAP method and the growth of yeast was measured spectrophotometrically at 600 nm. *Candida tropicalis* H was found to be highly effective for the removal of phenol. Phenol degradation was approximately 92.89 % of an initial concentration of 2000 mg phenol l^{-1} at 30°C in shaken culture (100 rpm) after 3 days incubation. After the application of Plackett-Burman design, 99.91 % degradation of phenol was achieved. The most effective factors influencing phenol degradation, among studied 11 variables, were K_2HPO_4 and culture volume. *Candida tropicalis* H could be a good candidate for the bioremediation of phenol contaminants in heavily polluted sites. A phytotoxicity study revealed that the biodegradation of phenol resulted in its detoxification, which indicated a possible use of phenol containing-effluents in the irrigation of plants after bioremediation process.

Introduction

The bio-control of pollutants resulted from the manufacture of many products such as pharmaceutical drugs, plastics, paints, detergents and others, has occupied an important interest in the scientific investigations in order to attenuate their high impact on the environment as well as on human health (Kulkarni and Kaware, 2013). Phenol is one of the dangerous pollutants spread worldwide in the environment (EPA, 2002). Its toxicity resides in the hydrophobicity of the compound and its ability to form free radicals (Hansh *et al.*, 2000). This toxicity is translated not only by its effect on different body organs and systems like skin, nervous system, eyes, liver and kidneys (Sinha *et al.*, 2011), blood components (Michalowicz and Duda, 2007), hormones (Jung *et al.*, 2004 and Aoyama *et al.*, 2005), mutations (Michalowicz and Duda, 2007), immunotoxicity (McCue *et al.*, 2003 and Yao and Hou, 2004), lethality (EPA, 2002), but also by its effect on plants (Flocco *et al.*, 2002 and Ucisk and Trapp, 2006).

Howevver, the carcinogenicity of phenol is inadequate for an assessment of the potential for phenol to cause cancer in humans (EPA, 2002), biological processes are widely used to remove phenol from different polluted areas (Arutchelvan *et al.,* 2006; Geng *et al.,* 2006 and Christen *et al.,* 2012), in addition to several physical and chemical remediation methods such as polymerization, photodecomposition, membrane based separation (Kulkarni and Kaware, 2013), electro coagulation (El-Ashtoukhy *et al.,* 2006), extraction (Khanahmedzadeh *et al.,* 2012), advanced oxidation processes (Saeed and Ilyas, 2012), adsorption and ion exchange method (Qadeer and Rehan, 2002 and Kulkarni and Kaware, 2013).

The non-biological processes have serious drawback like high cost and formation of hazardous byproducts, whereas the bioremediation furnishes economical and environmental advantages which make it the better approach for phenol removal (Banerjee and Ghoshal, 2010 and Christen *et al.,* 2012). Many studies were done to screen for appropriate microorganisms that can tolerate the inhibitory effects of phenol and effectively degrade it at relatively high concentration (Jiang *et al.,* 2007a, Liu *et al.,* 2011 and Basak *et al.,* 2013). In the present investigation, an efficient isolate of yeast was able to detoxify phenol-containing water.

Materials and Methods

Phenol stock solution and culture medium

Phenol stock solution of a concentration 50000 mg/l was prepared by dissolving 10 g of phenol into 200 ml of sterile distilled water. The solution was then filter sterilized using bacterial filter (0.2 µm pore-size) and stored in a sterilized dark bottle (250 ml) (Liu *et al.,* 2013). Ingredients of culture medium were all of analytical grade and obtained from Oxoid, Ldt. Mineral salt medium (MSM) contained (g/l) (Liu *et al.,* 2013): K_2HPO_4, 0.4; KH_2PO_4, 0.4; NaCl, 0.1; $MgSO_4.7H_2O$, 0.2; $MnSO_4.H_2O$, 0.01; $Fe_2(SO_4)_3.H_2O$, 0.01; $Na_2MoO_4.2H_2O$, 0.01; $(NH_4)_2SO_4$, 1. Final pH: 7 ± 0.2. For solid media, 15 g of agar/liter were added.

Isolation and identification of a phenol-degrading microorganism

Candida tropicalis H was isolated from petroleum-contaminated soil samples collected from different small local landfills in Beirut, Lebanon. Inocula were introduced into MSM supplemented with 1000 mg/l of phenol as sole carbon source. Shaken cultures (150 rpm) were incubated at 30 °C for a week. Transfers from liquid medium to solid medium allowed the separation of morphologically different isolates and further purification was done to obtain pure colonies. Isolates were tested to compare their phenol-degrading ability and detect the most potent strain. Fifty ml of MSM supplemented with 1000 mg/l phenol were dispensed in each 250 ml Erlenmeyer flasks, then inoculated with 5 % (v/v) from seed culture (OD 600nm = 1). The fermentation process was conducted under shaken condition at 30 °C for 5 days. Seed culture was prepared by transferring inocula from a yeast slant (24 hrs old) into 250 ml Erlenmeyer flasks containing 50 ml MSM supplemented with 1 g/l peptone (Tiwari and Mondal, 2015). The culture was grown aerobically by shaking (150 rpm) at 30 °C for 18 hrs until reached an absorbance equivalent to 1 (A 600nm = 1). A potent yeast isolate was selected and identified.

DNA was isolated according to the protocol of GeneJet genomic DNA purification kit (Thermo) number K0721. The yeast 28S rDNA was amplified from the total genomic DNA by using Maxima Hot Start PCR Master Mix (Thermo) number K1061 and by using universal specific primers designated to amplify 1500 bp fragment of the 28S rDNA

regions. The primers were: NL 1 (5'-GCATATCAATAAGCGGAGGAAAAG) and NL 4 (5' GGTCCGTGTTTCAAGACG G). The PCR was carried out for 35 cycles, denaturation at 94 °C for 1 min, annealing at 50 °C for 2 min and extension at 74 °C for 1.5 min.

An initial 3 min denaturation at 94 °C and a final 5 min extension at 74 °C were applied. Then PCR clean up to the PCR product was conducted using GeneJET™ PCR Purification Kit (Thermo) number K0701. The sequences obtained were aligned with known 28S rDNA sequences in the Genbank database using the basic local alignment search tool (BLAST) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/) and percent homology scores were generated to identify the yeast isolate.

The phylogenetic tree was constructed with MEGA 3 (Molecular Evolutionary Genetics Analysis) 3 software.

Quantitative determination of phenol

The concentration of phenol in MSM was determined by 4-AAP method (Martin, 1949 and Neumann, 2004): After removing of yeast cells by centrifugation at 6000 rpm for 20 min., the presence of phenol in the supernatant was revealed by the appearance of a pink color. The intensity of the pink color was measured using a spectrophotometer at 492 nm. The concentration of phenol in the medium was calculated based on the standard curve previously drawn using different concentrations of phenol. The percentage of phenol degradation was calculated by the mean of the following formula:

$$\frac{\text{(Initial phenol concentration – Residual phenol concentration)}}{\text{Initial phenol concentration}} \times 100$$

Nutritional and environmental factors affecting growth and phenol degradation by *Candida tropicalis* H

The effects of static and shaken conditions (100 and 150 rpm), incubation temperature (25, 30 and 35 °C), pH of culture medium (6.0, 6.5, 7.0, 7.5 and 8), incubation period (1, 2, 3, 4 and 5 days) and initial phenol concentration (1000, 1250, 1500, 1750, 2000 and 2250 mg/l), known for their impact on the activity of phenol degradation mechanism of the microorganism, were investigated. For this purpose, fifty ml of autoclaved MSM were dispensed in Erlenmeyer flasks 250 ml, inoculated with 5 % (v:v) from seed culture (OD 600nm = 1), then fortified with adequate phenol concentration (filter-sterilized) and incubated under the tested conditions. The residual concentration of phenol in the medium was evaluated and the percentage of the degraded phenol was calculated. The growth of the isolate was determined spectrophotometrically, the absorbance was measured at 600 nm against a blank derived from uninoculated medium incubated under the same conditions.

Optimization of phenol degradation by *Candida tropicalis* H. using multi-factorial statistical design: Plackett-Burman design

Plackett-Burman design was applied to reflect the relative importance of eleven various factors (K_2HPO_4, KH_2PO_4, NaCl, $MgSO_4.H_2O$, MnSO4.H2O, $Fe_2(SO_4)_3.H_2O$, $Na_2MoO_4.2H_2O$, $(NH_4)_2SO_4$, pH, inoculum size (%), and volume of culture medium (ml)) affecting the phenol degradation. MSM dispensed in 250 ml Erlenmeyer flasks and supplemented with 2000 mg/l phenol, was incubated under shaken condition (100 rpm) at 30 °C. At the end of the incubation time (3 days), the phenol measurement assay was done. All experimental conditions (trials) were performed in triplets. The main effect

that was estimated as a difference between both averages of measurements made at high level (+1) and the low level (-1) of each examined factor affecting phenol degradation, was determined using the following equation: $E_{xi} = (\Sigma M_{i+} - \Sigma M_{i-}) / N$. Where E_{xi} is the variable main effect, M_{i+} and M_{i-} are the percentages of phenol degradation in trials where the independent variable (x_i) was present in high or low level, respectively, and N is the number of trials divided by two. A main effect figure with positive sign indicates that the high level of the investigated variable is nearer to optimum and a negative sign indicates that the low level of this variable is nearer to optimum. Using Microsoft Excel, statistical t-values for equal unpaired samples were calculated for determination of variable significance.

Time course degradation of phenol under optimized conditions

Time course degradation of phenol under optimized conditions was investigated to minimize the incubation period. The optimized MSM (75 ml/flask) was dispensed in 250 ml Erlenmeyer flasks, supplemented with 2000 mg/l phenol and inoculated with 7 % (v/v) from a seed culture (OD_{600nm} = 1), then incubated under shaken condition (100 rpm) at 30 °C. Analyses were performed at different time intervals (1, 2, 3 or 4 days).

Phytotoxicity study

The toxicity of degraded phenol (2000 mg/l) in the optimized MSM by *Candida tropicalis* H was evaluated. The phytotoxicity study was carried out at room temperature, five grains of *Triticum aestivum* (wheat) and five seeds of *Lens culinaris* (lentils) were planted in pots of the same size. For each plant, two pots of each set were cultivated; the first set was irrigated with MSM (control), the second set was irrigated with treated MSM (supplemented with 2000 mg/l of phenol, inoculated with *C. tropicalis* H and incubated under optimized conditions), the third set was irrigated with untreated MSM (supplemented with 2000 mg/l of phenol). After 7 days of germination, shoot and root lengths were measured. The seedlings were dried at 60 °C till constant dry weights were recorded.

Results and Discussion

Isolation and identification of a phenol-degrading microorganism

A yeast isolate that can thrive in MSM supplemented with 1000 mg/l phenol, was selected for optimization of phenol degradation. The phylogenetic analysis based on 28S rRNA gene sequence showed 96 % similarity of yeast isolate with various *Candida* genotypes and the phylogenetic tree was presented in figure 1. Based on these phylogenetic characterization results, the selected isolate was identified as member of the species *Candida tropicalis* and it was named *Candida tropicalis* H. Many studies reported that isolated microorganisms from phenol polluted areas were adapted to phenol removal (Schie and Yong, 1998; Soudi and Kolahchi, 2011). In the present study, a yeast isolate showed a high potentiality to remove phenol from its culture medium.

Nutritional and environmental factors affecting growth and phenol degradation by *Candida tropicalis* H

The investigation on the effect of nutritional and environmental factors on the efficiency of phenol degradation by *Candida tropicalis* H revealed that the optimal degradation (approximately 92.89 %) of an initial concentration of 2000 mg phenol l^{-1} was obtained in culture incubated under shaken condition (100 rpm) at 30 °C for 3 days using MSM (pH 7.5) (Fig. 2, 3, 4 and Table 1). The

moderate shaken condition (100 rpm) within 3 days explored the moderate demand of oxygen level. Molecular oxygen is required as a co-substrate for the microbial degradation of wide variety of organic chemicals; including hydrocarbons and aromatic ring compounds (Basha *et al.,* 2010). Our finding matches with that of Kuntiya *et al*. (2013) where the moderate shaking speed of 100-125 rpm was used for phenol degradation by *Candida tropicalis* no. 10.Numerous researches carried out similar experiments to investigate the effect of temperature and pH on phenol degradation by microorganisms (Kim and Armstrong, 1981; Robertson and Alexander, 1992; Pakula *et al.,* 1999; Annadurai *et al.,* 2002; Kuntiya *et al.,* 2013 and Sivasubramania and Namasivayam, 2014). The increase of temperature from 28 °C to 30 °C improved the growth yield of *C. tropicalis* PHB5 and the biodegradation process (Basak *et al.,* 2013). These findings are in coincidence with the results obtained in the current study, where the optimal temperature was 30 °C. Decline in phenol degradation at higher temperature can be attributed to low biomass, cell decay or to the thermal deactivation of phenol degrading enzymes. In the current study, results showed that pH 7.5 led to the better phenol degradation. Similar results for phenol degradation were observed with *Candida tropicalis* HP 15 (Krug and Straube, 1986). Phenol concentration above 2000 mg/l resulted in a great inhibition of cell growth and phenol degradation. The maximum tolerable phenol concentration that *Candida tropicalis* H can degrade efficiently was 2000 mg/l. These findings are in good agreement with those found by Jiang *et al*. (2007b), where *Candida tropicalis* was able to degrade up to 2000 mg/l of phenol. The toxicity threshold concentration of phenol against microbial strains is different from a strain to another. This toxicity resulting in cell decay can be attributed at the cellular level, according to Heipieper *et al.,* 1994) to a feasible suggestion that phenolic compounds degrade the cell membrane integrity, reducing the membrane's efficacy as a selective. At the molecular level, dehydrogenase enzymes needed in catalyzing the biological oxidation of organic compounds, are membrane associated (Kewelo *et al.,* 1990 and Heipieper *et al.,* 1991 and 1992). Hence, the high concentration of phenol inhibits these enzymes leading to the decrease of degradation potential. The percentage of phenol degradation was also influenced by the incubation period; *Candida tropicalis* H degraded 25.67 % of the initial phenol concentration (2000 mg/l) within 24 hrs, the percentage increased till 92.98 % after 3 days. After 3 days, phenol degradation remained almost constant, which may be due to the saturation of active sites of utilizing enzymes (Supriya and Neehar, 2014). Also, Farag and Abd-Elnaby (2014) registered that *Fenellia flavipes* could consume 1500 mg/l of phenol during 72 hrs.

Optimization of phenol degradation by *Candida tropicalis* H. using multi-factorial statistical design: Plackett-Burman design

Plackett-Burman design was applied to reflect the relative importance of eleven various factors (Tables 2 and 3). The modified and optimized culture medium containing (g/l): K_2HPO_4, 0.6; KH_2PO_4, 0.2; NaCl, 0.15; $MgSO_4.H_2O$, 0.3; $MnSO_4.H_2O$, 0.005; $Fe_2(SO_4)_3.H_2O$, 0.015; $Na_2MoO_4.2H_2O$, 0.005; $(NH_4)_2SO_4$, 0.5, pH, 7; and volume of culture medium, 75 ml/250 ml flask, inoculated with 7 % inoculum and incubated for 3 days at 30°C under shaken condition at 100 rpm, showed phenol degradation 99.91 % which was greater than that recorded in the basal medium that showed 92.89 %. The main effect was calculated and presented graphically (Fig. 5). Statistical analysis of the data is demonstrated in table 4 as t-value for

the eleven experimental variables. The significance level was determined using the t-test. The factors which showed a high confidence percentage were K_2HPO_4 and volume of the culture medium, showing that they were the most positive significant variables affecting phenol degradation by *C. tropicalis* H (Fig. 6). Plackett-Burman design, applied in the current investigation, showed a great efficiency in the optimization of phenol degradation by *Candida tropicalis* H. The results demonstrated that the percentage of degradation was enhanced in one of the trials to 99.91 %. Culture volume with positive level was found to have an enhancement effect on phenol degradation by *Candida tropicalis* H, this finding is in agreement with that found by Kuntiya *et al*. (2013). In addition, the increase in the concentration of di-potassium hydrogen phosphate (K_2HPO_4) had a remarkable positive effect; this coincides with the results achieved in the study of Ghanem *et al*. (2009). Also, the high level of NaCl, $MgSO_4.H_2O$ and $Fe_2(SO_4)_3.H_2O$ (as obtained by Ghanem *et al*. (2009)) and the high level of KH_2PO_4 (as reported by Panidmadevi *et al*. (2014)), had positive effects on phenol degradation by *C. tropicalis* H. On the other hand, the high levels of $MnSO_4.H_2O$, $Na_2MoO_4.H_2O$, and $(NH_4)_2SO_4$ had negative effects on phenol degradation by *C. tropicalis* H contrarily to Ghanem *et al*. (2009), MnSO4.H2O and $NaMoO_4.2H_2O$ had a negative effect on phenol degradation by *C. tropicalis* H.

Table.1 Effect of pH on growth and phenol degradation by *Candida tropicalis* H

pH	Phenol degradation (%)	Growth (OD_{600nm})
6.0	90.36	1.17
6.5	91.23	1.21
7.0	92.82	1.27
7.5	92.98	1.43
8.0	91.81	1.11

Table.2 Variables and their levels employed in Plackett-Burman design for screening of some factors affecting phenol degradation by *Candida tropicalis* H

Symbol	Variable	Low (-)	Basal (0)	High (+)
A	**K_2HPO_4**	0.2	0.4	0.6
B	**KH_2PO_4**	0.2	0.4	0.6
C	**NaCl**	0.05	0.1	0.15
D	**$MgSO_4.H_2O$**	0.1	0.2	0.3
E	**$MnSO_4.H_2O$**	0.005	0.01	0.015
F	**$Fe_2(SO_4)_3.H_2O$**	0.005	0.01	0.015
G	**$Na_2MoO_4.2H_2O$**	0.005	0.01	0.015
H	**$(NH_4)_2SO_4$**	0.5	1	1.5
I	**pH**	7	7.5	8
J	**Inoculum size (%)**	3	5	7
K	**Culture volume (ml)**	25	50	75

Table.3 Randomized Plackett-Burman experimental design for evaluating factors influencing phenol degradation by *Candida tropicalis* H

Variable / Trial	A	B	C	D	E	F	G	H	I	J	K	Phenol degrada - tion (%)	Growth OD_{600nm})
1	+	-	+	-	-	-	+	+	+	-	+	**93.55**	1.52
2	+	+	-	+	-	-	-	+	+	+	-	**67.26**	0.81
3	-	+	+	-	+	-	-	-	+	+	+	**71.72**	1.24
4	+	-	+	+	-	+	-	-	-	+	+	**99.91**	1.89
5	+	+	-	+	+	-	+	-	-	-	+	**93.80**	1.53
6	+	+	+	-	+	+	-	+	-	-	-	**72.52**	0.85
7	-	+	+	+	-	+	+	-	+	-	-	**58.08**	0.78
8	-	-	+	+	+	-	+	+	-	+	-	**38.52**	0.77
9	-	-	-	+	+	+	-	+	+	-	+	**71.76**	1.26
10	+	-	-	-	+	+	+	-	+	+	-	**63.23**	0.88
11	-	+	-	-	-	+	+	+	-	+	+	**72.74**	1.36
12	-	-	-	-	-	-	-	-	-	-	-	**38.02**	0.64
13	0	0	0	0	0	0	0	0	0	0	0	**92.89**	1.04

Table.4 Main effect and t-test of variables affecting phenol degradation by *Candida tropicalis* H

Factors	Main effect	t-test
A	**23.23**	**2.48**
B	5.18	0.44
C	4.58	0.38
D	2.92	0.24
E	-3.00	-0.25
F	5.89	0.50
G	-0.21	-0.01
H	-1.40	-0.11
I	1.68	0.14
J	-2.39	-0.20
K	27.47	3.42

Table.5 The influence of irrigation with treated and untreated phenol supplemented MSM on the growth of *Triticum aestivum* (monocot plant) and *Lens culinaris* (dicot plant)

Test plant	*Triticum aestivum*			*Lens culinaris*		
Growth parameter / Irrigation with	Shoot length (cm)	Root length (cm)	Dry weight (g)	Shoot length (cm)	Root length (cm)	Dry weight (g)
MSM (control)	13.1	14.2	0.19	13.8	10.2	0.29
Treated MSM	12.5	13.3	0.18	12.2	8.1	0.25
Untreated MSM	No germination of both plants					

Fig.1 Phylogenetic relationships among representative experimental yeast isolate and the most related strains of *Candida tropicalis*

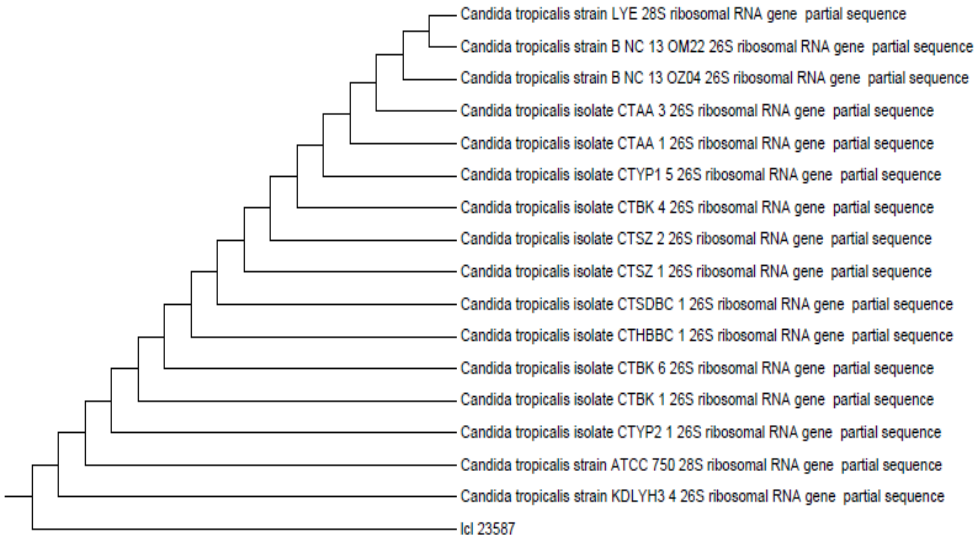


Fig.2 Effect of cultivation conditions on growth and phenol degradation by *Candida tropicalis* H

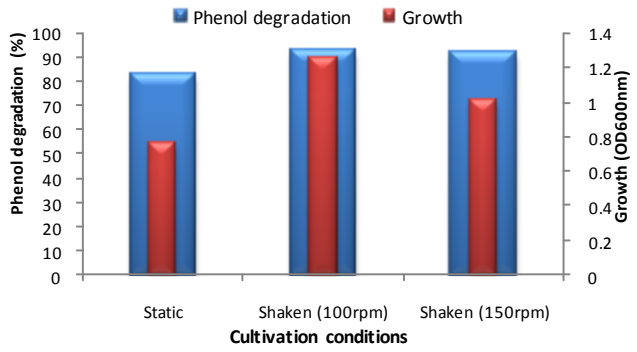


Fig.3 Effect of incubation temperature on growth and phenol degradation by *Candida tropicalis* H

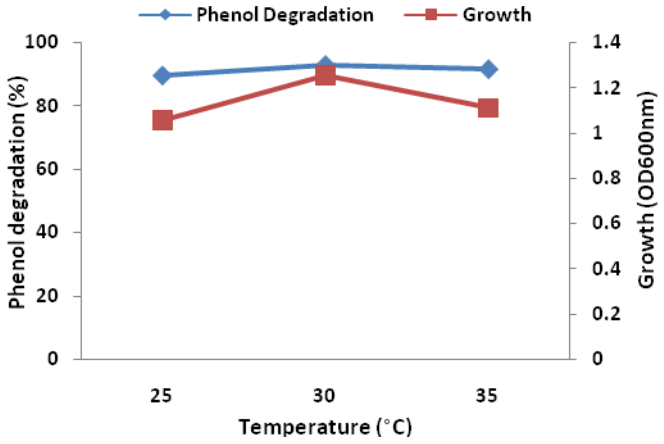


Fig.4 Effect of initial phenol concentration and incubation period on phenol degradation by *Candida tropicalis* H

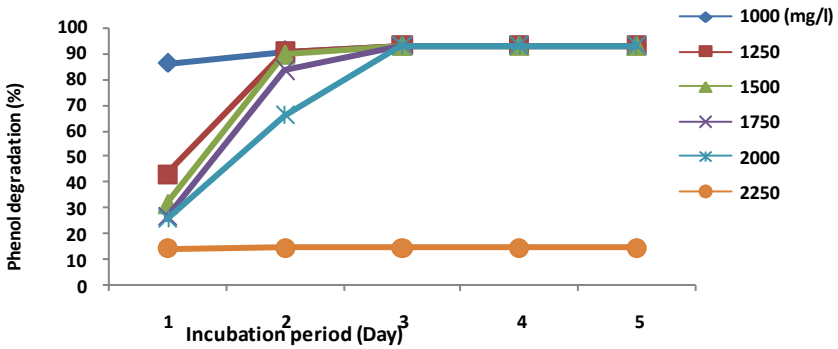


Fig.5 Main effect of variables affecting phenol degradation by *Candida tropicalis* H

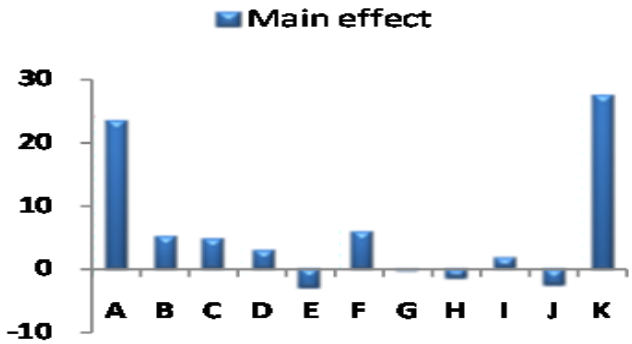


Fig.6 Effect of K2HPO4 and volume of the culture medium on phenol degradation by *Candida tropicalis* H

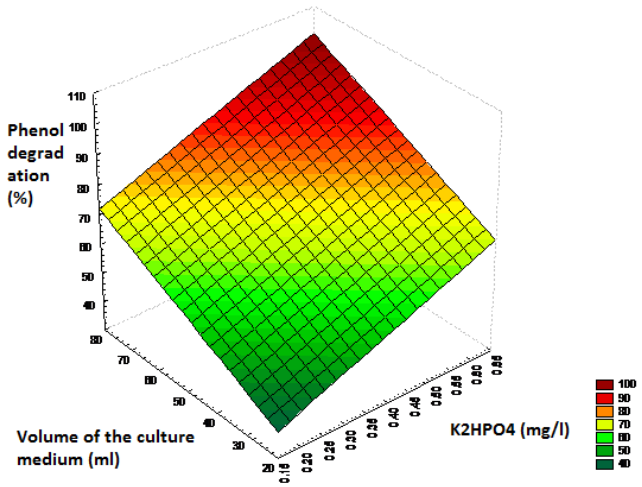
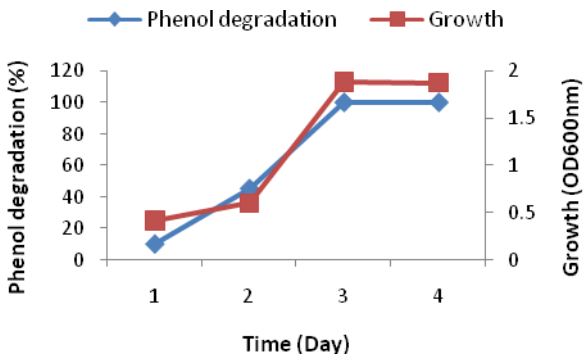


Fig.7 Time course degradation of phenol by *Candida tropicalis* H under optimized conditions



Time course degradation of phenol under optimized conditions

Time course degradation of phenol under optimized conditions was investigated to monitor the daily degradation process. The growth and percentage of phenol degradation by *Candida tropicalis* H were recorded (Fig. 7). The data showed that an incubation period shorter than 3 days was not enough to eliminate the concentration of phenol under investigation.

Phytotoxicity study

The influence of irrigation with treated and untreated phenol supplemented MSM on the growth of *Triticum aestivum* and *Lens culinaris* was demonstrated in table 5. Both plants failed to germinate when irrigated with untreated phenol supplemented MSM (2000 mg/l). However, they grew well when irrigated with treated phenol supplemented MSM by *Candida tropicalis* H and reached their maximum growth after 7 days. The growth of plants was compared with the

control, which were the plants irrigated with MSM. Dry weights determined were in accordance with the observable plant growth (shoot and root lengths).

Phenol-containing effluents are known to cause environmental and health hazards and they are discharged in the natural water sources like rivers, unfortunately used to irrigate plants. Thus, assessing the phytotoxicity of the treated and the untreated phenol containing media was an important concern. In the present study, the results were found to be in accordance with the findings of Wolski *et al*., (2012), who proved that the degraded phenol (400 mg/l) by the fungal strain *Penicillium chrysogenum* Thom ERK1 was not toxic for the germination and growth of wheat.

In the present study concluded that the isolated yeast *Candida tropicalis* H was able to degrade (after optimization of the nutritional and environmental factors affecting phenol degradation process) 99.91 % of 2000 mg/l phenol in MSM (pH 7.5), within 3 days at 30 °C under shaken condition (100 rpm). A phytotoxicity study revealed that biodegradation of phenol by *C. tropicalis* H resulted in its detoxification. Therefore, phenol containing industrial and municipal effluents can be treated using *C. tropicalis* H in order to be used in the irrigation of non-edible plants.

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