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Identification of *Yarrowia lipolytica* Y103 and Its Degradability of Phenol and 4-Chlorophenol

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Abstract A nonconventional yeast strain Y103 capable of degrading several aromatic hydrocarbons was isolated from the wastewater of the Yocheon industrial complex. The strain Y103 was identified as *Yarrowia lipolytica* on the basis of its unique dimorphic and biochemical characteristics as determined by a Biolog test. *Y. lipolytica* Y103 was found to degrade phenol and 4-chlorophenol to produce catechol. The catechol then will be further degraded to produce 2-hydroxymuconic semialdehyde via *meta*-cleavage. These results indicate that strain Y103 degrades 4-chlorophenol, phenol, and catechol through a consecutive reaction to produce 2-hydroxymuconic semialdehyde. The most active degradation of phenol by *Y. lipolytica* Y103 occurred with a 0.5 mM phenol concentration in an MM2 medium at 30°C and pH 7.0.

Key words: *Yarrowia lipolytica* Y103, identification, degradation, phenol, 4-chlorophenol

Aromatic hydrocarbons, particularly chlorinated aromatics, are one of the most recalcitrant groups of pollutants in nature. Their recalcitrant characteristics are due to the chemical structure of a benzene ring and chlorine substituent. Therefore, studies on the microbial degradation of xenobiotic aromatic compounds, including polychlorinated biphenyls and various pesticides, have been focused on the biochemical reactions of ring-cleavage and dechlorination [6, 8, 26], and one-ring aromatic compounds substituted with a hydroxyl, methyl, nitro group, or chlorine have been used as model compounds for studying the mechanism of biodegradation [12, 20]. The degradative pathways of these compounds have been elucidated in various microorganisms isolated from contaminated natural environments [1, 5]. Most of the organisms degrading benzene, catechol, xylene, toluene, and phenol have been identified as bacterial species [27]. The bacterial enzymes and genes responsible for the degradation of aromatics have also been extensively studied [2, 10, 17].

As a result, some eukaryotic organisms such as yeast and fungi have been found to be capable of degrading aromatic compounds [3, 4, 11]. Dimorphic *Yarrowia lipolytica*, previously classified as *Candida lipolytica*, has been reported to be involved in the degradation of fatty acids (*beta*-oxidation) and alkanes [16, 28]. There have also been a few reports on hydrocarbon degradation by these organisms [4, 15]. In contrast, *Candida tropicalis* has been reported as a phenol degrader [13, 14, 18, 25]. Nonetheless, there has been no report on the degradation of phenol and chlorophenol by *Yarrowia lipolytica*.

We isolated the nonconventional dimorphic yeast, *Yarrowia lipolytica*, from contaminated wastewater as a phenol degrader, and the properties of its degradability of several aromatic compounds including phenol and 4-chlorophenol were carried out.

Materials and Methods

Isolation and Cultivation of Strain Y103

Wastewater samples from the Yocheon industrial complex were incubated in an LB (Luria-Bertanni) broth medium at 30°C for 24 h. The enriched culture was further incubated in an MM2 broth supplemented with 0.5 mM phenol in a shaking incubator at 30°C for 24 h, as described by Seo *et al.* [24]. Several strains were isolated by plating the enriched culture on an MM2 agar supplemented with 0.5 mM phenol. The isolate of strain Y103 was selected based on its degradability of phenol and other aromatic hydrocarbons.

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Identification of Strain Y103

The isolate Y103 was examined for its biochemical and morphological properties using a Biolog test and microphotography. The Biolog test was performed with Yeast identification test panels (Biolog Inc., Hayward, CA, U.S.A.) as previously described by Kim *et al.* [15]. The cell suspension of Y103 was inoculated on a MicroPlate™ which had been pre-heated at 28°C and then incubated at 30°C for about 30 min. The MicroPlate™ was read at 590 nm, and then the results were analyzed using the MicroLog™ 3 computer software (Biolog Inc., Hayward, CA, U.S.A.).

The colonies of strain Y103 grown on the MM2 agar containing 0.5 mM phenol were pre-fixed with 2.5% glutaraldehyde for 4 h, and then post-fixed with 1% osmium tetraoxide, as previously described by Seo *et al.* [24]. The colonies were dehydrated by conventional procedures and then dried with a critical point dryer. The dried colonies were gold coated using a Sputter coater (Giko Engineering Co., Japan), and then observed with a scanning electron microscope (Hitachi Co., Japan). The cells taken from the Y103 culture in the YM broth [3] at an appropriate time were stained with crystal violet, and then observed with a light microscope for microphotography.

Degradation of Aromatics by Strain Y103

The degradation of phenol and other aromatic hydrocarbons by *Y. lipolytica* was examined using a resting cell assay [9]. The cells were incubated in an MM2 broth supplemented with 0.5 mM each of aromatic hydrocarbon. The cells were then harvested and washed twice with 10 mM potassium phosphate buffer (pH 7.0). The cell pellets were resuspended in the same buffer containing 0.2 mM of each respective aromatic. The degradation of each aromatic compound was finally examined by scanning UV-absorbance at a wavelength of 200 to 400 nm [23].

The dechlorination activity of the chloroaromatics by strain Y103 was examined using the colorimetric method described by Tsoi *et al.* [26]. The cells were inoculated

Table 1. Biochemical characteristics of *Yarrowia lipolytica* Y103 examined by the Biolog assay.

Characteristics	Reaction	Characteristics	Reaction	Characteristics	Reaction
α-Cyclo-dextrin	-	D-Tagatose	-	α-Hydroxybutyric acid	+
β-Cyclo-dextrin	+	D-Trehalose	-	β-Hydroxybutyric acid	-
Dextrin	-	Turanose	+	γ-Hydroxybutyric acid	-
Glycogen	+	Xylitol	+	*p*-Hydroxyphenyl acetic acid	+
Inuline	+	D-Xylose	+	α-Keto glutaric acid	+
L-Arabinose	-	Acetic acid	-	α-Keto valeric acid	+
D-Arabitol	-	Lactamide	+	Mono-methyl succinate	-
Arbutin	+	D-Lactic acid methyl ester	-	Propionic acid	+
Cellobiose	+	L-Lactic acid	+	Pyruvic acid	-
D-Fructose	+	D-Malic acid	+	Succinamic acid	-
L-Fucose	+	Methyl pyruvate	-	Succinic acid	-
α-D-Lactose	+	Alaninamide	+	N-Acetyl-L-glutamic acide	-
Lactulose	+	D-Alanine	+	Glycyl-L-glutamic acid	-
Maltose	+	L-Alanine	+	L-Pyroglutamic acid	-
Maltotriose	+	L-Alanyl-glycine	+	L-Serine	-
D-Mannitol	+	L-Asparagine	+	Putrescine	+
D-Mannose	-	L-Glutamic acid	-	2,3-Butanediol	+
β-Methyl-D-glucoside	-	Adenosine	+	Glycerol	+
α-Methyl-D-mannoside	+	2-Deoxy adenosine	+	Thymidine-5-monophosphate	+
Palatinose	+	Inosine	+	Uridine-5-monophosphate	+
D-Psicose	-	Thymidine	+	Fructose-6-phosphate	+
D-Raffinose	-	Uridine	-	Glucose-1-phosphate	+
L-Rhamnose	-	Adenosine-5-mono-phosphate	+	Glucose-6-phosphate	-
Mannan	+	L-Malic acid	+	D-L-α-Glycerol phosphate	-
Tween 40	-	Gentiobiose	-	3-Methyl glucose	-
Tween 80	+	D-Gluconic acid	+	α-Methyl D-glucoside	+
N-Acetyl-D-glucosamine	+	α-D-Glucose	+	D-Ribose	-
N-Acetyl-D-mannosamine	+	M-Inositol	+	Salicin	-
Amygdalin	-	D-Melezitose	+	Sedoheptulosan	+
D-Galactose	+	D-Melibiose	+	D-Sorbitol	+
D-Galacturonic acid	-	α-Methyl-D-galactoside	+	Stachyose	+
Sucrose	-	β-Methyl-D-galactoside	-		

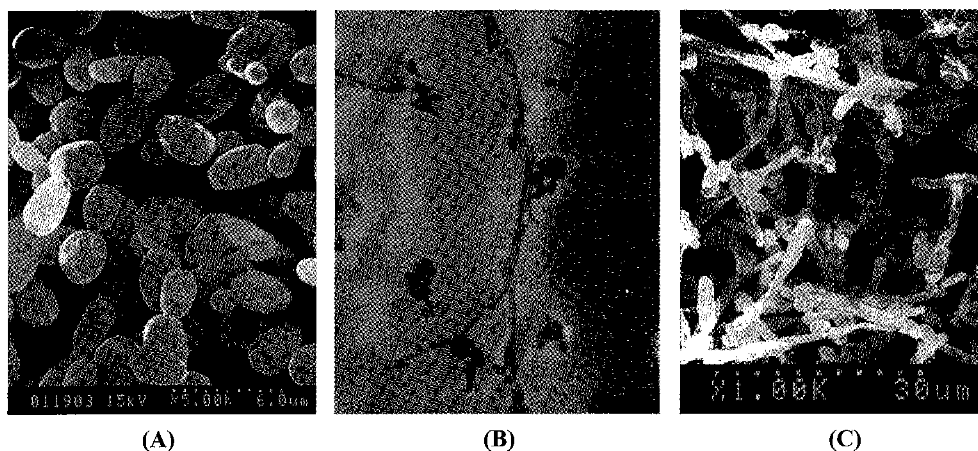

Fig. 1. Scanning electron- and light-micrographs of *Yarrowia lipolytica* Y103 during cultivation in YM medium for 60 h.
A, electron-micrograph of 6 h culture; B, light-micrograph of 30 h culture; C, electron-micrograph of 60 h culture.

in a chloride-free medium containing 0.5 mM each of chloroaromatic compounds and then examined for the production of chloride ions after incubation for 12 h.

Growth of Free and Immobilized Cells on Phenol

The strain Y103 was examined for its growth on 0.5 mM phenol with freely suspended and polyurethane-immobilized cells using the method described by Manohar and Karegoudar [19]. The freely suspended cells were added to 250-ml Erlenmeyer flasks containing an MM2 medium with 0.5 mM phenol and incubated at 30°C on a rotary shaker at 150 rpm. Culture samples taken at the indicated times were examined for cell growth (viable cell count and culture turbidity) and for the degradation of phenol. The immobilized cells on polyurethane were inoculated in a 250-ml Erlenmeyer flask containing an MM2 medium with 0.5 mM phenol, and examined for their growth and the degradation of phenol by replacing the spent medium with a fresh one every 10 h.

Effects of Environmental Factors on Phenol Degradation

The degradation of phenol by *Y. lipolytica* Y103 was examined as a function of temperature, pH, and concentration of phenol [23]. The cells inoculated in an MM2 broth supplemented with 0.5 mM phenol were inoculated at different temperatures and pH values. The samples taken from each culture after 24 h of incubation were then examined for the relative degradation of phenol and growth of the cells. The effect of the phenol concentration on its degradation was also tested at 30°C and pH 7.0.

RESULTS AND DISCUSSION

Identification of *Y. lipolytica* Y103

The biochemical characteristics of strain Y103 examined by the Biolog test are shown in Table 1. The organisms showed a yeast-shaped form, as seen in Fig. 1A, when cultivated in an MM2 medium supplemented with 0.5 mM phenol at 30°C for 24 h. However, as shown in Figs. 1B and 1C the cells exhibited a filamentous form in an old culture incubated for over 30 h in MM2 with phenol and YM media. Accordingly, the morphology of the cells in the culture gradually changed from a yeast form to a filamentous form as a function of the cultivation time, exhibiting a typical dimorphism (Fig. 2). The Biolog data of the dimorphic Y103 strain showed a high confidence level (% identity=99.8) to *Yarrowia lipolytica.* On the basis of these results, strain Y103 was classified as *Yarrowia lipolytica* and referred to as *Y. lipolytica* Y103 hereafter.

The genus *Yarrowia* has been previously classified as *Candida* [3], and *Yarrowia lipolytica* has been commonly reported as a fatty acid degrader [28]. A few reports have

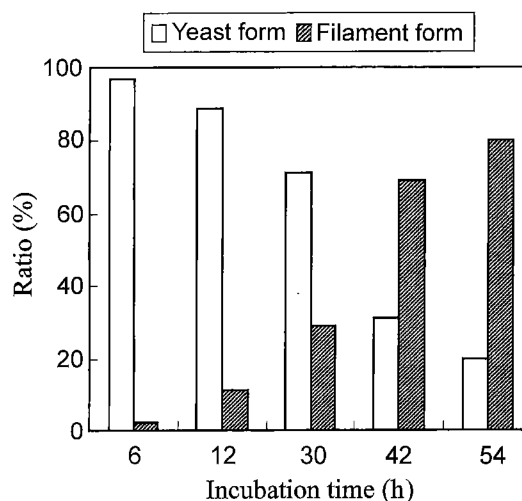

Fig. 2. Change of morphological forms in *Yarrowia lipolytica* Y103 during cultivation in YM medium for 60 h.

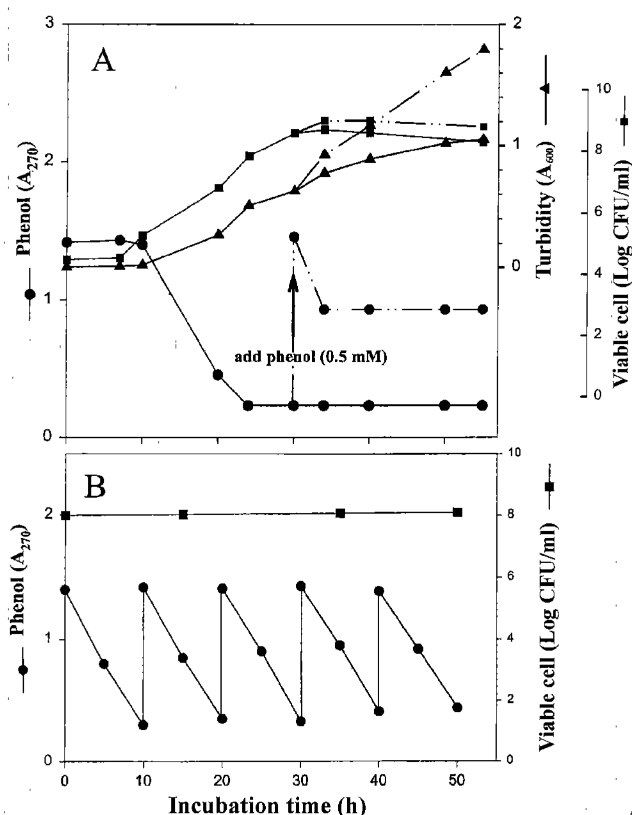

Fig. 3. Degradation of phenol in free cell culture (A) and semicontinuous culture (B) of immobilized cells of *Yarrowia lipolytica* Y103 on polyurethane and their growth on phenol.

When the free cell culture was supplemented with 0.5 mM phenol after 30 h of incubation (dotted lines), the organisms did not grow as well and the phenol was not degraded as actively as in the initial culture. However, the immobilized cells continued to degrade phenol very actively in the semicontinuous culture by keeping their yeast-shaped morphology over 50 h.

indicated that *Yarrowia* sp. degrades alkanes [22] and aliphatic hydrocarbons in crude oil [16]. However, the *Y.* lipolytica strain Y103 in this study was shown to be capable of degrading phenol and 4-chlorophenol.

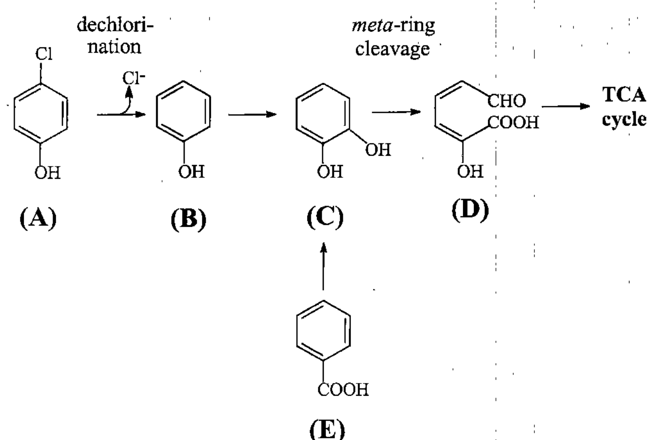

Fig. 5. Proposed pathway for degradation of phenol and 4-chlorophenol by *Yarrowia lipolytica* Y103.

A, 4-chlorophenol; B, phenol; C, catechol; D, 2-hydroxymuconic semialdehyde; E, benzoate.

Degradative Characteristics of Phenolics

The degradation of phenol by freely suspended and polyurethane-immobilized cells was examined in an MM2 broth containing 0.5 mM phenol (Fig. 3). The phenol was completely degraded and the cells grew about 2 orders of magnitude after incubation at 30°C for about 20 to 30 h, as shown in Fig. 3A. When 0.5 mM phenol was added to an old culture after 30 h of incubation, the degradation was markedly reduced thereafter and the organisms did not grow as well (Fig. 3A), and most of the cells in the culture exhibited a filamentous shape. This means that *Y. lipolytica* cells in a yeast-shaped morphology would appear to have a more active degradability of phenol than those in a filamentous form. However, *Y. lipolytica* cells immobilized on polyurethane maintained their viability and yeast-

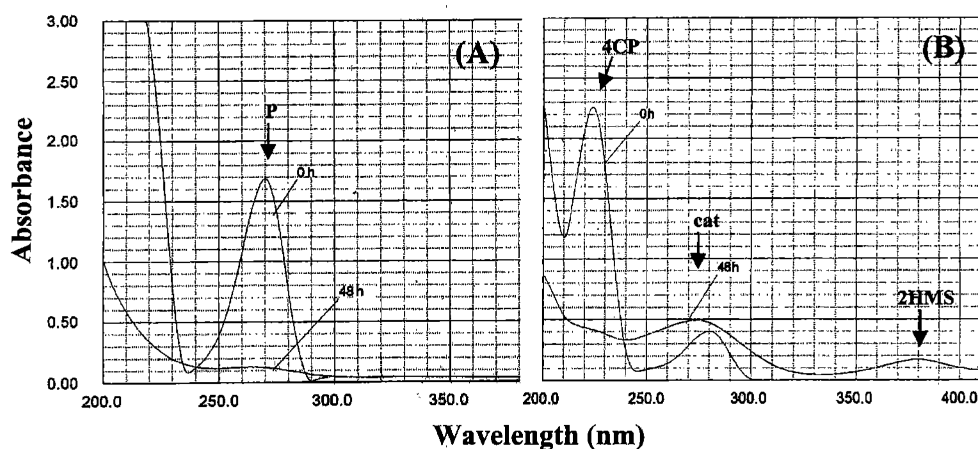

Fig. 4. Degradation of phenol (A) and 4-chlorophenol (B) by *Yarrowia lipolytica* Y103.

Table 2. Degradation of several aromatic hydrocarbons by *Yarrowia lipolytica* Y103.

Substrate	Degradation
Phenol	+
4-Chlorophenol[†]	+
Catechol	+
4-Chlorocatechol	+
Benzoate	+
2-Chlorobenzoate	-
3-Chlorobenzoate	-
4-Chlorobenzoate	-
3,4-Dichlorobenzoate	-
4-Hydroxybenzoate	-
Protocatechuate	-

[†]Degradation of 4-chlorophenol and other chloroaromatics was examined using the $Fe(SCN)^{++}$ colorimetric assay described by Tsoi *et al.* [26] for dechlorination.

shaped morphology (data not shown) along with an active degradability of phenol for over 50 hours when the spent medium was replaced with a fresh medium every 10 h (Fig. 3B).

The spectrophotometric results of phenol and 4-chlorophenol (4CP) degradation are shown in Fig. 4. During incubation for 48 h at 30°C (Fig. 4B), the Y103 strain degraded 4CP to catechol and 2-hydroxymuconic semialdehyde (2HMS) which absorbed UV light at 275 nm and 384 nm, respectively. Such metabolites were detected by UV-spectrophotometry in previous reports [13, 24]. Fujita *et al.* [7] and Powlowski and Shingler [21] described earlier that the degradation of phenol starts with the formation of catechol under aerobic conditions. In this study, a *meta*-cleavage product, 2HMS, found from catechol in a 48 h culture, was also detected at 284 nm (Fig. 4B). This meant that the catechol produced from phenol was further degraded to form 2HMS via *meta*-cleavage. Catechol was also produced from benzoate by this organism, and then it was degraded (data not shown). Therefore, the possible pathway for the degradation of phenol and 4-chlorophenol is depicted in Fig. 5. Dechlorination and *meta*-ring cleavage reactions are also thought to be involved in the catabolic degradation.

Table 3. Effects of environmental factors on the degradation of phenol in MM2 medium by *Yarrowia lipolytica* Y103 after 24 h incubation and its growth on phenol.

Environmental factor		Relative degradation (%)	Relative growth (%)
Temperature (°C)	20.0	53.2	61.5
	30.0	100.0	100.0
	37.0	19.8	23.0
pH	5.0	46.6	61.5
	7.0	100.0	100.0
	9.0	33.3	38.4
	11.0	6.5	15.3
Concentration (mM)	0.3	100.0	96.5
	0.5	100.0	100.0
	1.0	53.2	69.2
	3.0	26.6	23.0

The degradation of several other aromatic hydrocarbons by *Y. lipolytica* strain Y103 is shown in Table 2. The strain was also able to degrade catechol and benzoate. However, chlorinated aromatics, except for 4-chlorophenol, were not degraded by this organism. The effects of certain environmental factors on the degradation of phenol were examined in an MM2 medium and the results are shown in Table 3. Strain Y103 exhibited its highest active degradability of phenol at 0.5 mM phenol concentration, at 30°C and pH 7.0.

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