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Analysis of Enzymes of *Stenotrophomonas maltophilia* LK-24 Associated with Phenol Degradation

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The analysis of enzymes associated with metabolism of phenolics by *Stenotrophomonas maltophilia* LK-24 was conducted. To identify metabolites of phenol and phenolic compound, we investigated enzymes of *S. maltophilia* LK-24 associated with degradation of phenolics. We found that phenol hydrolase, catechol-2.3-dioxygenase, 2-hydroxymuconic semialdehyde dehydrogenase, 2-hydroxymuconic semialdehyde hydroxylase and acetaldehyde dehydrogenase were activated. The results showed that phenolics were gone through the *meta*-pathway ring cleavage. The results will contribute greatly to understand metabolic pathways of phenol and it is possible to make some assessment of the feasibility of using *S. maltophilia* LK-24 for the treatments of phenolic-contaminated waste streams.

Key words: *Stenotrophomonas maltophilia*, biodegradation, phenol, *meta*-pathway

The production and usage of man-made chemicals in industry has led to the entry of many xenobiotics into the environment. One such group of xenobiotics is phenol and its derivatives that are pollutants of environmental apprehension due to their use a wide range of agriculture and industrial processes [18]. The influx of such phenolic compounds into open water has led to become priority pollutants for the acute toxicity and resistance to degradation. The recalcitrance of phenolic compounds is cleaved with great difficulty, the stability of their aromatic structure and the relatively high water solubility has led to their contamination of many environments and accumulation nature.

Microbes to primary metabolites that are chandelled further into a few central metabolic pathways such as the catechol pathway, where the metabolites are broken down to common intermediates, were used to degrade recalcitrant phenolic compounds. Nevertheless, the more detailed investigations of degradative pathway have been made on very few bacteria species mostly of the genus *Pseudomonas* [11-13, 15, 33], *Pseudomonas putida*, in particular, has been the subjects of abundant studies in the filed of biodegradation. Useful characteristics of *P. putida* are their ability to utilize a wide variety of xenobiotic and phenolic compounds for growth thus making it attractive for use in waste treatment [6, 17, 22]. Similar studies have also been reported in the bacteria *Alcaligens eutrophus* [2], *Bacillus stearothermophilus* [8], *Comamonas testosteroni* [32], *Rhodococcus opacus* [34], and in the yeast *Yarrowia lipolytica* [21].

The degradation of phenol may occur using either the *ortho*-pathway or *meta*-pathway, following their transformation to catechol, although the *meta*-pathway is more common [14]. The initial step in aerobic degradation of phenol is their transformation to catechol by phenol hydroxylase. After production of catechol, cleavage of the aromatic ring occurs using either the *ortho*- or *meta*-cleavage pathway.

In this study, the naturally isolated strain, *Stenotrophomonas maltophilia* LK-24 [20] was examined for its capability to grow on relatively high phenol concentrations together with the investigation of enzyme related with phenol degradation and metabolism under aerobic conditions. In addition, the *ortho* or *meta*-ring cleavage pathway of catechol transformation was studied with identification of key metabolites.

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MATERIALS AND METHODS

Chemicals

The Carbon sources used in the degradation studies, as well as minimal medium components, were purchased from Sigma-Aldrich Chemical Co. in USA. Their purities were examined by thin layer chromatography (TLC) and confirmed to be homogenous.

Microorganism and Culture Conditions

Stenotrophomonas maltophilia LK-24 isolated from contaminated [20] was used in this study and the biodegradation experiments were mostly performed in 500 ml Erlenmeyer flask containing 200 ml minimal medium with an initial pH 6.0 at 37. The minimal medium (MM) used in the degradation studies was composed of NH_4Cl, 760 mg; KH_2PO_4, 680 mg; $MgSO_4 \cdot 7H_2O$, 71.2 mg; $CaCl_2 \cdot 6H_2O$, 5.5 mg; $FeSO_4 \cdot 7H_2O$, 4.98 mg; $CuSO_4 \cdot 5H_2O$, 0.785 mg; $MnSO_4 \cdot 4H_2O$, 0.81 mg; $ZnSO_4 \cdot 7H_2O$, 0.44 mg; $Na_2MoO_4 \cdot 4H_2O$, 0.25 m; and deionized water 1,000 ml [23]. Phenolic compounds were supplied at a concentration of 5 mM into the medium after sterilization. *S. maltophilia* LK-24 grown overnight in Luria broth (LB) medium containing (gram per litter) pancreatic digest of casein, 10 g; NaCl 5, g; and yeast extract 5 g was centrifuged with 14,000 rpm for 20 min, washed twice with 10 mM sodium phosphate buffer with pH 6.0, and resuspended to give an optical density (OD) at 660 nm of 0.5. The flasks for biodegradation experiments were inoculated with a 5% (v/v) inoculum, which corresponding to approximately 5×10^7 cells per ml, and were incubated at 37°C and pH 6.0 under aerobic conditions. Uninoculated control flasks were incubated in parallel. For examine of phenol or catechol concentrations, samples were detached aseptically at regular intervals.

HPLC Analysis of Phenol Concentration

The concentrations of phenol in bacterial culture were determined by isocratic reversed-phased high performance liquid chromatography (HPLC) at room temperature by modifications of the method described by Erikson et al. [10]. Samples were centrifuged at 20,000 rpm for 20 min under 4°C to discarded cells and supernatants were kept at -20°C until analysis. The assays were carried out by HPLC (Waters 600E) equipped with a reversed-phase PEGASIL ODS column (25×0.45 cm) and SSC-5200 UV-detector (Senshu Scientific, Tokyo, Japan). The flow rate was 1 ml min^{-1} and the solvent system was acetonitrile/water (40:60, v/v). To decide intracellular phenol concentrations, the cells grown in minimal medium were centrifuged and the pellets were washed twice with 10 mM sodium phosphate buffer with pH 6.0. Moreover, the washed cells were suspended in methanol, sonicated for 3 min by MSE 100 watt Ultrasonic Disintegrator (MSE, London, UK), and centrifuged to remove cell debris. The supernatant was filtrated through a 0.22 m membrane-filter (Millipore Co., Mass., USA) and an aliquot was analyzed by HPLC.

The degradation ratio of phenol was calculated as follows; Degradation (%) = [(initial concentration of phenol) (the concentration after incubation)/(initial concentration of phenol)] 100.

Preparation of Cell-Free Extracts

Cells of strain LK-24 were harvested by centrifugation at 20,000 rpm and 4°C for 20 min, and washed twice with 50 mM Tris-HCl buffer pH 7.2 and resuspended in the same buffer. The cells was homogenized by sonication (MSE 100 watt Ultrasonic Disintegrator, MSE, London, UK) and centrifuged at 20,000 rpm for 20 min under 4°C. The cell-free extracts were kept at 20 until used

Phenol Hydroxylase

The oxidation of NADH in the presence of phenol by hydrogen peroxide-treated cell extract was monitored at 340 nm for analysis of phenol hydroxylase [8]. Reaction mixture of 3 ml containing 50 mM Tri-HCl buffer with pH 7.2, 1.0 mol NADH, and 100 nmol phenol were equilibrated at 37°C before the addition of cell-free extracts of 100 µl. One unit of activity was defined as the amount of enzyme catalyzing the oxidation of 1 µmol NADH min^{-1}.

Catechol-1,2-dioxygenase and Catechol-2,3-dioxygenase

Determinations of catechol 1,2- and catechol 2,3-dioxygenase activities were performed by modifications of the method described by Feist and Hegeman [14]. The formation of *cis, cis*-muconic acid that the *ortho*-cleavage product of catechol was measured for catechol-1,2-dioxygenase activities. 2 ml of 50 mM Tris-HCl buffer with pH 8.0, 0.7 ml distilled water, 0.1 ml of 100 mM mercaptoethanol, and 0.1 ml of cell-free extract were mixed thoroughly and 0.1 ml of 1 mM catechol was added

to make total volume 3 ml. In addition, the contents were mixed again. Catechol-1,2-dioxygenase activities were measured a spectrophotometer (Specord 10, UV/Visible spectrophotometer, Carl Zeiss Technology, Jena, Germany) at 260 nm for detection of *cis*, *cis*-muconic acid.

Catechol-2,3-dioxygenase activities were measured by the formation of 2-hydroxymuconic semialdehyde that the *meta*-cleavage product of catechol. The reagents, which containing 2 ml of 50 mM Tris-HCl buffer with pH 7.5, 0.6 ml of distilled water and 0.2 ml of the cell free extract were added 0.2 ml of 100 mM catechol to make up 3 ml of total volume and was then mixed with the reagents completely. The production of 2-hydroxymuconic semialdehyde was scanned at 375 nm with spectrophotometer to investigate catechol-2,3-dioxygenase activities.

Specific activities of catechol-1,2- and 2,3-dioxygenase were expressed as 1 mol catechol converted min^{-1} mg $protein^{1}$ using molar absorption coefficients for *cis*, *cis*-muconic acid and 2-hydroxymuconic semialdehyde of 16,800 mol^{-1} cm^{-1} at 260 nm and 36,000 mol^{-1} cm^{1} at 375 nm, respectively [32].

2-Hydroxymuconic-semialdehyde Hydroxylase or Dehydrogenase and Acetaldehyde Dehydrogenase (Acylating)

Assays for 2-hydroxymuconic-semialdehyde hydroxylase (HMSH) and dehydrogenase (HMSD) were done using the ring cleavage products of catechol as respective substrate [28]. The absorbance for catechol was measured at 375 nm and then 2-hydroxymuconic semialdehyde hydroxylase activities were assayed by measurement of the decrease of A_{375} in the presence of 0.1 mM NAD^{+}. The enzymic assay of acetaldehyde dehydrogenase (acylating, ADA) was performed according to the methods described by Shingler *et al.* [31]. ADA was also made an analysis by spectrophotometrically by observing the reduction of NDA^{+} to NADH at 340 nm coupled to the conversion of acetaldehyde to acetyl-CoA. 50 µl of 4 mM dithiotheritol, 50 µl of 1.5 mM NAD, 50 µl of 2 mM CoASH, and 40 µl of cell-free extract were added to 800 µl of 50 mM Tris-HCl buffer (pH 9.5). To start the reaction, 10 µl of 1 M acetaldehyde was added. For ADA, one unit of enzyme activity is defined as 1 nmol of NADH formed per minute. Protein concentrations in the cell-free extract were determined using the Bio-Rad protein assay reagent according to the method of Bradford [7]. Bovine serum albumin (Sigma-Aldrich Co., Fraction V, USA) was used as a standard.

Production of Acetaldehyde and Pyruvate from 4-oxalocrotonate (2-hydroxymuconate) by *Stenotrophomonas maltophilia* LK-24

0.3 mM 4-oxalocrotonate (2-hydroxymuconate) was incubated for 180 min with cell-free extract of *S. maltophilia* LK-24 in 50 mM Tris-HCl buffer (pH 8.0) containing 1 mM $MgSO_4$ in a final volume of 1 ml. At the end of the incubation, the mixture was diluted with an equal volume of distilled water and was boiled for 5 min. After the addition of NADH, acetaldehyde was determined by measuring the decrease in absorbance at 340 nm concomitant with NADH oxidation in the presence of alcohol dehydrogenase [28]. Pyruvate was determined with a diagnostic kit (Kit 726; Sigma-Aldrich Co., USA) dependent on the action of lactate dehydrogenate with the concomitant decrease in NADH.

Detection of CO_2 by GC

Twenty milliliter of sterile minimal medium (MM) was incubated with a 12 hour-old culture of *S. maltophilia* LK-24, having a cell density of 5×10^{7} cells ml^{-1}, in 60 ml serum bottles. The bottles were then sealed by self-sealing butyl-rubber stoppers and aluminum seals and incubated at 37°C for 96 h together with uninoculated controls under aerobic conditions. The gas in the headspace from inoculated and uninoculated bottles was analyzed for the presence of CO_2 at 12 h-intervals in a gas chromatograph (GC; Model 263-50, Hitachi Ltd., Tokyo, Japan) equipped with a thermal conductivity detector, hydrogen as the carrier gas at a flow rate of 30 ml min^{-1}, and a HP Vocol capillary column (3 m × 2 mm). The injector temperature 150°C and the detector temperature is 250°C.

Growth on Aromatic Carbon Sources

Phenol, benzoate, *p*-hydroxybenzoate, *o*-cresol, *m*-cresol, *p*-cresol, toluene, *o*-xylene, *m*-xylene, and *p*-xylene were tested for their ability to support growth of the bacterium. To ensure that the chosen concentrations were not toxic for strain LK-24, growth of the culture in MM in the presence of the substrates was tested. Since no toxic effects were observed at concentrations of 1 mM of phenolic substrates, all aromatic carbon sources were tested at this concentration.

Elucidation of the Mode of Ring Cleavage Phenolic Compounds

S. maltophilia LK-24 was incubated overnight on LB

agar plates containing 1 mM phenol. A 0.5 M catechol solution was sprayed onto the colonies. The formation of an intense yellow color indicated the presence of catechol-2,3-dioxygenase, as described by Buswell [9]. For detection of the *ortho*-cleavage pathway, Rothera reaction was carried out according to the method of Reichardt [26]. The exhibition of a purple color was detected, demonstrating that strain LK-24 degrades catechol by catechol-1,2-dioxygenase.

RESULTS

Growth Characteristics of *Stenotrophomonas maltophilia* LK-24 on Phenol as a Sole Carbon Source

Strain LK-24 was able to utilize phenol as a sole carbon and energy source. The degradation of phenol and the cell growth of strain LK-24 under aerobic culture conditions in MM are shown in Figs. 1 and 2. 2.5 mM, 5 mM, and 10 mM phenol were degraded completely after 18 h inoculation of strain LK-24. After 9 h of incubation, the degradation of phenol by *S. maltophilia* LK-24 was approximately over 80%, although strain LK-24 degraded phenol completely at 10 mM concentration and the cell growth of strain LK-24 was inhibited entirely in MM at 40 mM higher than 20 mM. When cell was monitored, the cell growth in minimal medium containing 10 mM phenol concentration showed a maximum density at 12-15 h of incubation. Upon continued incubation for 24 h, the degradation of phenol was completely done and cells exhibited good growth. After 15 h incubation, the degradation of phenol at 20 mM concentration was reached up to 50%, although the growth of cells was too low to be detected by measuring turbidity. The degradation rate of phenol was depended upon the growth phase of the precultured cells, as shown in Fig. 2. The degradation rate of phenol at early log phases (5-10 h incubation), exponential phases (10-15 h incubation), and stationary phases (15-20 h incubation) of strain LK-24 was 2.5 nmol mg^{-1} min^{-1}, 10.7 nmol $mg^{-1}min^{-1}$, and 4.6 nmol $mg^{-1}min^{-1}$, respectively. Moreover, the degradation was decreased with the initial concentration of phenol added in MM. The degradation of phenol, thus, was observed only at blow of 20 mM concentration.

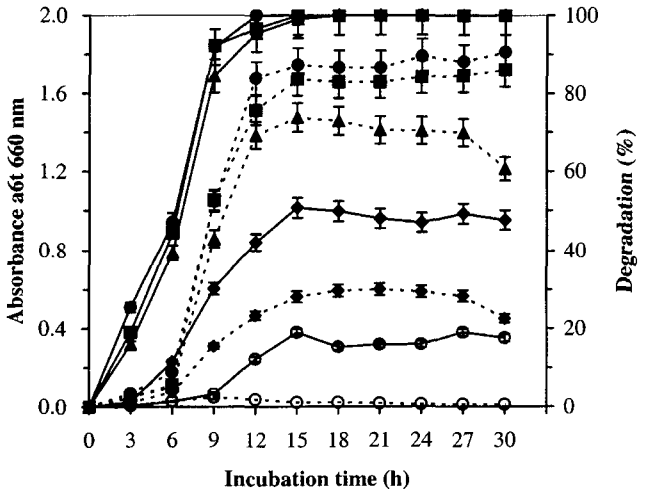

Fig. 1. Relationship between phenol degradation ratio (solid line, ——) and cell growth (dotted line, -------) of *Stenotrophomonas maltophilia* LK-24 cultivated in minimal medium (MM) containing initial concentrations of phenol 2.5 mM (●), 5 mM (■), 10 mM (▲), 20 mM (◆), and 40 mM (○) at 37°C and pH 6.0.

Induction of Phenol Hydroxylase and Catechol-1-2, or -2,3-dioxygenase

Periodic monitoring of the cell growth, catechol-2,3-dioxygenase activity, and extracellular phenol concentrations were exhibited significant differences in cell toxicity and induction specificity. In Fig. 3, *S. maltophilia* LK-24

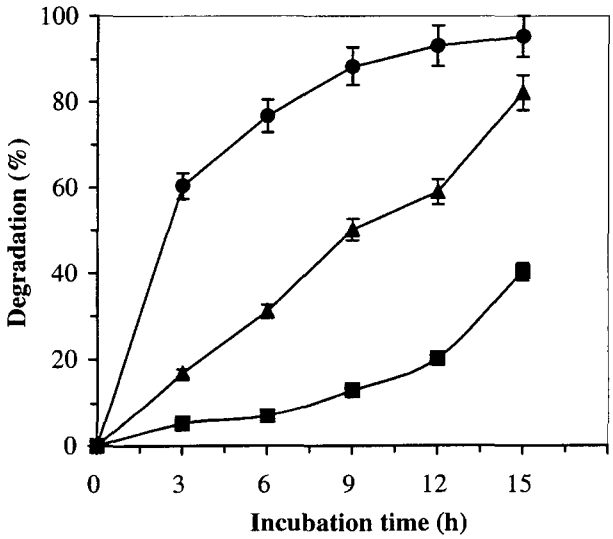

Fig. 2. Relationship between phenol degradation and cell concentrations of *Stenotrophomonas maltophilia* LK-24 incubated in minimal medium (MM) containing 2.5 mM phenol at 37°C and pH 6.0. The initial cell concentration of 5% (v/v) adjusted to approximately 0.5 at 660 nm was used as inoculum. Symbols are (▲), 5-10 h cultivated cells; (●), 10-15 h cultivated cells; and (■), 15-20 h cultivated cells. Phenol concentration was measured by HPLC. The degradation ratio of phenol was calculated as follows; Degradation (%) = [(initial concentration of phenol) − (the concentration after incubation)/(initial concentration of phenol)] × 100.

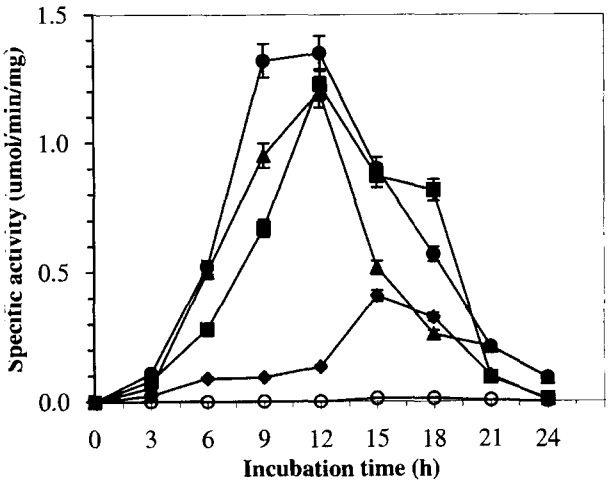

Fig. 3. Time course of the specific activities of catechol-2,3-dioxygenase in *Stenotrophomonas maltophilia* LK-24 cultures supplemented with phenol at 2.5 mM (●), 5 mM (■), 10 mM (▲), 20 mM (◆), and 40 mM (○) at 37°C and pH 6.0.

exhibited strong induction of catechol-2,3-dioxygenase activity at 2.5-20 mM phenol concentration. The delay in enzyme induction for 3-9 h incubation, and the decay of activity through the stationary phases were greatly affected by phenol concentrations in the medium. However, the maximal specific activities achieved at 1.2-1.5 μmol min^{-1} mg^{-1} protein. The rapid loss of catechol-2,3-dioxygenase activity in the stationary phases is a characteristic of enzyme systems under the induction of a specific degradation pathway. The activity of catechol-1,2-dioxygenase was not induced or somewhat induced at very low leve, suggesting that phenol is degraded via the *meta*-pathway by strain LK-24.

After colonies of cell grown on LB agar plate were sprayed with catechol, an intense yellow color appeared with a maximum 375 nm, demonstrating that the enzymes for phenol degradation were constitutively induced and than phenol was degraded through the *meta*-cleavage pathway. In the Rothera reaction, any purple color was not detected; representing that the *ortho*-pathway for cleaving catechol does not activated in strain LK-24 (Table 3).

Table 2. Activity was measured in cell free extracts after *Stenotrophomonas maltophilia* LK-24 was grown in minimal medium containing 1 mM phenol, *p*-Hydroxybenzoate or succinate for 12 h at 37°Cunder aerobic culture conditions.

Carbon source	Enzyme activity (μmol min^{-1} mg^{-1} protein)		
	HMSH	HMSD	ADA
Phenol	3	17.5	16
p-Cresol	13.8	3	5.1
p-Hydroxybenzoate	ND	0.3	0
Succinate	ND	BD	0

BD, blow limit of detection; ND, Not determined; HMSH, 2-hydroxymuconic-semialdehyde dehydrogenase; HMSD, 2-hydroxymuconic-semialdehyde hydrolase; ADA, acetaldehyde dehydrogenase (acylating).

Table 3. Growth of *Stenotrophomonas maltophilia* LK-24 on minimal medium containing various phenolic compounds as a sole carbon source and induction of catechol-1, 2- and catechol-2, 3-dioxygenase activities.

Phenolic compound	Growth	C-1, 2-D	C-2, 3-D
Phenol	++	BD	ED
Benzoate	+	BD	BD
p-Hydroxybenzoate	+	BD	BD
o-Cresol	++	BD	ED
m-Cresol	++	BD	ED
p-Cresol	++	BD	ED
Toluene	-	ND	ND
o-xylene	-	ND	ND
m-xylene	-	ND	ND
p-xylene	-	ND	ND
Catechol	+	ND	ED

Growth of *S. maltophilia* LK-24 and catechol-1,2- and catechol-2,3-dioxygenase by the formation of visible colonies and color on agar plates after 24 h incubation at 37°C on LB medium at pH 6.0: -, no visible growth; +, slightly visible growth; ++, good growth; C-1,2-activity, catechol-1,2-dioxygenase activity; C-2,3-D activity; catechol-2,3-dioxygenase activity. BD, blow limit of detection; ND, enzyme activity was not detected because there was no growth on substrates; ED, easily detected.

Table 1. Carbon source range, identification of reaction products, and induced phenol hydrolase specific activity from *Stenotrophomonas maltophilia* LK-24.

Carbon source	PH specific activity (μmol min^{-1} mg^{-1} protein)	Product identified	Cleaved by C-2,3-D Specific activity
Phenol	2.705	Catechol	+
o-Cresol	3.610	3-Methylcatechol	+
m-Cresol	2.907	3-Methylcatechol	+
p-Cresol	4.216	4-Methylcatechol	+

PH, phenol hydrolase; C-2,3-D, catechol-2,3-dioxygenase.

In Table 3, nevertheless, *o*-, *m*-, and *p*-xylene were not ruined by strain LK-24 with any additions of complex substrates such as yeast extract or peptone. It is notable that *p*-cresol showed the highest levels of catechol-2,3-dioxygenase induction. When *p*-hydroxybenzoate and benzoate were degraded, catechol-2,3-dioxygenase, in the *meta*-cleavage pathway, was detected at very low levels (0.1-0.2 μmol min^{-1} mg^{-1} protein), but an almost constant level of 2-hydroxymuconic semialdehyde kept in the culture was undetectable (Table 2).

To identify the reaction products, the supernatants of *S. maltophilia* LK-24 reactants with different phenolic componds supplied at a final concentration of 1 mM, were extracted with acetonitrile, and then analyzed by reverse-phase HPLC. In Table 1, the catechol-2,3-dioxygenase were identical to those determined for ring cleavage products obtained from authentic standards of catechol, 3-methyl-, and 4-methylcatechol. The identification of the reaction products suggested that *o*-, *m*- and *p*-cresol were mainly converted to 3-methyl and 4-methylcatechol, although phenol hydroxylase reaction products were completely disappeared.

Analysis of 2-Hydroxymuconic-semialdehyde hydroxylase or -dehydrogenase and Acetaldehyde dehydrogenase (acylating)

In strain LK-24, the activity of each *meta*-pathway enzymes, HMSD and HMSH, were induced when phenol was being used as a carbon source, representing that the induction of these enzymes was controlled by the regulatory system responsive to phenol or its metabolites. In Table 2, enzymatic conversion of 2-hydroxymuconic semialdehyde by strain LK-24 was completely depend on NAD^+, demonstrating that strain LK-24 does not have NAD^+-independent HMSH and has only NAD^+-dependent HMSD. 2-Hydroxymuconic-semialdehyde was ruined to 2-hydroxymuconate by HMSD. Nevertheless, HMSH was strongly activated by *p*-cresol. Moreover, ADA activity in strain LK-24 grown in the presence of phenol was detected and was higher than in *p*-hydroxybenzoate and succinate.

Conversion of Acetaldehyde and Pyruvate from 4-Oxalocrotonate (2-Hydroxymuconate) by *Stenotrophomonas maltophilia* LK-24

The experiment was carried out to investigate further metabolism of 4-oxalocrotonate (2-hydroxymuconate) as a substrate of 2-hydroxymuconic semialdehyde. The cell free-extract of *S. maltophilia* LK-24 accumulated the acetaldehyde and pyruvate at very low levels, 0.1 mM, and 0.37 mM, respectively, after 3 h incubation.

Growth of *Stenotrophomonas maltophilia* LK-24 on Various Phenolic Compounds

The cell growth of *S. maltophilia* LK-24 on various phenolic substrates was shown in Table 3. This bacterium was unable to utilize both toluene and xylene, but was able to metabolite benzoate and *p*-hydroxybenzoate. In ironical, however, the cells of strain LK-24 was slightly grew in minimal medium containing catechol. Comparing with previous reports [2, 11, 12, 15, 21, 25], the cell growth of strain LK-24 was good in catechol used as a major sole carbon source.

DISCUSSION

The cell growths of *S. maltophilia* LK-24 at various phenol concentrations were studied in this work. At phenol concentration of 2.5-10 mM, the cell growth was not limited, and product inhibition was observed at much higher than 20 mM substrate concentrations. *Bacillus* sp. strain Cro3.2 [27] was capable to degrade phenol and tolerated at concentration up to 0.1% (w/v) without apparent inhibition of growth. Park *et al.* [25] reported that *Rhodococcus* sp. EL-43P showed the highest growth in a culture containing 1,000 ppm of phenol. At higher phenol concentrations than 1,000 ppm, the growth was greatly inhibited. Moreover, the yeast *Yarrowia lipolytica* Y103 [22] was able to degrade several aromatic hydrocarbons including phenol, catechol, and benzoate. Then strain Y103 exhibited the highest active degradability of phenol at 0.5 mM concentration. On base of this research, therefore, *S. maltophilia* LK-24 might be able to exhibit relatively high tolerance and efficiency at waste treatment contaminated with high concentration of phenol than previous reports [21, 25, 27]. In Fig. 1, the phenol degradation activity of phenol hydroxylase in strain LK-24 was induced after 3-6 h incubation with phenol or cresols, implying that induction and activation of enzymes were needed enough time to initiate adapted for degradation of substrates.

The capabilities of bacteria in degradation phenols have been well studied and the metabolic pathways of phenol were mostly well discussed [3-5, 11, 12, 16]. On basis of

analysis of the induced enzymes, *S. maltophilia* LK-24 exhibit the *meta*-pathway of catechol-2,3-dehydrogenase for degradation of phenol and catechol under aerobic conditions, and its regulation is related indirectly to phenol concentrations. A less than 20 mM, phenol was undergone the *meta*-pathway since the metabolites were analyzed by HPLC. But, they could not able to be detected by thin layer chromatography (TLC). The formation of 2-hydroxymuconic semialdehyde, a metabolite of catechol through the *meta*-pathway, in culture medium was certified due to an induction of catechol-2,3-dioxygenase by inducers such as phenol or cresols. *Pseudomonas putida* [15] and *Alcaligenes eutrophus* [2, 19, 24] exhibited both the *ortho*- and *meta*-cleavage pathway under aerobic culture conditions. However, the *meta*-pathway is common. An almost exclusive expression of the *ortho*-cleavage pathway was observed at initially low growth rates in *Alc. eutrophus* and *P. putida*. Nevertheless, the present works demonstrated that phenol could not experience the *ortho*-pathway to be degraded under aerobic culture conditions.

Adams and Ribbons [1] described that the addition of yeast extract or peptone on the medium was necessary to most of the phenols-degrading bacteria for the ruin of phenol during reaction. In precise, *P. putida* [15] also did not utilize catechol as a sole carbon source induced after phenol degradation. It means that catechol could not be an effective functional elicitor of catechol-1,2- and catechol-2,3-dioxygenase. In our case, however, strain LK-24 slightly grew on catechol as a sole carbon source owing to catechol as an intermediate of phenol or cresols degradation. This means that catechol may be an effective functional inducer of catechol-2,3-dioxygenase under aerobic culture conditions for degradation of phenol. The growth of strain LK-24 on catechol was observed. It is supposed that the enzymes induced by phenol are the necessary for transformation of catechol and catechol to strain LK-24 grown on phenol could be a kind of triggers to cause the *meta*-pathway.

Shinger [31] have attributed that HMSH was an essential

Table 4. Detection of CO_2 as an end product by *Stenotrophomonas maltophilia* LK-24 in minimal medium containing 1 mM phenol under aerobic culture conditions.

Incubation time (h)	CO_2 (%)	
	Uninoculated	Inoculated
12	-	-
24	-	+
36	-	+
48	-	+
60	-	+
72	-	+
84	-	+
96	-	-

-, not detected; +, detected

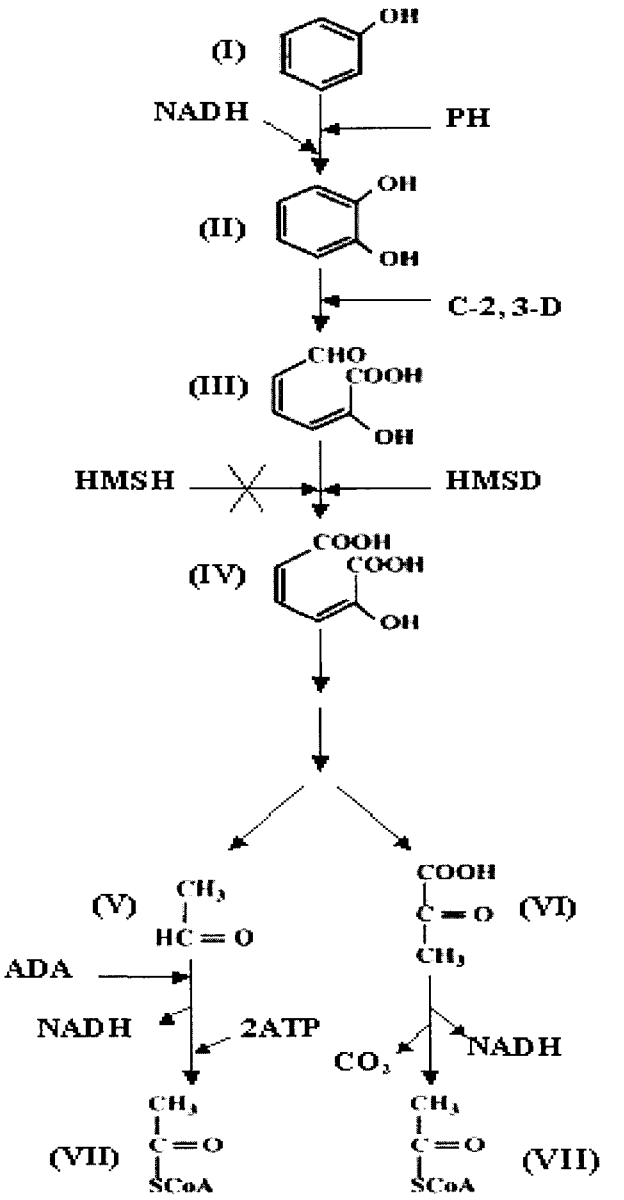

Fig. 4. Possible metabolic pathway of phenol by *Stenotrophomonas maltophilia* LK-24. The metabolites are (I), phenol; (II), catechol; (III), 2-hydroxymuconic semialdehyde; (IV), 4-oxalocrotonate (2-hydroxymuconate); (V), acetaldehyde; (VI), pyruvate; and (VII), acetyl-Coenzyme A. The abbreviations are phenol hydrolase, (PH); catechol-2.3-dioxygenase, (C-2,3-D); 2-hydroxymuconic semialdehyde dehydrogenase, (HMSD); 2-hydroxymuconic semialdehyde hydroxylase, (HMSH); and acetaldehyde dehydrogenase (acylating), (ADA).

for degradation of 2-, and 3-methylated phenols. Namely, the hydrolytic branch involving the step catalyzed by HMSH was required for degradation of 2- and/or 3-methylated phenols. In the results obtained from this work, strain LK-24 could consume methylphenol causing the activation of HMSH in *p*-cresol mixed culture. The 4-oxalocrotonate branches were mostly catalyzed by HMSD in degradation of phenol (Table 1), though HMSH was detected due to accumulation of very low level. Therefore, HMSD and HMSH may be activated in the cells of strain LK-24 and the works are depending upon supplied substrates as a sole carbon source. Accordingly, the entire metabolic pathway of phenol by *S. maltophilia* LK-24 is functional for 2-hydroxymuconic semialdehyde and its metabolites, and the pathway for phenol metabolism is similar that known for the *meta*-pathway for catechol.

In cell-free extracts of *S. maltophilia* LK-24, the metabolic rate of 2-hydroxymuconic semialdehyde was relatively low as compared to previous reports [4, 17, 21] due to the instability of substrate 4-oxalocrotonate (2-hydroxymuconate) in strain LK-24. On observation that 4-oxalocrotonate (2-hydroxymuconate) was converted to pyruvate and acetaldehyde in cell free extracts of *S. maltophilia* LK-24, the metabolism of 4-oxalocrotonate (2-hydroxymuconate) less than stoichiometric conversion might be due to the existence enzymes in cells of *S. maltophilia* LK-24 that catalyze the further metabolism of pyruvate and acetaldehyde. The results exhibited that 4-oxalocrotonate (2-hydroxymuconate) was further degraded by cells of *S. maltophilia* LK-24 via a series of reactions identical those of the *meta*-pathway for catechol [6, 8, 21, 24, 27, 32]. Moreover, the degradability of *S. maltophilia* LK-24 for metabolizing pyruvate and acetaldehyde to acetyl-Coenzyme A was concerned. The experiments were carried to determine whether enzyme in strain LK-24 could catalyze pyruvate and acetaldehyde to acetyl-Coenzyme A. The ADA was as well induced in *S. maltophilia* LK-24 by phenol. That is to say, the primary assimilations of acetaldehyde and pyruvate were formed during the cleavage of phenols with the *meta*-pathway.

The gas in the headspace from culture medium inoculated by *S. maltophilia* LK-24 was analyzed for the presence of CO_2 at 12 h intervals in a gas chromatograph. The consequences of the analysis were shown in Table 4. CO_2 was detectable (retention time = 1.11 min) after 24 h of incubation and was persisted for 72 h. Since *Pseudomonas mendocina* [29] was also a phenol degrading culture, it further degraded phenol compound with the intermediate and terminal oxidation. CO_2, was formed terminal. This study derived similar results. The degradation of phenol by *S. maltophilia* LK-24 includes the *meta*-cleavage pathway with CO_2 as the end product. According to the conclusions obtained this investigation, a possible scheme for the degradation of phenol by *S. maltophilia* LK-24 is summarized in Fig. 4. Thus, the phenol was mineralized to CO_2 through several metabolites. From the results presented here, it is possible to make some assessment of the feasibility of using *S. maltophilia* LK-24 for the treatments of phenolic-contaminated waste streams.

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국문초록

Stenotrophomonas maltophilia LK-24의 페놀분해 관련 효소

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Stenotrophomonas maltophilia LK-24가 페놀화합물을 분해하는데 관련된 효소들과 페놀의 분해 산물을 분석한 결과 phenol hydrolase, catechol-2.3-dioxygenase, 2-hydroxymuconic semialdehyde dehydrogenase, 2-hydroxymuconic semialdehyde hydroxylase, 및 acetaldehyde dehydrogenase를 확인하였다. 이러한 효소들의 활성으로 보아 페놀은 *meta*-pathway ring cleavage를 거치면서 분해되는 것으로 사료된다. 이러한 결과는 페놀화합물의 metabolic pathways를 이해하는데 많은 도움이 되며, phenolic-contaminated waste streams에 *S. maltophilia* LK-24를 사용할 수 있으리라 여겨진다.

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