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Degradation of naphthalene by thermophilic bacteria *via* a pathway, through protocatechuic acid

Research Article

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Abstract: A number of thermophilic bacteria capable of utilizing naphthalene as a sole source of carbon were isolated from a high-temperature oilfield in Lithuania. These isolates were able to utilize several other aromatic compounds, such as anthracene, benzene, phenol, benzene-1, 3-diol, protocatechuic acid as well. Thermophilic isolate G27 ascribed to *Geobacillus* genus was found to have a high aromatic compound degrading capacity. Spectrophotometric determination of enzyme activities in cell-free extracts revealed that the last aromatic ring fission enzyme in naphthalene biotransformation by *Geobacillus* sp. G27 was inducible *via* protocatechuate 3, 4-dioxygenase; no protocatechuate 4, 5-dioxygenase, protocatechuate 2, 3-dioxygenase activities were detected. Intermediates such as o-phthalic and protocatechuic acids detected in culture supernatant confirmed that the metabolism of naphthalene by *Geobacillus* sp. G27 can proceed through protocatechuic acid *via ortho*-cleavage pathway and thus differs from the pathways known for mesophilic bacteria.

Keywords: *Thermophilic bacteria • Geobacillus • Naphthalene • Dioxygenases*

Abbreviations

Mineral salt medium (MSM), protocatechuate 2,3-dioxygenase (2,3-PCDase), protocatechuate 3,4-dioxygenase (3,4-PCDase), protocatechuate 4,5-dioxygenase (4,5-PCDase), retention time (R_t), retardation factor (R_f).

1. Introduction

Pathways by which mesophilic bacteria degrade aromatic hydrocarbons under aerobic conditions as well as enzymes involved in the metabolism of these hydrocarbons are well-studied [1-4]. On the other hand, little is known on the growth of thermophilic microorganisms on aromatic hydrocarbons, such as benzoic acid, cresols and phenols [5-7]. These microorganisms contain enzymes that function at elevated temperatures and are more stable. Such enzymes are of great interest for industrial application. The only enzyme described in considering aromatic hydrocarbon metabolism of thermophilic bacteria is catechol 2, 3-dioxygenase of thermophilic, phenol-degrading *Bacillus thermoleovorans* [8]. It has been established [9] that the species *Bacillus thermoleovorans* utilizes naphthalene and its degradation pathways differ from those characteristic of mesophiles. As far as we know, there is no information about the enzymes participating in naphthalene degradation by thermophilic bacteria.

The aim of the present study was to identify the main naphthalene metabolites produced by thermophilic *Geobacillus* sp. G27, isolated from geothermal oil field of Lithuania, and to determine the key enzymes activities of naphthalene degradation, involved in the last ring fission by thermophilic bacteria.

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2. Experimental procedures

2.1. Microorganism isolation and cultivation

Thermophilic bacterial isolate designated as G27 was isolated from the oil fields of Lithuania. Crude oil samples (1 ml) were suspended in mineral medium containing (g l^{-1}) NaCl – 28.4, K_2HPO_4 – 4.74, KH_2PO_4 – 0.56, $MgSO_4$ – 0.5, $CaCO_3$ – 0.1, NH_4NO_3 – 2.5. The flasks were incubated at 60°C for 48 h. Enriched cultures were plated onto nutrient agar containing (g l^{-1}) tryptone – 10.0, meat extract – 5.0, NaCl – 5.0, agar – 20.0. All plates were incubated at 60°C for 72 h. The ability of bacterial isolate to grow on various aromatic compounds was examined by auxanographic screening on a solid medium [10].

For enzymes assays and metabolites determination thermophilic isolate was grown in mineral salt medium (MSM) [9] containing (g l^{-1}): NH_4Cl – 1.0, $Na_2HPO_4 \cdot 2H_2O$ – 0.42, $NaH_2PO_4 \cdot H_2O$ – 0.38, $MgCl_2 \cdot 7H_2O$ – 0.1, $CaCl_2 \cdot 2H_2O$ – 0.1, KCl – 0.04, $FeSO_4 \cdot 7H_2O$ – 0.008 and supplemented with 0.03 g l^{-1} yeast extract and 2.5 mM naphthalene. The pH of the medium was adjusted to 7.0 with 2M KOH. The flasks were inoculated with 10% (v/v) of pre-culture grown on overnight in MSM with 0.03 g l^{-1} yeast extract and 0.3 mM ammonium acetate at 60°C to an OD_{590} of 0.4. Batch cultures were incubated in the dark at 60°C. Bacterial growth was determined turbidimetrically at 590 nm. The enzyme assays control consisted of the same MSM with glucose (0.6%) instead of naphthalene, giving the same OD as G27 growth on naphthalene. An abiotic control, sterile medium without inoculation, was also run in all metabolites determination experiments.

2.2. Microorganism identification

The identification of subsurface bacterial isolate, designated as *Geobacillus* sp. G27, was done on the basis of its cultural, physiological, biochemical and molecular characteristics. The shape and size of the living and stained cells were under an Olympus AX70 microscope (magnification 1000x). The Gram reaction was determined using a Gram-staining kit according to recommended protocol (Merck). The effect of temperature on the growth of isolate was tested in nutrient broth. The effect of pH on growth was determined in media adjusted to the appropriate pH with HCl or NaOH and recorded after 3 days at 60°C. Anaerobic growth was tested by incubation of the cultures in 100-ml rubber-sealed screw-cap bottles containing anoxic medium [11]. Gelatin liquefaction, starch hydrolysis, casein and tyrosine decomposition, phenylalanine deamination, the Voges-Proskauer reaction, nitrate and nitrite reduction, catalase, oxidase reactions, acid production from carbohydrates, citrate and propionate utilization were all examined by the methods described by Claus and Berkeley [12]. The organic acid utilization tests were performed in a basal medium [11] supplemented with a separately sterilized solution of one of the substrates.

The total G27 DNA was isolated by the protocol of Ronimus *et al.* [13] and purified according to Sambrook *et al.* [14]. Amplification of 16S rDNA sequence was performed with eubacterial primers 27f and 1495r [15], synthesized by MBI Fermentas (Lithuania). PCR amplification mixture (50 µl) contained PCR buffer with $(NH_4)_2SO_4$, 10 ng of isolated DNA, 150 µM of each dNTP, 1.5 mM $MgCl_2$, 0.5 µmol of each primer and 1.25 U Taq DNA polymerase (MBI Fermentas, Lithuania). The amplification was performed using 35 cycles in Eppendorf thermocycler (2 min initial denaturation at 94°C, 1 min denaturation at 94°C, 2 min annealing at 56°C, 3 min extension at 72°C and 10 min final extension). The PCR products were visualized by 1% agarose gel electrophoresis and purified from the gel using a Perfectprep Gel Cleanup kit (Eppendorf AG, Germany). The PCR product was directly sequenced with the PCR primers 27f, 400f, 440r, 765r, 1495r [15] on an ABI PRISM 377 automated sequencer (Applied Biosystems) using the BigDye® Terminator v3.1 Cycle Sequencing Kit. Sequences were edited with DNASTAR package (DNASTAR Inc., USA). The GenBank databases were searched for matching known eubacterial sequences using the BLAST software.

For the determination of isolate G27 genomic DNA base composition, DNA was extracted from 1 g wet wt of the culture cell pellet. The cells were disrupted with a French pressure cell and the DNA was purified on hydroxyapatite according to Cashion *et al.* [16]. For the DNA base composition, the genomic DNA was hydrolyzed according to Mesbah *et al.* [17]. The resulting deoxyribonucleosides were analyzed by HPLC (Shimadzu Corp., Japan). The calibration was done using non-methylated λ DNA (Sigma) and DNA from *Bacillus subtilis* DSM 402), *Xantomonas campestris* (DSM 3586) and *Streptomyces violaceoruber* (DSM 40783). The G+C content was calculated according to Mesbah *et al.* [17].

2.3. Naphthalene degradation curve

Naphthalene degradation was obtained by monitoring the fluorescence intensities on a fluorescence spectrophotometer (850, Hitachi, Tokyo, Japan). A 12 nm slit was used for excitation (284 nm) and a 9 nm slit for emission (328 nm). Naphthalene concentration was measured by extracting 50 ml of batch culture two times with 10 ml of hexane.

2.4. Enzyme assays

Cells were harvested by centrifugation at 5 000 x g for 10 min at 4°C, washed twice with 3 ml of 50 mM sodium phosphate buffer, pH 7.0, containing 10% (v/v) glycerol and resuspended in the same volume of the buffer. The cells were disrupted by ultrasonic treatment for 3 min at 22 kHz, 65 μA by using a sonicator (UR 105 P, Tomy Seiko Co. Ltd., Tokyo, Japan). The residue was removed by centrifugation at 4°C for 25 min at 14 000 × g. The supernatant was stored at –20°C prior to analysis.

Enzyme activities were assayed by monitoring the disappearance of substrate or appearance of products with a UV-visible spectrophotometer (SF-46, LOMO, Saint-Petersburg, Russia) with thermojacketed cuvette holder at 60°C in a total volume of 3.0 ml. One unit of enzyme activity was defined as that catalyzing either the degradation of 1 μmol of substrate or the formation of 1 μmol of degradation product per minute. Protein was measured by the standard method with bovine serum albumin [18].

Protocatechuate 2, 3-dioxygenase (2, 3-PCDase; EC 1.13.11.x) was assayed by observing accumulation of 2-hydroxymuconic semialdehyde at 375 nm [19]. Protocatechuate 3, 4-dioxygenase (3, 4-PCDase; EC 1.13.11.3) activity was determined by monitoring the decrease in absorbance at 290 nm as described by Iwagami *et al.* [20]. Protocatechuate 4, 5-dioxygenase (4, 5-PCDase; EC 1.13.11.8) was monitored by an increase in absorbance at 410 nm [21].

2.5. Identification of metabolites

All organic solvents were HPLC grade or re-distilled before use (Lachema, Neratovice, Czech Republic). Protocatechuic acid (97%) and 3-methylcatechol were from ACROS Organics (Geel, Belgium). Naphthalene (Scintillation Grade), 1- and 2-naphthols (>99%), 2, 3-naphthalenediol, catechol (>99%) and phthalic acid were supplied by Merck (Darmstadt, Germany). 1, 2-Dihydroxynaphthalene was obtained from Sigma-Aldrich (Stenheim, Germany).

2.5.1. TLC

The metabolites were isolated from culture supernatant of the bacteria grown on naphthalene by extraction three times with 10 ml ethyl acetate after acidification with 2M HCl to pH 2. The ethyl acetate was evaporated and the residue was re-dissolved in ethyl alcohol. The extracts were analyzed on TLC plates (Silica Gel F_{254}, Merck, Germany) using the petroleum ether – benzene – methanol – acetic acid solvent system (80:20:10:4) [22]. The metabolites were visualized under UV light at 254 nm and by exposure to iodine vapor. The compounds were identified by their retardation factor (R_f) and absorbance spectra in comparison with those of authentic compounds.

2.5.2. GC-MS

To identify the metabolites, the entire cultures were centrifuged at 5 000 × g for 10 min at 4°C. The supernatant was extracted four times (1:1, v/v) with ethyl acetate or chloroform, twice at neutral pH and twice after acidification to pH 2.0. The acidic and neutral extracts were dried over Na_2SO_4 and analyzed separately. The volatile compounds of the extract were analyzed as obtained or after methylation with dimethylsulfate according to the method of Bask *et al.* [23]. For gas chromatography (GC) analysis, 5 μl aliquots of extract was taken. Analyses were carried out on a HP 5890 (Hewlett Packard) gas chromatograph equipped with a flame ionization detector. The separation was performed on a silica capillary column, CP-Sil 8CB (50 m x 0.32 mm i.d., film thickness 0.25 μm). The GC oven temperature was programmed as follows: from 60°C (isothermal for 1 min) to 160°C at a rate of 5°C min^{-1} and to 250°C at a rate of 10°C min^{-1} and the final temperature was kept for 7 min. The temperatures of the injector and the detector were 250°C and 280°C, respectively. The flow rate of carrier gas (helium) was 1 ml min^{-1}. The same capillary column and temperature program as in the GC analysis was used in GC-MS. Mass spectra in electron mode were generated at 70 eV. Qualitative analysis was based on retention indexes, mass spectra comparison with data in the literature and mass spectral libraries, comparison of the mass spectra with those of commercially available compounds.

3. Results and discussion

Most of the aerobic microorganisms use different enzymes for the initial attack on the diverse aromatic substrates, but the catabolic pathways converge on

just a few central intermediates such as catechol or substituted catechols (protocatechuic and gentisic acids). Generally, two mechanisms are available for biological fission of the aromatic ring. The mechanisms have been designated as *ortho* and *meta*. However, the *ortho* pathway seems to be more common in the biodegradation of unsubstituted aromatic hydrocarbons by bacteria [2].

3.1. Microorganism identification

The subsurface bacteria isolated from geothermal oilfield in Lithuania were moderate thermophilic, aerobic, Gram-staining positive, motile, spore-forming rods, singly or in short chains. The cells were 2.1-7.4 μm in length and 06-1.1 μm in width. Oval terminal to subterminal endospores were produced within the slightly distended sporangia. Colonies were round, smooth, light-brown and varied in diameter from 1 to 4 mm.

The optimal growth temperature ranged between 45 – 70°C with an optimum at 60°C. Growth at 60°C occurred between pH 6.0 and 7.5, with a pH optimum at 7.0. Oxidase and catalase tests were positive. G27 was a heterotrophic, facultative anaerobic organism, and grew at salt concentrations from 0 to 1.0 % NaCl. Nitrate and nitrite were reduced without formation of gaseous products. Acetate and citrate were utilized. The Voges-Proskauer reaction was negative. Acids were produced from D-glucose, D-mannose, glycerol and cellobiose.

Most of the thermophilic *Bacillus* species previously assigned to the rRNA group 5 have been placed to a new genus *Geobacillus. Geobacillus* species form a phenotypically and phylogenetically coherent group of thermophilic bacilli with high levels of 16S rRNA sequence similarity (98.5-99.2%). This group comprises established species of thermophilic bacilli [24, 25]. Most of these thermophilic strains have been found to grow at 55°C.

The bacterial 16S rDNA nucleotide sequence was aligned manually against representatives of the genus *Bacillus* and *Geobacillus* from the GenBank databases. 1074 bp sequence has been submitted to GenBank under accession number AY946034. The level of 16S rDNA sequence similarity of isolate G27 to bacilli of group 5 was approximately the same as those between species of this group (97.9-99.5%). The isolate G27 appeared to be the closest neighbor to species of Geobacillus genus (99.3-98.0% sequence similarity). A search of the GenBank database revealed the highest level of similarity (99.3%) with those of strain *G. stearothermophilus* R-19048.

The G+C content of isolate G27 total DNA was 54.9 mol %. This corresponds to the G+C values of

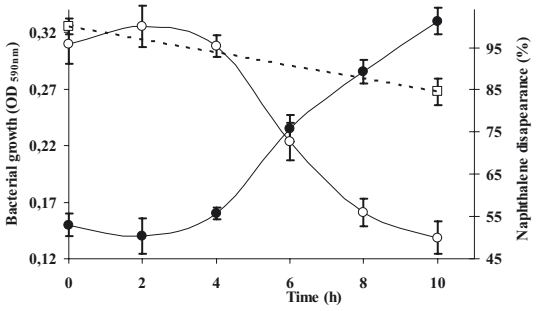

Figure 1. Biomass concentration (●) and naphthalene biodegradation (O) in the short time scale by thermophilic isolate G27 in batch culture on 2.5 mM naphthalene. Dotted line (**- -**) indicates abiotic loss of naphthalene.

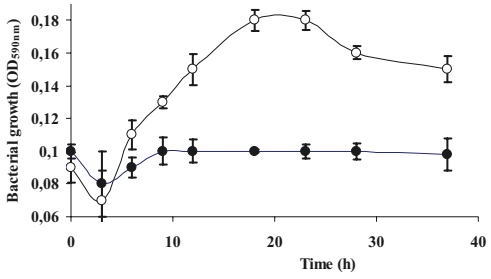

Figure 2. Growth of isolate G27 on 5 mM o-phthalic acid (O) and salicylic acid (●) in batch culture.

Geobacillus genus (49.0 – 58.0 mol %) [25]. It was similar to *G. thermocatenulatus* (55.2 mol %) and *G. thermoleovorans* (53.7 mol %) species. However, the G+C content of isolates of the same species can differ 5 mol %. Thus, phylogenetic analysis based on 16S rDNA sequences showed that this bacterial isolate indeed belongs to *Geobacillus* genus [25].

3.2. Growth on aromatic carbon sources

Thermophilic *Geobacillus* sp. G27 was able to grow on anthracene, protocatechuic acid, benzene-1, 3-diol, phenol and benzene. This isolate showed a poor growth on benzene-1, 2-diol (catechol), although the catechol pathway is known to be a common route for the degradation of naphthalene by mesophilic microorganisms [26, 27]. Moreover, in this study, methylcatechols were found to be growth inhibitors. Growth characteristics on naphthalene of the thermophilic isolate G27, sampled for further investigations are given in Figure 1.

During the first growth hours when concentration of biomass remained almost constant, the amount of degraded naphthalene was insignificant, while a large part was assimilated on the tenth hour of the exponential growth phase. Cultivation of *Geobacillus* sp. G27 in mineral salt medium (MSM) supplemented with 5 mM o-phthalic or salicylic acids was performed (Figure 2).

Geobacillus sp. strain grew on o-phthalic acid but no growth was observed on salicylic acid. When this strain was grown on o-phthalic acid, the 3, 4-PCDase activity was observed. Thus, it is quite possible that naphthalene is metabolized by thermophilic isolates *via* protocatechuic acid pathway. To confirm this suggestion, we found the *Geobacillus* sp. G27 grown on naphthalene accumulated o-phthalic acid, but no salicylic acid. The TLC of supernatant extract showed nine spots at R_f 0.04, 0.07, 0.1, 0.18, 0.21, 0.25, 0.35, 0.45 and 0.52. The isolated products from their R_f and UV spectra were identified as protocatechuic acid (R_f 0.25), o-phthalic acid (R_f 0.35), 2, 3-dihydroxynaphthalene (R_f 0,45) and benzoic acid (R_f 0,52). It is known that phthalic acid is further metabolized in aerobic mesophilic bacteria through the common intermediate, protocatechuic acid [28].

Retention time (min)	Compound	Mass to charge ratio (m/z) of major ion peaks (%)[a]
11.2	Naphthalene (substrate)	128(M^+) (100),122(2),102(7)101(2),81(1), 75(3),74(3),64(4),51(8)
14.5	o-Phthalic acid	166(M^+) (2),148(37),104(100),76(85), 50(43)
20.3	2-Naphthol	144(M^+) (100),145(10),116(28),115(62), 89(8),72(2),63(9),57(10)
20.8	1-Naphthol	144(M^+) (100),145(11),116(42),115(76), 88(10),74(4),63(10),54(7),52(5)
26.4	Protocat-echuic acid	154(M^+) (100),138(11),137(10) 109(34), 81(12),63(7),55(6),51(9)

Table 1. Detection of naphthalene metabolites by GC-MS. [a] *The ion abundance percentages are shown in parentheses.*

3.3. Identification of naphthalene degradation metabolites

Six metabolites were detected in GC-MS chromatograms of derivatized or non-derivatized extracts from naphthalene grown culture. Four compounds (o-phthalic acid, 2-naphthol, 1-naphthol and protocatechuic acid) were detected directly (Table 1), and as methylated derivatives. Additionally, two compounds were detected as methylated derivatives, i.e. 1,2-dihydroxynaphthalene as 1,2-dimethoxynaphthalene [MS fragments:188(M^+) (66), 174(11), 173(100), 145(24), 130(10), 115(10), 114(7), 102(11), 76(10), 63(4), 51(5)] and benzoic acid as benzoic acid methyl ester [MS fragments: 136(M^+) (28), 105(100), 106(8), 77(75), 51(40), 50(16)]. Identification of all of these compounds was carried out by comparing their MS data and retention time (R_t) with those of standards.

Two metabolites of naphthalene degradation were identified as 1-naphthol and 2-naphthol (Table 1), which have been described as intermediates of naphthalene degradation by *B. cereus* [29] and *B. thermoleovorans* [9]. Monohydroxylated naphthalenes could be intermediate compounds, but also a side reaction, because most of bacterial naphthalene cleavage reactions occur at the 1,2-position coursed by dioxygen-

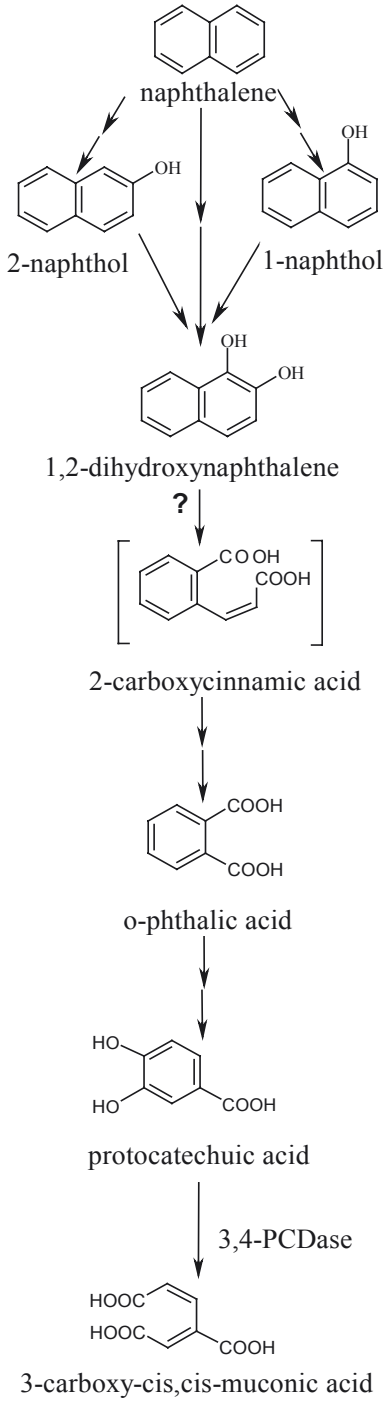

Figure 3. Proposed scheme of precursor-product relationship for naphthalene degradation by thermophilic isolate *Geobacillus* sp. G27. Brackets indicate metabolites, proposed elsewhere (Annweiler *et al.* 2000).

ation and dehydrogenation reactions [27]. It is possible primary oxidation reactions were catalysed by both dioxygenases and monooxygenases [30]. Naphthalene metabolism could be initiated by the formation of naphthalene oxide mediated by cytochrome P450-dependent monooxygenases. Subsequent rearrangement forms 1-naphthol and 2-naphthol which could be hydroxylated to 1,2-dihydroxynaphthalene. Several researchers [31] confirmed this capability detecting o-phthalic acid as 1-naphthol transformation metabolite by *Pseudomonas* sp. 2-Carboxycinnamic acid was not detected in our studies, but was assumed as a ring fission product by Annweiler *et al.* [9] in studies with thermophilic *B. thermoleovorans* (now *Geobacillus thermoleovorans*) bacteria. GC-MS analysis of ethyl acetate or chloroform extracts of culture medium revealed the major metabolites of naphthalene had a molecular weight and fragmentation pattern identical to the molecular weight and fragmentation pattern of protocatechuic acid, o-phthalic acid (Table 1) and benzoic acid. Benzoic acid could form by decarboxylation of o-phthalic acid. Known metabolic pathway of o-phthalic acid through protocatechuic acid enters the central metabolism as succinic acid by *orto*-cleavage of protocatechuic acid [1, 2]. This was confirmed by detection of 3, 4-PCDase enzymatic activity. Protocatechuic acid, 1-naphthol and o-phthalic acid were identified by mass spectra, which were analogous to those reported for phenanthrene ring cleavage products by Pseudomonas strain sp. DLC-P11 [31]. Recently Herwijnen *et al.* [32] reported for a partially new anthracene degradation pathway in *Mycobacterium* LB501T through o-phthalic and protocatechuic acids. As seen from above, these metabolites are common for biodegradation of polyaromatic hydrocarbons by bacteria. Protocatechuic acid is a well-known compound of the lower pathway key intermediate, never before detected in naphthalene metabolism by bacteria.

3.4. Key enzymes activities

Theoretically, further degradation of protocatechuic acid could proceed by one of the few pathways. Enzymatic activities of naphthalene-induced cells were compared. The enzymatic studies have shown naphthalene to degrade *via* protocatechuic acid by the *ortho*-cleavage pathway, because the cell-free extracts of the *Geobacillus* sp. G27 grown on naphthalene contained the activity of 3,4-PCDase, but no 4,5-PCDase, 2,3-PCDase. Activity of 3, 4-PCDase reached 350 units mg protein^{-1} on the tenth hour of the exponential growth phase. The pathway was inducible since the glucose-grown cell free extract did not contain activities of these enzymes.

The observation of 1-naphthol, 2-naphthol, 1, 2-dihydroxynaphthalene, protocatechuic acid, o-phthalic acid and enzyme activity of 3, 4-PCDase specifies provides a possibility of a pathway for naphthalene degradation by thermophilic bacteria (Figure 3). The naphthalene degradation by the thermophilic *Geobacillus* sp. G27 differs from the known pathways found for the mesophilic bacteria which degraded naphthalene through salicylic acid and catechol or gentisate. The pathway is more similar to that reported by Annweiler *et al.* [9] for *B. thermoleovorans* where catechol or salicylic acid were not detected as a naphthalene metabolites, but alternative 2-carboxycinnamic acid and phthalic acid were identified.

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