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Effect of pH and NaCl Concentration on Benzoate Utilization of Anoxygenic Photosynthetic Bacteria

DWI SURYANTO[1‡] & ANTONIUS SUWANTO[1,2*]

[1]*South East Asian Regional Center for Tropical Biology, Bogor 16001*
[2]*Department of Biology, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University, Bogor 16144*

Total genomic analysis of three anoxygenic photosynthetic bacteria, DS-1, DS-4 and Cas-13, was done using Macro-Restriction Fragment Length Polymorphism technique employing pulsed-field gel electrophoresis. The constructed phylogenic tree of the profile analysis showed that DS-1 and Cas-13 were closely related rather than to DS-4. Their ability to use benzoate as their sole C source were optimum at NaCl concentration of 0.5% (w/v) and pH of 7.5, respectively. The isolates could grow in benzoate-containing media up to 10 mM. Growing in other C-sources was optimum in 3.0% casamino acid and 1.0% dextrine, but exhibited different response for other compounds.

Key words: anoxygenic photosynthetic bacteria, benzoate degradation, MFLP analysis

In the last few decades, many hydrocarbon compounds especially aromatic hydrocarbons have been introduced in large quantity and accumulated in soil, aquatic environment, anaerobic sediment, or even in deep-ground water (Kuo & Genthner 1996). They become a serious problem since they are toxic and carcinogenic (Leahy & Colwell 1990).

The persistence of the aromatic hydrocarbon compounds in environment depends on the structure and the complexity of the compounds. Haloaromatic and polycyclic aromatic hydrocarbon in general are relatively recalcitrant. It is known that mineralization rate (degradation of the compounds to CO_2 and H_2O) of higher-molecular-weight complex aromatic hydrocarbon, such as resin, and asphaltenes is much slower than degradation of lower-molecule-weight aromatic hydrocarbon such as monocyclic aromatic compounds. However, previous studies showed that complex hydrocarbons were rapidly degraded in optimum condition (Leahy & Colwell 1990).

Although previous works showed anaerobic degradation of many monocyclic aromatic (Kuo & Genthner 1996, Crowford *et al.* 1998), anaerobic catabolism of benzoate has gotten more attention. Previous studies on anaerobic benzoate degradation in various bacteria were done by Kuo and Genthner (1996) and Warikoo *et al.* (1996). Other studies showed that a group of bacteria, anoxygenic phototrophic bacteria (APB), photoanaerobically catabolize benzoate and its derivatives or homologs (Harwood & Gibson 1988, Kamal & Wyndham 1990, Wright & Madigan 1991, Gibson & Gibson 1992, Shoreit & Shaheb 1994). Since the APB demonstrate biochemical versatility, they are relatively easier to study rather than any other obligately anaerobic bacteria.

‡ Alamat kini: Department of Biology, Faculty of Mathematics and Natural Sciences, North Sumatra University, Medan 20155
* Penulis untuk korespondensi, Fax. +62-251-315107, E-mail: asuwanto@indo.net.id

For the purpose of employing such microorganisms for bioremediation purposes, the effect of environmental factors such as pH, temperature, and salinity should be determined, otherwise the ability of microorganisms to utilize aromatic hydrocarbon compounds might not be optimized. In this study, we observed the ability of three isolates to grow in different concentration of benzoate and NaCl as well as in different initial pH was examined. We also identified the isolates based on their Macro-Restricted Fragment Length Polymorphism profiles (MFLP).

MATERIALS AND METHODS

Three isolates of APB (DS-1, DS-4 and Cas-13) were used in this study. The two formers of APB strains were isolated from Java and the last was isolated from Molucca (Suryanto & Suwanto 2000).

For testing the anoxygenic photosynthetic bacteria (APB) growth on aromatic compounds and the ability to degrade aromatic compounds, the isolates were grown in modified Sistrom (Suwanto & Kaplan 1989) by omitting all carbon sources including nitrilo-triacetic acid, with 5 mM benzoate as C-source supplemented with or without vitamins in 100 ml completely filled screw-cap tubes. All cultures of APB were illuminated with 40 W of tungsten bulb in a distance of 30 cm. Unless it mentioned otherwise, all media were adjusted to pH 7.2.

To examine degrading ability of three isolates in different conditions, the isolates were grown in modified Sistrom medium with different NaCl concentrations (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0%), benzoate concentrations (2.5, 5.0, 7.5, 10.0 mM) and 5.0 mM benzoate without vitamin supplementation, and at different initial pH (4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0). Either NaOH or HCl solutions were used to adjust pH. The ability of the isolates to grow in benzoate with different condition was measured at 120 hours of incubation time. Utilization of other C-sources was measured at 72 hours of inoculation.

Growth of the isolates in other C sources was performed in modified Sistrom by omitting all carbon sources including nitrilotriacetic acid but vitamins with either 5.0 mM Na-citrate, 5.0 mM glucose, 5.0 mM glutamate, 5.0 mM mannitol, 1.0% dextrin, 3.0% ethanol, 5.0 mM Na-tartrate, 3.0% casamino acid, and 3.0% glycerol added with 1% $CaCO_3$ as sole carbon sources. All media were adjusted to pH 7.2.

For all inoculation, the seed cultures were taken from 2-days old culture of aerobic bacteria or 3-days old culture of APB of 5 mM Na-benzoate medium. For other C sources, inoculations were taken from 2 days old culture. The cultures were grown with the initial cell concentration of approximately 5 x 10^6 cell/ml. Benzoate compounds were filter-sterilized.

Cell density was measured as absorbance at 660 nm (Harwood & Gibson 1988, Kamal & Wyndham 1990) using *Novaspec II* (Pharmacia, Uppsala, Sweden) spectrophotometer. Benzoate concentration was measured at its absorption maximum of 276 nm using Hitachi Model U-2010 UV/Vis spectrophotometer (Hitachi Instrument, Inc. Japan) following the establishment of standard curve relating benzoate concentration to UV absorbance (Shoreit & Shaheb 1994).

Intact genomic DNA and restriction digestion was performed as follows. Gel inserts for total genomic analysis and DNA restriction were prepared as described by Suwanto and Kaplan (1989). Pulsed-field gel electrophoresis to obtain MFLP profiles was utilized for DNA separation using CHEF-DR® II (Bio-Rad, Richmond, CA). Treecon computer program (Yves Van de Peer of Department of Biochemistry, University of Antwerp) was used to determine their relatedness in phylogenic tree based on their MFLP profiles obtained from pulsed-field gel electrophoresis.

RESULTS AND DISCUSSION

The result on MFLP analysis (Figure 1) showed that isolate DS-1 was phylogenetically related to isolate Cas-13 rather than to DS-4 (Figure 2). It also showed that *Rhodobacter sphaeroides* 2.4.1 was relatively not closed to the other three APB isolates. The MFLP technique has been very useful in characterizing strains within a species which otherwise exhibit similar morphological and physiological properties rather than other typing techniques including auxotyping, serotyping, restriction endonuclease analysis (REA), ribotyping, and random amplified polymorphic DNA (RAPD) (Saulnier *et al.* 1993, Suwanto 1994).

Unlike *R. sphaeroides* 2.4.1, all of the three isolates were able to metabolize benzoate (Suryanto *et al.* 2001). Among the members of the APB group, *Rhodopseudomonas palustris* is the most common species that capable of utilizing benzoate (Harwood & Gibson 1988, Kamal & Wyndham 1990, Gibson & Gibson 1992, Shoreit & Shaheb 1994). It was observed in previous study that 65.8% of tested isolates of the APB were able to use benzoate as their sole C-source (Suryanto & Suwanto 2000).

The ability to grow in aromatic compound media such as benzoate with no vitamins (Figure 3, 4, 5) indicated that the APB were able to synthesize their own vitamins. However, supplemented vitamins in the media could increase their potential in metabolizing benzoate (Suryanto *et al.* 2001).

Similar profiles of growth and degradation were observed at different NaCl concentration, benzoate concentration, and initial pH. Optimum NaCl concentration and pH for growing were 0.5% (w/v) and 7.5, respectively (Figure 3, 4, 5). Sathappan (1997) showed that the fresh water anoxygenic photosynthetic bacteria, *R. palustris* strain B1, had optimum NaCl concentration of 0.5% (w/v) and pH of 5.5-8.

Obviously, benzoate was toxic to these three isolates. Lower absorbance at 660 in 10.0 mM benzoate (Figure 3, 4, 5) indicated that these three isolates might poorly grow in benzoate concentration exceed 10.0 mM. Many workers used up to 5.0 mM for degradation test in APB (Gibson & Gibson 1992, Shoreit & Shaheb 1994). Increases in cell tolerance against toxic substrates were crucial to improve the degradation capabilities. Alteration of *cis* to *trans*-fatty acid of cell membrane might improve cell tolerance to toxic substrates (Hiepieper *et al.* 1992).

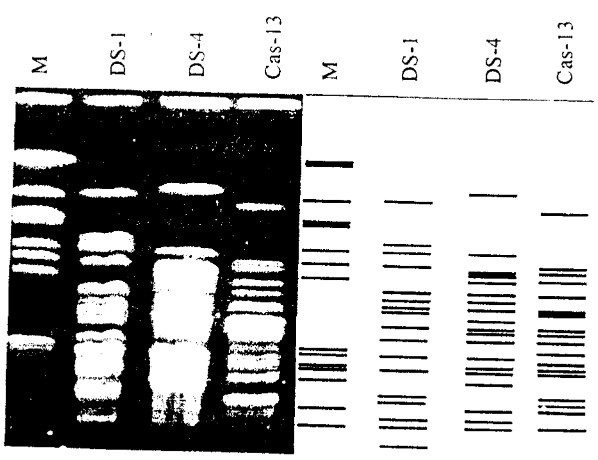

Figure 1. MFLP profiles of total genome digested with *AseI*. Molecular standard (M) is total genome of *Rhodobacter sphaeroides* 2.4.1.digested with *AseI*.

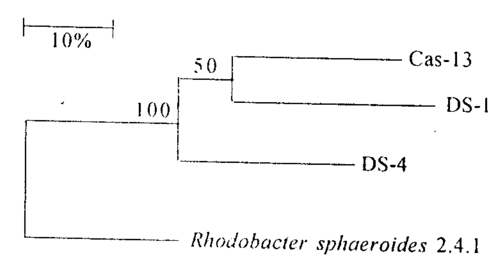

Figure 2. Phylogenic tree of MFLP profiles generated from total genomic DNA digested with *AseI*. The number at the tree lines represented bootstrap values.

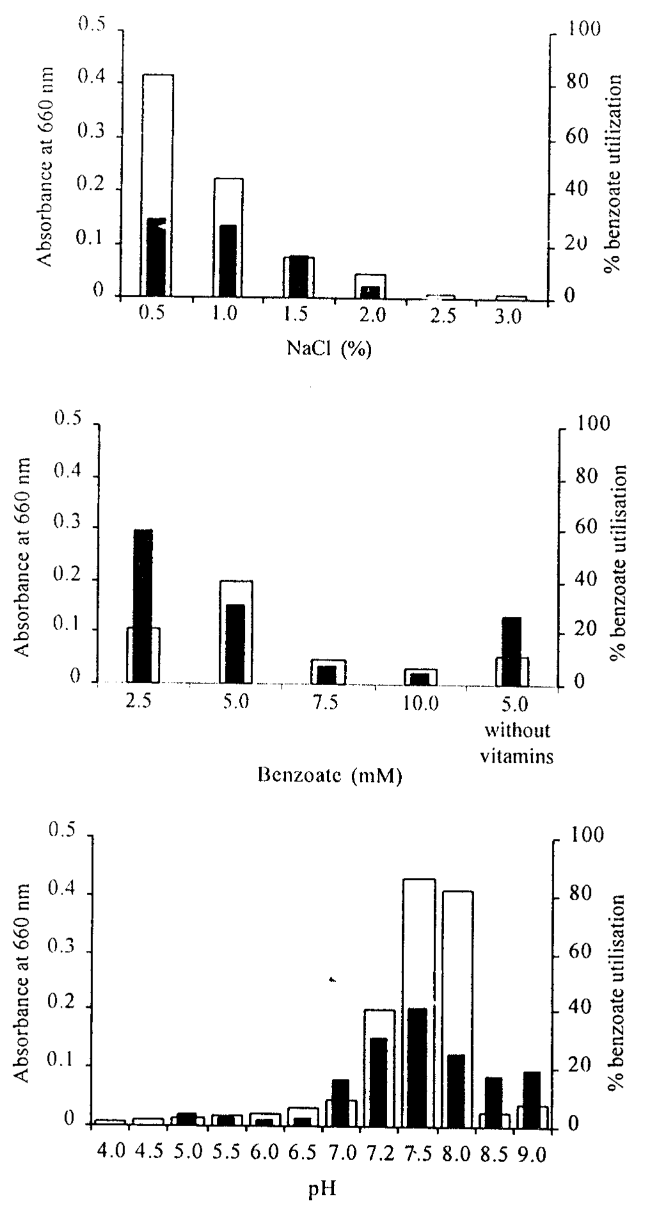

Figure 3. Histogram of cell density and benzoate utilization of isolate DS-1. ■ Cell density, ❑ Benzoate.

There was a relationship between cell density and benzoate utilization for all isolates, which the cell density was relatively high in pH of 7.5-8.0 and in NaCl concentration of 0.5% (w/v). Previous studies showed that the effect of pH on degradation of organic compounds might vary. However, extreme pH would be expected to have a negative influence on the ability of microbial populations to degrade hydrocarbons (Leahy & Colwell 1990). Dibble and Bartha (1979) observed an optimal pH of 7.8 in the range 5.0 to 7.8 for mineralization of oily sludge in soil. Hambrick *et al.* (1980) showed that the rate of microbial mineralization of octadene and naphthalene to be depressed at pH 5.0 compared with pH 6.5 (Venkateswaran *et al.* 1993). The cell growth might be indirectly affected by pH by change enzyme activity.

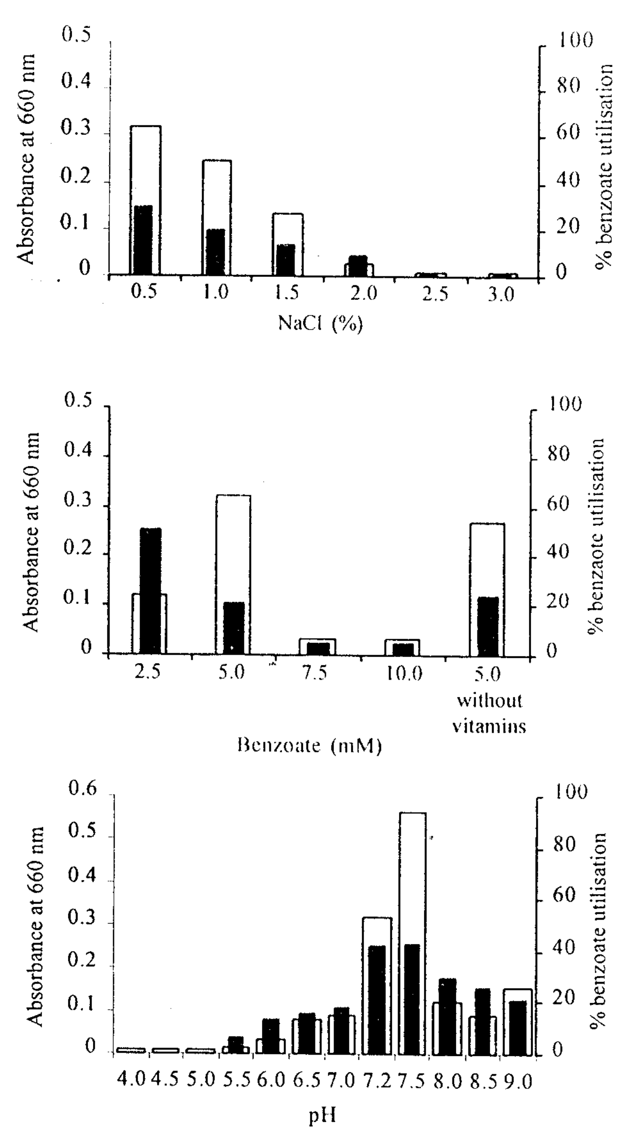

Figure 4. Histogram of cell density and benzoate utilization of isolate DS-4. ■ Cell density, ❑ Benzoate.

Few studies were done on effect of salinity on the microbial degradation of hydrocarbons (Leahy & Colwell 1990). The reduction of cell density as it observed at higher NaCl concentration cultures might be due to cell lysis. Shiaris (1989) noticed a generally positive correlation between salinity and rates of phenanthrene and naphtalene degradation in estuarine sediments. Ward and Brock (1978) observed that rates of hydrocarbon metabolism decreased with increasing salinity in the range 3.3 to 28.4% and attributed the results to a general reduction in microbial metabolic rates of hypersaline salt evaporation ponds.

The experiments with other organic compounds showed that, in general, casamino acid and dextrin supported the cell growth. The growth in other C sources might vary (Figure 6). However, it is well known that purple non-sulfur bacteria grow better in media with C4 dicarboxylic acids such as malate or succinate (Tabita 1995).

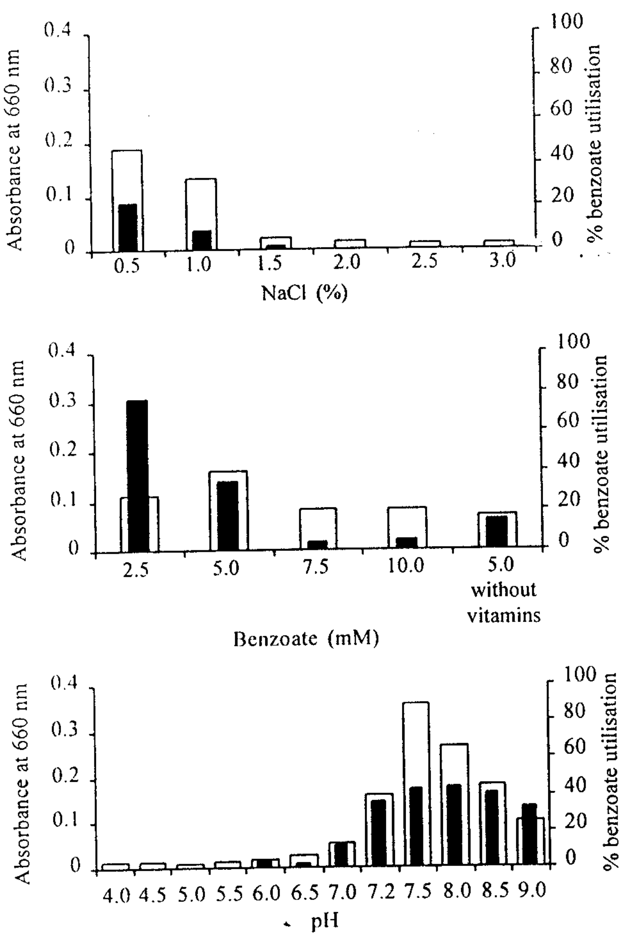

Figure 5. Histogram of cell density and benzoate utilization of isolate Cas-13. ■ Cell density, ❑ Benzoate.

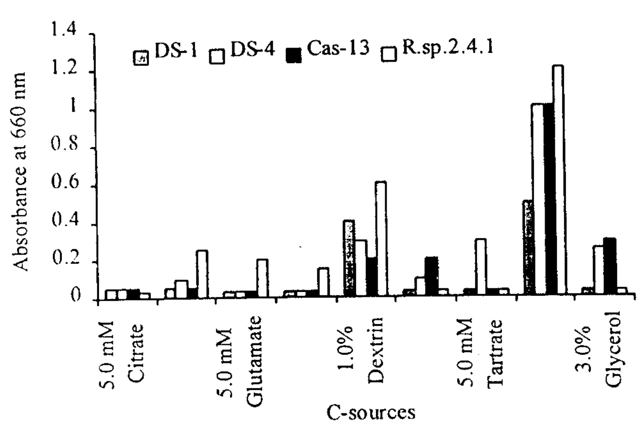

Figure 6. Histogram of cell density of other C-source utilization of isolate DS-1, DS-4, Cas-13, and *Rhodobacter sphaeroides* 2.4.1 (R.sp.2.4.1).

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