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High potential of thermotolerant *Candida tropicalis* no. 10 for high concentration of phenol biodegradation

Ampin Kuntiya[1], Shinji Takenaka[2] and Phisit Seesuriyachan[1,*]

Abstract

A thermotolerant *Candida tropicalis* strain No. 10 was isolated from a chemical contaminated soil sample. The strain was capable of degrading 100 mg/L phenol completely from 20-42°C, a wider temperature range than those previously reported in yeasts. It could also completely degrade phenol at an initial concentration up to 1,000 mg/L in a minimum mineral salt medium. The optimum temperature and initial pH were 35°C and 8, respectively. Phenol degradation was inhibited when oxygen in the medium was gradually decreased. Most tested sugars such as fructose, galactose, glucose, maltose, mannose, sorbitol and sucrose of 10 mM concentration, inhibited phenol degradation completely, whereas most organic acids; citric, lactic and succinic acids of 10 mM concentration, showed no effect. At the level above 0.31 mM glucose, phenol removal decreased in an opposite direction of glucose. Metal ions such as Co^{2+}, Cu^{2+} and Ni^{2+} showed strong inhibition to phenol degradation whereas Zn^{2+} showed slightly inhibition. The strain metabolized phenol via catechol *ortho*-clevage pathway and the catechol 1,2-dioxygenase specific activity was comparable to other *Candida* spp.

Keywords: *Candida tropicalis*, catechol degradation, ortho-cleavage pathway, phenol, thermotolerant

1. Introduction

Degradation of phenol, a common toxic pollutant derived from a wide variety of industries, using a biological process has been extensively studied because of several advantages such as effectiveness, minimal cost of operation and potential of degrading the pollutant to innocuous products. Numerous microorganisms, especially bacteria and fungi, have been employed in such processes (Shawabkeh et al., 2007; Jones et al., 1995). Several yeasts have been shown to degrade phenol (Adav et al., 2007; Bastos et al., 2000), *C. tropicalis* is a candidate of genus *Candida* being the most studied.

Many microbial strains have been reported with the ability of phenol biodegradation at a mesophilic range of 29-30°C (Wang and Li, 2007; Chen et al., 2002; Bastos et al., 2000) and thermophilic range of 50-70°C (Ali et al., 1998; Mutzel et al., 1996). Unfortunately, little

[1]Division of Biotechnology, Faculty of Agro-Industry, Chiang Mai University, Chiang Mai 50100, Thailand

[2]Department of Agrobioscience, Graduate School of Agricultural Science, KobeUniversity, 1 1 Rokkodai, Nada Ku, Kobe 657 8501, Japan

*Corresponding author. Tel/Fax: +66 5394 8201 E-mail: phisit.s@cmu.ac.th, phisit.seesuriyachan@gmail.com

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informationis available on those which could degrade phenol at a wide range of temperature (Kotresha and Vidyasagar, 2008; Shawabkeh et al., 2007; Adav et al., 2007), especially a thermotolerant type (Jones et al., 1995). The thermotolerants aremesophiles which can grow at high temperatures, 37-40^{o}C (Saeki et al., 1997) and are considered superior in many aspects than the non-thermotolerant counterparts. One of these advantages includes the cultivation of thermotolerants in South East Asian Countries such as Thailand with the typical room temperature of 35-40^{o}C.

In this paper, we reported an isolation, selection and characterization of thermotolerant yeast strains from chemical contaminated soil samples. One among them, identified as *C. tropicalis*, showed the high potential of phenol degradation in a mineral medium. The strain was a thermotolerant type capable of degrading phenol at temperature range of 20-42^{o}C. In addition, the effects of various factors on phenol degradation were also reported.

2. Materials and Methods

2.1 Medium

The medium used was a mineral salt medium (Ralston and Vela, 1974) which contained (g/l): $NaHCO_3$ 0.125; KH_2PO_4 0.1; NH_4Cl 0.07; Na_2SiO_3 0.02; $FeSO_4.7H_2O$ 0.007; $MnCl_2.4H_2O$ 0.01; $ZnSO_4.7H_2O$ 0.0015; casamino acid 0.01. The pH was adjusted to 8.0 using NaOH solution. When a solid medium was prepared, an agar was added (15 g/L).

2.2 Isolation, selection and identification of phenol degrading microorganism

The soil samples were serially diluted with water before plating onto a mineral salt agar medium containing 200 mg/L phenol and incubated at 35^{o}C for 24-48 h. Total number of 10 morphologically different colonies were selected and purified. For the selection of the strains having high potential for phenol degradation, an inoculum was prepared by transferring individual isolate from the mineral salt agar slant to nutrient broth containing 200 mg/L phenol and incubated at 35^{o}C for 24 h. Phenol degradation test was conducted by inoculation a 100 mL medium salt medium containing phenol of varying concentrations (250-1,000 mg/L) with 1 mL nutrient broth inoculum and incubation at 35^{o}C under shaking condition (120 rpm). Samples were periodically taken and assayed in duplicate for pH, remaining phenol and biomass concentrations. The strain showing the highest phenol degradation potential was identified as *Candida tropicalis* using biochemical tests (Larone, 2002) and later confirmed by 26S rRNA gene sequence analysis. On the agar medium used in this study, the strain colonies were rounded small, pale yellow colored and smooth appearance.

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2.3 Effect of temperature on phenol degradation

One hundred milliliter volumes of the mineral salt medium in 250 mL Erlenmeyer flasks containing 100 mg/L phenol was inoculated with 1 mL inoculum and incubated at 20, 25, 30, 35, 40, 42 and 45^{o}C on a rotary shaker (120 rpm). All experiments were done in triplicate and the average values were reported.

2.4 Effect of various factors on phenol degradation

The mineral salt medium of 100 mL volumes in 250 mL Erlenmeyer flasks with 1,000 mg/L phenol and 1% (v/v) inoculum was used as a standard condition in the investigation of factors affecting phenol degradation. The flasks were incubated at 35^{o}C on a rotary shaker with shaking speed of 120 rpm. The examined factors included; phenol concentration (350, 500, 750, 1,000 and 1,250 mg/L), pH (5-9), oxygen concentration (50, 100, 150 and 200 mL of medium volume), sugars (10 mM of D-arabinose, D-fructose, D-galactose, D-glucose, myo-inositol, lactose, maltose, D-mannose, D-sorbitol and sucrose), organic acids (10 mM of citric, D,L-malic, lactic and succinic acids), metal ions (0.04 mM of Co^{2+}, Cu^{2+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, Ni^{2+}, Zn^{2+}) and glucose concentration (0–10 mM).

2.5 Enzyme assays

Cells were disrupted by quartz sand in a grinding bowl for preparation of cell extracts. Catechol 1,2-dioxygenase and catechol 2,3-dioxygenase were assayed according to published procedures (Aoki et al, 1984; Murakami et al., 1998).

2.6 Analytical methods

Biomass concentration was determined by measuring optical density at 600 nm. Phenol concentration was measured by the 4-aminoantipyrine colorimetric method (APHA, 1998) on a supernatant drawn from samples centrifuged at 15,000 *g*.

3. Results and Discussion

3.1 Isolation and selection of phenol-degrading microorganisms from soil samples

Ten phenol-degrading strains from chemical contaminated soil samples collected from chemical laboratory effluent manholes at the Faculty of Agro-Industry, Chiang Mai University, were isolated from the mineral salt medium containing 200 mg/L phenol. Among 10 morphologically different colonies, 5 strains grew well under high concentration of phenol (Table

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1). The efficient phenol degradation of strain No. 10 was evident and was therefore used for further experiments.

Table 1. Phenol degradation by isolated microorganisms in mineral salt medium containing different concentrations of phenol.

Strain No.	% degradation (cultivation time in days)			
	350 ppm	500 ppm	750 ppm	1,000 ppm
3	70(2)	35(3)	0[a]	0[a]
4	98(2)	96(3)	9(3)	0(4)
8	87(2)	54(3)	0(3)	0(4)
9	100(2)	0(3)	0[a]	0[a]
10	100(2)	100(3)	100(3)	100(4)

[a] The isolates did not grow in each test medium.

3.2 Identification of the strain No.10

Biochemical and morphological properties were examined by using previously cited methods (Larone, 2002.). The nucleotide sequence of the 26S rRNA gene of strain No.10 (558 bp; accession number AB375431) appeared identical to that of *Candida tropicalis* type strain.

3.3 Effect of temperature on phenol degradation

In this study, *C. tropicalis* strain No.10 showed an ability to degrade phenol in a mineral salt medium containing 100 mg/L phenol at 20-42^{o}C (Fig. 1a). The strain completely degraded phenol at 30, 35, and 40^{o}C within 8 h and at 20 and 42^{o}C within 16 and 24 h, respectively. The microbe was thus classified as a thermotolerant strain (Saeke, et al., 1997). Completed degradation at 25-40^{o}C was achieved when aerobic granules of *C. tropicalis* were cultivated (Adav et al., 2007). *Klebsiellaoxytoca* could degrade phenol at 25-42^{o}C (Shawabken et al., 2007) whereas *Pseudomonas aeruginosa* strain (MTCC 4996) at 15-45^{o}C (Kotresha and Vidyasagar, 2008). To the best of our knowledge, therefore, strain No.10 showed wider range of temperature for phenol degradation than those previously reported in yeast. An optimum temperature for phenol degradation rate and biomass concentration was 35^{o}C as shown in Fig. 1a and 1b, respectively.

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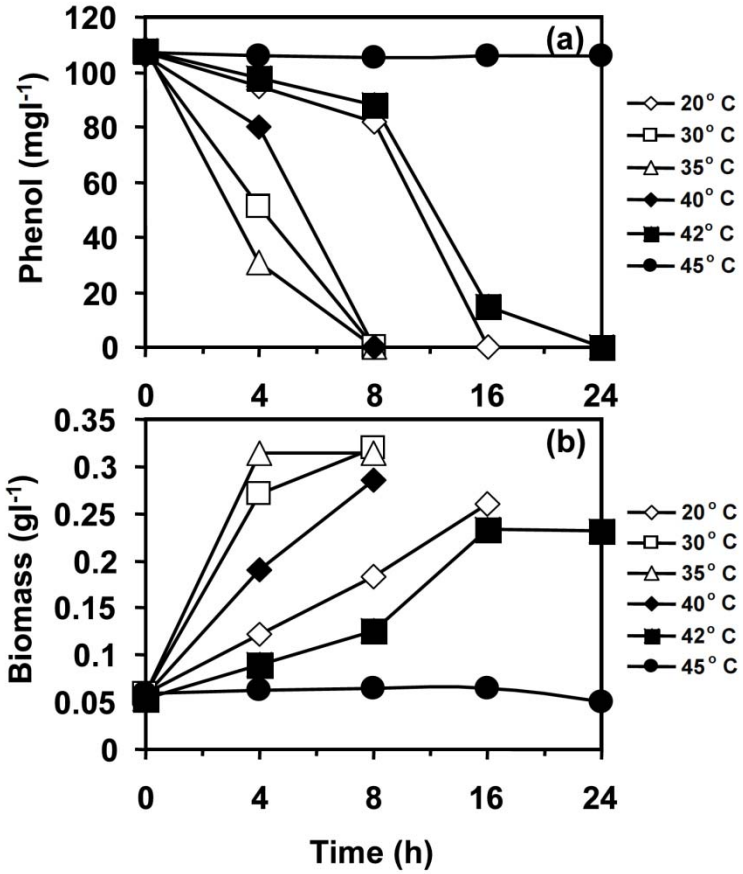

Figure 1. Effect of temperature on phenol degradation by *C. tropicalis* strain No.10

a) Phenol degradation b) Biomass concentration

3.4 Effect of various factors on phenol degradation

To test the tolerance of microbe against phenol, the cultivation in various phenol concentrations (350-1,250 mg/L) was performed. At the concentration up to 1,000 mg/L, the strain was able to degrade phenol completely in a sole mineral salt medium without additional supplement, whereas at 1,250 mg/L 72% degradation was achieved (Fig. 2a). The comparison was made to a number of microbes capable of degrading phenol at high concentrations (> 500 mg/L), it was evident that those microbes required certain supplements (e.g. yeast extract and peptone) to attenuate phenol toxicity (Kotresha and Vidyasagar, 2008; Lob and Tar, 2000). In order to achieve the highest degree of degradation, the concentration of 1,000 mg/L phenol was chosen for further study.

An initial pH of the mineral salt medium containing 1,000 mg/L phenol on phenol degradation by *C. tropicalis* strain No. 10 were adjusted from 5-9. The isolated strain grew well and degraded phenol efficiently under weak alkali and neutral conditions (Fig. 2b). This was in

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contrast with a typical yeast in which the growth preference was under weak acidic condition. Complete degradation of 1,000 mg/L phenol was achieved within 2.5 days at initial pH of 8.0. The faster phenol degradation was observed in this experiment relative to free cells of *C. tropicalis* NCYC 1503 which was able to remove 1,000 mg/L phenol within 180 h (7.5 days) at pH level of 7.2 (Chen et al., 2002). An ability of *C. tropicalis* to tolerate a broad pH range of 3-9 (Bastos et al., 2000) and to grow at the optimal pH range of 5-7 had been reported (Adav et al., 2007; Kotresha and Vidyasagar, 2008).

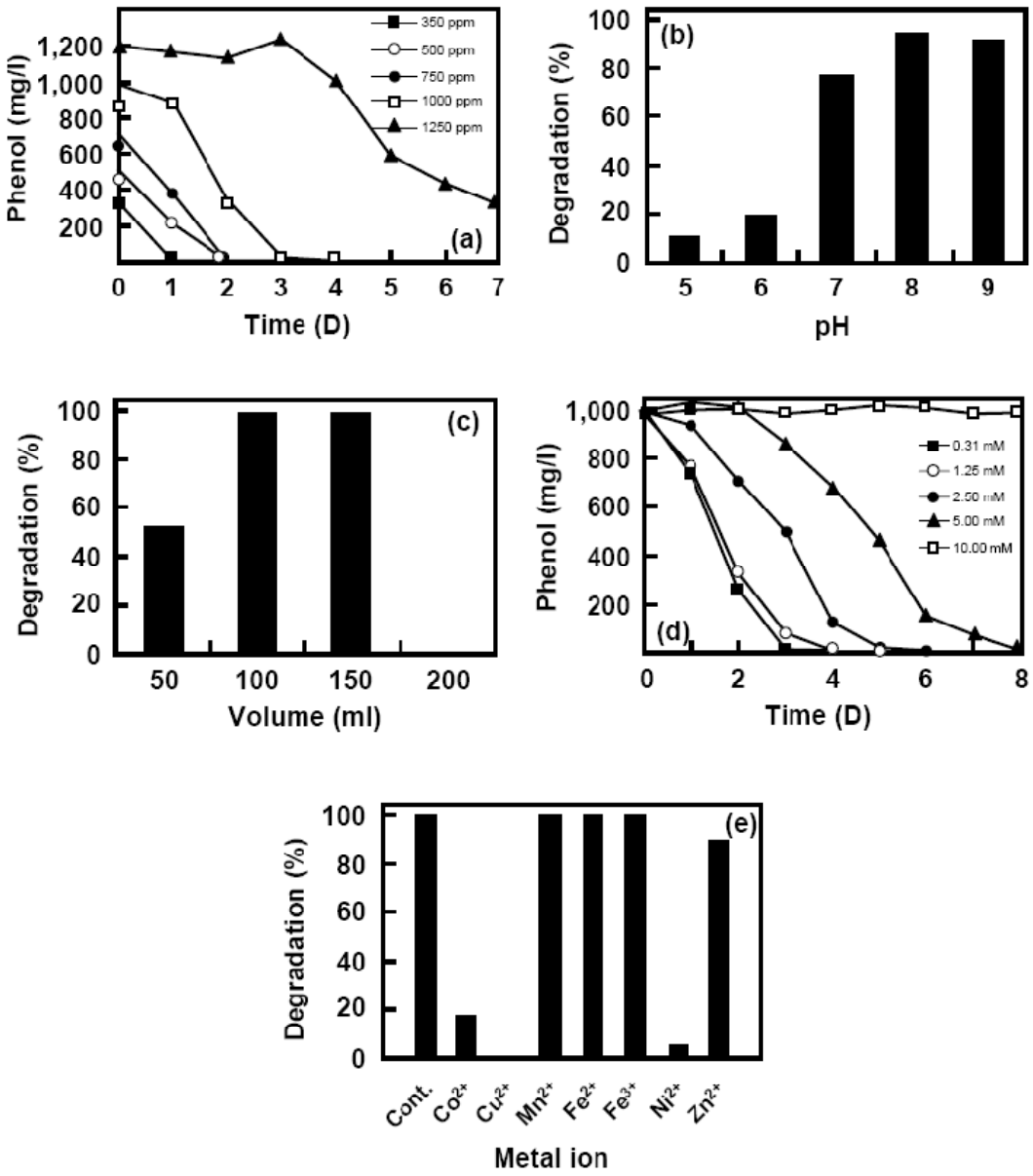

Figure 2. Effect of various factors on phenol degradation. a) Phenol concentration, b) Initial pH, c) Oxygen, d) Glucose concentration, and e) Metal ions

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The effect of oxygen concentration was investigated by using different volumes of medium. Some enzymes involved in phenol degradation pathway, such as phenol monooxygenase and catechol dioxygenase, required oxygen to complete the catalytic reaction. Under low concentration of dissolved oxygen, the activity was absent. In 200 mL-culture, the level of dissolved oxygen decreased gradually and eventually led to the inhibition of phenol degradation (Fig. 2c). Vigorous shaking of 50 mL culture probably contributed to the oxidation of phenol as evident by the brown color appearance of the medium. It has been shown that the percentage of phenol degradation by *K. oxytoca* was increased with an elevation in agitation speed from 50 to 250 rpm (Shawabkeh et al., 2007) while a shaking speed of 100-125 rpm was optimal for phenol degradation by *Pseudomonas aeruginosa* (Kotresha and Vidyasagar, 2008).

Another possible factor that could influence the phenol degradation potential of microorganisms was the presence of an alternative carbon/energy source. The effect of sugars (D-arabinose, D-fructose, D-galactose, D-glucose, myo-inositol, lactose, maltose, D-mannose, D-sorbitol and sucrose) and organic acids (citric acid, lactic acid, D,L-malic acid, and succinic acid) on phenol degradation were investigated. Most tested sugars were able to inhibit phenol degradation completely with the exception of arabinose, inositol and lactose where slight phenol degradation was observed (data not shown). The presence of organic acids, except malic acid, did not stall the degradation reaction (data not shown). Some studies also reported carbon catabolite repression by glucose and organic acid on phenol degradation (Lob and Tar, 2000; Muller et al., 1996).

The addition of D-glucose at the concentration higher than 0.31 mM (55.8 mg/L) resulted in the decrease of phenol degradation (Fig. 2d). Various literatures suggested that the impact of glucose on phenol degradation depended on microbial strains and level of glucose concentrations. Phenol removal efficiency by biological sludge was increased in the presence of low glucose concentration (50 mg/L) and decreased when the concentration was increased to 125-500 mg/L (Movahedian et al., 2005). There existed two stage growth in the cultivation of *C. albicans* TL3 with 0.67% D-glucose and phenol in which the consumption of D-glucose occurred rapidly in the first stage and phenol in the latter (Tsai et al., 2005). This was compared to the study of phenol degradation rate at 750 mg/L initial level in the presence of glucose using *P. putida* ATCC 49451 by Lob and Tar (2000). Phenol removal rate increased with D-glucose concentration below 1.0 g/L while the opposite was observed with increasing D-glucose concentration. Similar finding was also reported in the case of *P. aeruginosa* MTCC 4996 whose phenol degradation rate was diminished when the glucose concentration was elevated beyond 0.25 g/L (Kotresha and Vidyasagar, 2008). In our study, one possible reason

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of phenol degradation depression in the presence of higher glucose concentrations could be the associated rapid drop in pH to the level as low as 3.07, at which phenol degradation was completely inhibited (Zhang et al., 2013; Singh et al., 2008).

Among the variety of metal ions tested for the impact of phenol degradation, Co^{2+}, Cu^{2+} and Ni^{2+} strongly inhibited degradation whereas Zn^{2+} showed slight inhibition (Fig. 2e). Nakamura and Sawada (2000) reported the stronger inhibitory effect by Cu^{2+} than Zn^{2+} on phenol consumption by *Acinetobactercalcoaceticus* AH strain. The presence of metal ions such as Fe^{2+}, Cu^{2+}, Zn^{2+} and Mn^{2+} at low concentrations appeared to stimulate and enhance phenol degradation rate of *P. aeruginosa* MTCC 4996 (Kotresha and Vidyasagar, 2008).

3.5 Characterization of enzymes involved in phenol-degrading pathway

Catechol 1,2-dioxygenase activity in the crude extracts prepared from the cells grown in the medium with and without the addition of phenol was determined. The specific activity (0.79 U/mg of protein) was significantly higher in the extracts where phenol was also added. The microbe metabolized phenol via catechol *ortho*-cleavage pathway followed by the induction of dioxygenase production. The specific activity of strain No. 10 was similar to those of *C. tropicalis* (Bastos et al., 2000) and *C. albicans* (Tsai et al., 2005).

4. Conclusion

Candida tropicalis strain No.10 was isolated and identified as a thermotolerant yeast capable of degrading 100 mg/L phenol completely at a wide range of temperature at 20-42^{o}C in a minimum mineral medium. Up to 1,000 mg/L of phenol was degraded completely and the strain appeared to grow well under the slightly alkali condition. Low concentration of glucose, the presence of certain organic acids and heavy metals showed no effect on phenol degradation. Enzyme characterization revealed that phenol was utilized via the *ortho*-cleavage pathway and the catechol 1,2-dioxygenase specific activity was comparable to those reported previously.

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