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Isolation of Yeasts utilizing Phthalic Compounds as a Sole Carbon Source

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프탈산 화합물을 탄소원으로 이용하는 효모의 분리 및 동정

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ABSTRACT: Three isolates of yeast utilizing phthalic compounds as a sole carbon source were obtained from the surface waters exposed to the industrial effluents near Cheong Ju city. On the basis of microscopic observations on morphology and various biochemical characterizations, the three isolates were identifed as a species of *Rhodotorula*, *Candida* or *Torulopsis*. A number of aromatic chemicals including phthalic compounds would support the growth of these yeasts as a sole carbon source, Thus, the yeast isolates would have potentials in reduction of environmental burden due to industrial wastes of aromatic hydrocarbons.

KEYWORDS: Phthalic compounds, *Candida*, *Rhodotorula*, *Torulopsis*.

Phthalic compounds, such as diesters of o-phthalic acid, isophthalic acid and terephthalic acid are greatly used in plastic and synthetic fabric industries. Industrial waste products of these compounds have brought ubiquitous environmental contaminants and problems of public health (Peakall, 1975). Thus, there is a special concern on phthalates as well as other aromatic compounds adsorbed to particulate matter and deposited in natural sediments, leading to their accumulation in our environments.

Some of soil microorganisms facilitate their growth by degrading aromatic chemicals and by utilizing their carbon products as substrates. Ecologically, this process helps the reentry of carbons of aromatic compounds into the natural biological cycles, preventing their accumulation in our environments. Studies on metabolic pathways on degradation of aromatic compounds are made with a wide range of bacteria (Dagley, 1971; Parke and Ornston, 1986; Evans and Fuchs, 1988), but a little studies with the fungi (Gaal and Neujahr, 1979; Anderson and Dagley, 1980). Nontheless, a few of studies on the microbes utilizing phthalate compounds are reported (Aftring, *et al.*, 1981; Nozawa and Maruyama, 1988a; 1988b). But the researches on degradation of phthalate compounds by yeast are very rare, as compared with those on bacteria.

Here, we report the yeasts capable of utilizing phthalates and various aromatic compounds as a carbon substrate for growth, which were isolated from the surface waters received from the industrial effluents.

Materials and Methods

Chemicals and media

Chemicals used in the experiments were all reagents grade unless otherwise noted. Culture

media used were products of either Difco laboratories (Detroit, MI) or BBL Microbiology Systems (Becton Dickinson and Co., Cockeysville, MD) unless otherwise noted.

Isolation of phthalate utilizing yeasts

Soil and/or water samples of the rice paddies receiving the wastes from the chemical industries in the areas of Cheong Ju city were employed for isolation of yeasts utilizing phthalates as a carbon source. One-tenth gram of the soil samples collected were suspended in several ml of nutrient broth and mixed thoroughly. One ml of particleless supernatant of soil preparation or water samples collected were inoculated to the minimal salt medium concocted with the 50 mM sodium-potassium phosphate (pH 6.5), 1 mM of $MgSO_4 \cdot 7H_2O$, 0.1 mM $CaCl_2$, 0.01 mM $FeSO_4 \cdot 7H_2O$, 0.2% (w/v) ammonium sulfate, and 0.2% (w/v) phthalic acid (Sodium salt, Sigma Chemical Co., St. Louis, MO.) as the sole carbon source, and placed in a shaking incubator at 30℃ for a few days. Then, the culture broth was appropriately diluted and 0.1 m*l* of the diluted culture broth was spread on minimal salt agar plates supplemented with 0.2% phthalate. Yeast-like colonies were picked and subcultured on phthalate-minimal salt agar plates until a single colony of one kind of yeast was obtained.

Cultural and growth characteristic of the yeast isolates

The morphology of each yeast colony was observed on Sabouraud Dextrose agar, Yeast Malt agar as well as phthalate-minimal salt agar. Metabolic versatility was studied by testing growth in mininal salt or mininal salt agar containing various aromatic chemicals (0.2%) as a sole source of carbon with or without the supplement of 0.1% casamino acids. Occurrence of polyphosphate deposits in the yeasts was observed by alkaline methylene blue staining (Gerhardt *et al.*, 1981). The cell growth of yeasts was measured by a spectrometer at 420 nm (Spectronic. 20, Milton Roy Co.,).

Biochemical characteriastics of the yeast isolates

Yeast nitrogen base without amino acids (Difco laboratories) was employed in the studies of biochemical properties of the yeast isolates as suggested by Barnett, *et al.* (1983). Urea hydrolysis test was done by mixing the yeast cultures to phenol red urea broth (Difco laboratories). Nitrate reduction test was performed by culturing the yeasts in nutrient broth containing 0.1% potassium nitrate and 0.7% agar for 24 hours, and, subseqently, adding the coloring reagents to the culture broth (Gerhardt, *et al.*, 1981).

Microscopic observation of the yeast isolates

The slide cultures of each yeast isolate was prepared on the 2% Malt agar or Potato Dextrose agar, to observe the natural state of conidiospore or conidia attachments (Kendrick and Carmichael, 1973, Fassatiova, 1986). The pieces of agar containing the mycelia or cells of the yeast were placed on the slide glass and smashed with the cover glass. Then, the mycelia were observed under the light microscope (80 x, or 800 x), and sometimes, after stained with 0.05% lactophenol tryphan blue.

Results and Discussion

Among the presumptive isolates of phthalate utilizing microorganims, three isolates (N-1, N-2, and G-1) were confirmed as the yeast by the colonial morphology and microscopic examination of the stained preparations. All of the isolates are more favour to grow at 30℃, but suppressed their growth under the anaerobic environments (i.e. Gas-Pack Anaerobic Jar). Thus, the yeast isolates employed in various experiments were aerobically incubated at 30℃, unless otherwise specified. Liquid culture of the yeast isolates was done in a shaking incubator with 150 strokes per min. The growth curves of the yeasts in minimal salt medium containing 0.2% phthalic acid were shown in Fig. 1. The isolate of N-1 grew slowly as compared with the others and its growth was reached to half of that of G-1 isolate after 40 hours' incubation (the point of each isolate indicated by the final hour, 12.5 hrs for G-1, 18.5 hrs for N-2 and 39.0 hrs for N-1, was on the initiating of the stationary phase at the yeast growth). The isolate of G-1 grew rapidly and reached to the stationary phase after 12 hours' incubation. The isolate of N-1 grew twice as slow as that of G-1. The growth

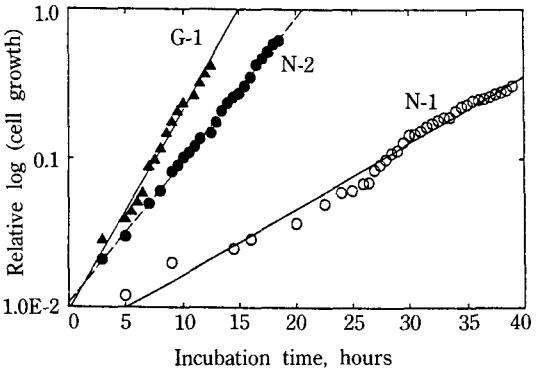

Fig. 1. Growth curves of yeast isolates grown in minimal salt broth containing both the phthalic acid (0.2 %, w/v) and casamino acid (0.1%, w/v). The lines of each isolate were resulted from the regression: Log (cell growth)=a constant+a specific growth rate/hour, with the 95% confidence level.

of yeast cell on the minimal salt medium indicated that the isolates were capable of utilizing phthalic acid as a carbon source.

Many of microorganisms including bacteria and fungi are kwown to dissimilate the aromatic compounds through the dihyroxylated intermediates of cathechol and protocathechuate, called to "β-ketoadipate pathway" or "ortho-cleavage" (Yeh and Ornston, 1981; Parke and Ornston, 1986; Anderson and Dagley, 1980; Gaal and Neujahr, 1979; Durham *et al.*, 1984). A few of Pseudomonads, for example, *Pseudomonas acidovorans*, are reported to dissimilate through meta-cleavage (Dagley, 19 71; Parke and Ornston, 1976; Shulka, 1989). Degradations of aromatic compounds by anaerobic microorganisms are known to be somewhat different from those by aerobic microorganisms; the former by the conversion of benzene ring to cyclohexane by reductase and the latter of dihydroxylation by oxygenase under aerobic environments (Berry *et al.*, 1987; Evans and Fuchs, 1988; Nozawa and Maruyama, 1988a). However, a dissimilar pathway from the known pathways was reported (Nozawa and Maruyama, 1988b) in the case of phthalate degradation by a denitrifying soil bacterium, and was asked for more attentions to study throughly. Nevertheless, a few of metabolic studies on degradation phthalic compounds by yeasts have been studied.

Table I. Growth of yeast isolates on various aromatic compounds[a].

Aromatic compounds	Yeast Isolates		
	N-1	N-2	G-1
Benzene	−[b](+)[c]	+(+)	+(+)
Toluene	−(+)	+(+)	+(+)
Xylene	−(−)	−(−)	−(−)
Phenol	−(−)	−(−)	−(−)
Resorcinol	−(+)	−(−)	−(−)
o-Cresol	+/−(+)	−(−)	−(−)
Benzoate	+/−(+)	+(+)	+(+)
Salicylate	−(+)	+/−(+)	−(+)
m-Hydroxybenzoate	−(+)	+/−(+)	+/−(+)
p-Hydroxybenzoate	−(+)	+(+)	+/−(+)
Phthalate	+(+)	+(+)	+(+)
di-Octylphthalate	−(+)	+/−(+)	+(+)
t-Cinnamic acid	−(−)	−(−)	−(−)
Hippuric acid	−(+)	−(+)	−(+)

[a]Growth was recorded on 24 hours' culture in the minimal salt medium supplemented with the 0.2% of the above compounds as a carbon source.
[b]The positive (+), negative (−) or (+/−) indicated an apperent growth, no growth, or slight growth, respectively.
[c]Symbols in the parenthesis were growth records when the 0.1% casamino acid was added to the medium to facilitate growth.

Results of studies on metabolic versatility of the isolates utilizing various aromatic compounds (Table I) showed that the isolates of N-2 and G-1 were more versatile in utilizing aromatic compounds, whereas the isolate of N-1 revealed a limit in utilization of different aromatic compounds. Particularly, the isolate of N-1 were not well grown on the mineral salts without the supplement of casamino acids. Interestingly, it seemed that the yeast isolates could not use phenolic compounds, i.e. phenol, cresol or resorcinol as a sole carbon source.

Assumed that our observations were correct for these yeasts, a new metabolic pathway or biological degradation of aromatic compounds would be speculative for the yeasts. And it is considered to be contributable in cleaning up the pollutants of

Table II. Some of biochemical properties of yeast isolates.

Tests	Yeast Isolates		
	N-1	N-2	G-1
GROWTH[a] on			
glucose	++	++	++
galactose	−	+	+
lactose	−	−	−
sucrose	−	++	++
melibiose	−	+/−	+/−
melezitose	−	++	+++
starch	−	+	+
gluconate	+	++	+
succinate	+	+	+
ethanol	+	+	+
cycloheximide (0.01%)[b]	−	+	+++
cycloheximide (0.1%)[b]	−	+	+
Nitrate reduction	+	+	+
Starch formation	−	−	−
Urea hydrolysis	−	+	−

[a] Growth was recorded on 24 or 48 hours' cultures: +++, ++, +; positive, +/−; weak positive, −; negative responses: the 0.5% (v/w) above compound was added to Yeast Nitrogen Base without amino acids (Difco lab. products).
[b] The 0.5% glucose was supplied as a carbon source.

plastic industries loaded to our environments. Since the cells of yeasts have been resource of single cell protein, to develop new foods or feedstuffs, the researches for these organisms would turn out to be valuable in development of new food/feedstuffs. Thus, we would attempt to pursue on catabolic pathway of phthalic compounds by the yeast isolates as well as on their physiology.

Morphlogical features of these isolates were noted on Table III and shown on Fig. 2-8. The cells of N-1 isolate were varied in shape, reproducing by multilateral budding and pseudomycelium was more or less abundantly developed and attached to blastospores. Thus, its genus was identified as

Table III. Morphological features of yeast isolates.

Morphological features on[a]	Yeast Isolates		
	N-1	N-2	G-1
SD agar	cream white dried colony	pink opaque colony	cream white colony
YM agar	cream white mucoid colony	pink mucoid colony	cream white mucoid colony
Phthalate-minmal agar	cream opaque colony	pink opaque colony	cream opaque colony
Size (Slide culture on Malt agar)	2−3×3−3 μ irregular blastospore	3−4×5−7 μ oval blastospore	2.0−2.5×2−3 μ (globose) 1.5−2×9−12 μ (rods)
Genus	*Candida*	*Rhodotorula*	*Torulopsis*

a species of *Candida* (Barron, 1963; von Arx, 1981). Cells of N-2 were round, oval or elongate. Yeast-like blastospores were lacking of hyphae and not borne on dentricles. Colonial pigmentation (pink or red) as well as cell morphology suggested that the isolate of N-2 was a species of *Rhodotorula* (Barron, 1963; von Arx, 1981). Cells of G-1 seemed to be reproduced by multilateral budding and generally round or oval, rarely somewhat elongate. These were only exceptionally capsulated, but the starch-like compounds were not observed in cell capsules. Thus, this was assumed as a species of *Toluropsis* (Barron, 1963; von Arx, 1981).

None of the yeast isolates was not revealed polyphosphate deposits of cells by alkaline methylene blue staining. Some of biochemical properties of the yeast isolates, such growth on various sugars, starch formation, nitrate reduction were tested accordig to the methods widely employed for yeast identifications (Barnett, *et al.*, 1983) and recorded on Table II. On the basis of biochemical properties of yeast isolates, the isolate of N-2 was speculated as *Rhodotorula glutinis* (Barnett, *et al.*, 1983), whereas the isolates of N-1 and G-1 were still very ambiguous in their identifications. Further biochemical works would be needed for exact identifications of species of the isolates.

摘 要

화학공장폐수가 유입되는 곳의 자연수에서 프탈산화합물을 탄소원으로 이용하여 성장하는 효모 3

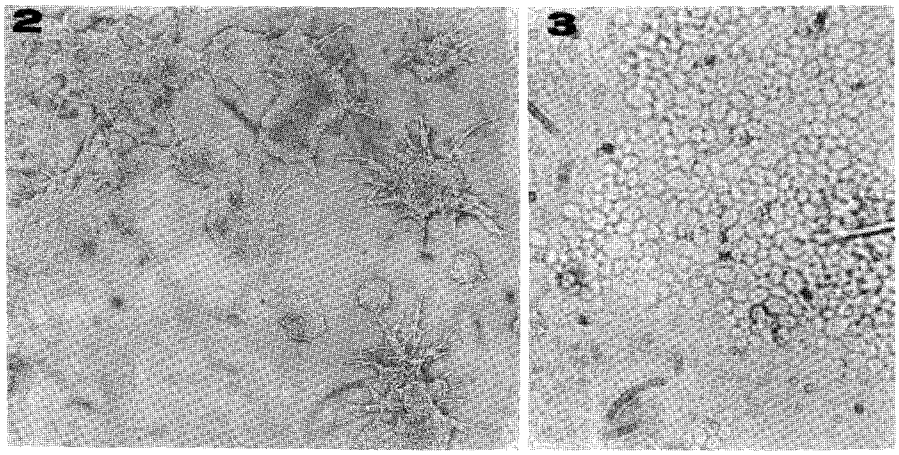

Fig. 2-3. The N-1 isolate identified as a species of *Torulopsis;* 2) Colonies (8×10), and 3) Cells (8×100).

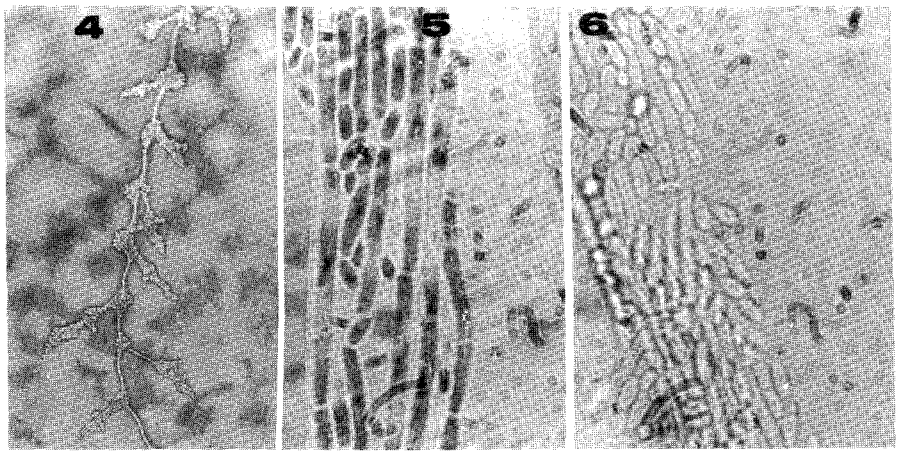

Fig. 4-6. The G-1 isolate identified as a species of *Candida;* 4) Colonies (8×10), 5) Psuedomycelia and cells stained (8×100), and 6) not stained (8×100).

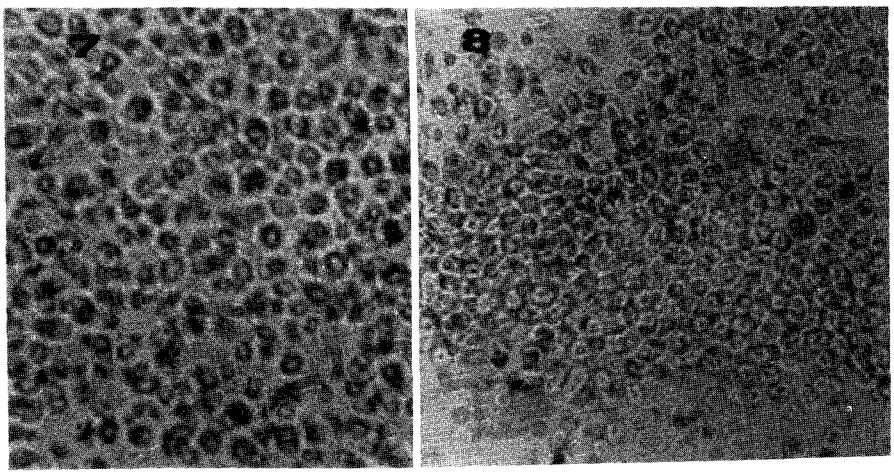

Fig. 7-8. The N-2 isolate identified as a yeast of *Rhodotorula glutinis;* 7) Cells not stained (8×100), and 8) Cell stained (8×100).

종을 분리하였는데, 형태학적 및 생화학적 특성의 연구결과 이들은 *Candida sp., Rhodotorula sp., Torulopsis sp.* 로 동정되었다. 분리한 효모들은 프탈산화합물 뿐만 아니라 톨류엔 등 다수의 방향족 화합물을 이용하여 성장하기에 화학공업의 발달로 인하여, 자연생태에 다량 유입, 축적되고 있는 난분해성 방향족화합물의 효율적 생분해 방법을 모색하는에 활용될 수 있으리라 추정되며, 따라서 이들 효모에 대한 다각적 연구가 필요하다고 사료된다.

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