Simple Protocol for Adaptation of *Ralstonia eutropha* toward *P*-nitrophenol Degradation

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Abstract— Growth adjustment for *Ralstonia eutropha* relevant to the biodegradation of *p*-nitrophenol (PNP) was investigated. Based on considering growth medium compositions capability of the bacterium for performing of this oxidative event was found to be related to presence of three growth medium nutrients namely, glucose, yeast extract and peptone. The experiments were directed to reduce levels of and then eliminate these common media ingredients. The processes were done through consecutive inoculum transfer program and trend of the operation(s) were monitored by use of HPLC. Complete decomposition of PNP (13 mg/l) by *R. eurtopha* was occurred in 20 hrs at 30°C and in absence of glucose, peptone while the level of yeast extract was decreased to 80%. The results were discussed in terms of chemotaxis behavior of this Gram$^-$ and motile bacterium (*R. eutropha*). This type of structural bacterial response could be understood when one considers membrane carrier as responsible proteins to respond to organics toxic or nutrient items.

Upon fluctuation of PNP concentration bacterial *R. eutropha* showed more or less same degradative behavior. *R.eutropha* species including H16 have flagella and the results have been discussed in terms of probable existence of pollutant carrier(s) such as PNP in the cells' membrane. Presence of sugar and phosphate transporters has been well documented in bacterial cells.

***Index Terms*— biodegradation, *p*-nitrophenol, *Ralstonia eutropha*..**

I. Introduction

Commercial production of various compounds such as pesticides, herbicides, explosives, dyes and plasticizers depends on use of PNP [1]. Because of severe environmental pollution (air, soil and ground water), US Environmental Protection Agency (EPA) has classified PNP as priority pollutant and its concentration in natural waters has been set at levels below 10 ng l^{-1}[2].

Capability of certain groups of bacteria (*Psedumonas, Arthrobacter, Burkholderia, Trichosporon, Rhodococcus*) towards degrading nitroaromatic compounds has been shown [3-7]. Degradative ability of the microbes towards organics present in environment at very low concentration may express by non-growing microorganisms or by organisms that their growth would be supported by organic compounds present at high a level [8] in the other hand growth of microbes is not dependent on pollutant as sole source of carbon for energy production and utilization. Biodegradation may also occur in presence of supportive substrate (glucose or yeast extract) in addition to the tested pollutant or with presence of only tested pollutant. It is important to mention thus to recognize occurrence of biodegradation either with the pollutant or without the polluted organic but with glucose or yeast extract alone at the present study both are indicate we characterization of metabolizing PNP by non-growing cells [9]. *R. eutropha* species have flagella and this protein machinery known as chemotaxis has been characterized in these species [10]. On the other hand presence of membrane protein carrier for transportation of different chemical items in a variety of microbes [11-13] should be taken into account when one searches chemotaxis behavior toward non-nutrient material.

In the present work *R. eurtopha* adaptation was studied in terms of decomposition ability of the bacterium towards PNP with or without presence of some selected growth medium nutrients. Pollutant removal issues are easier to handle with use of adapted microbes. The results of the study have been viewed under influence of chemotactic behavior of *R.eutropha* and probable existence of related carriers in the cells.

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II. Materials and Method

A. Microorganism and cultivation medium

R. eutropha supplied from Japan Collection of Microorganisms (JCM). Accession number of strain is JCM 20644 in 2007. The composition of the culture medium and its concentration used in this study, has been described elsewhere [14].This standard medium contained 310 mg phosphate/l with equimolar concentrations of KH_2PO_4 and K_2HPO_4, 0.76 g l^{-1} NH_4Cl and the following salts (mg l^{-1}):$MgSO_4.7H_2O$, 71.2 ;$ZnSO_4.7H_2O$, 0.44 ;$MnSO_4.H_2O$,0.61;$CuSO_4.5H_2O$, 0.78 ; $NaMoO_4.2H_2O$, 0.25 ; $Fe(SO_4)2.7H_2O$, 4.98.

B. Chemicals

PNP was obtained from Kanto Chemical Company (Japan). All chemicals used in this study were of analytical grades with the highest purities.

C. Preparation of the inoculum for the adaptation /study

For the bacterial adaptive purposes standard medium having PNP at the some specified initial concentration was used while glucose, peptone and yeast extract have been added to this medium (Table 1). Before the inoculation this medium was autoclaved at 121 ºC for 15 min. The inoculation of *R. eutropha* was performed. In fact the elimination of each of the three above named component was the aim of this part of the experiment (Table 1).Thus after nine transfer of the inoculum according to the defined stages in the Table 1 only yeast extract at 0.5 g/l was needed to be included in the standard cultivation medium having PNP at initial concentration of 13 mg/l the time recorded showed 14 hrs for complete degradation of PNP (i.e., no detection by use of HPLC analysis). 5 ml of the latter grown culture was transferred to the fresh autoclaved standard medium (50 ml) containing PNP at the pre-determined concentration for the biodegradation study. This broth medium had also yeast extract at the 0.25 g/l (pH 7, incubation conditions: reciprocal shaker at 30°C for 20 hrs). Of course PNP at the pre-selected concentration was included in the above standard cultivation. The grown culture in the broth then was centrifuged (2700 rpm, 20 min) by use of the sediment, an appropriate suspension of *R. eutropha* cells was prepared: adding the cells' sediments to the autoclaved standard medium containing PNP at the some specified concentration.

D. Analytical methods

To determine the amount of remaining PNP concentration, the cell free supernatants obtained after centrifuge at 12000 rpm for 20 min were analyzed by the HPLC system Shimadzu equipped with an Inertsil ODS-3V reverse phase column and a UV detector (280 nm). Acetonitril and $NaClO_4$ (the pH adjusted to 2.5) were the solvents system for this measurement. Cell growth was monitored spectrophotometrically at 546 nm.

III. Results and Discussion

Adaptation of bacterial biomass to PNP is crucial in biological treatment systems [15]. After adaptation experiments carried out were to determine if *R. eutropha* was able to grow and utilize PNP as sole carbon and energy sources. Elimination of the nitro group from the aromatic nucleus oxidatively, yielding nitrite, has been confirmed in some several bacteria and in the decomposition of various nitroaromatic compounds. According to the literature *p*-nitrophenol was degraded oxidatively while nitrite molecules were released in the medium [10, 16].

A. Biological adaptation of R. eutropha on PNP

Results of the *R. eutropha* adaptation on PNP are given in Table 1. Presence of glucose, yeast extract and peptone as some influential medium ingredients for the growth of this bacterium was evaluated through consecutive inoculum transfer operation. The basis was on developing a growth medium containing PNP with lowest amount of each of these three ingredients. The *R. eutropha* growth as a function of a single variable or PNP was aim of this adaptation study. Ultimately it was possible to define standard growth medium supplied with only yeast extract at the level of 0.5 g/l. By use of this medium then the lowest concentration of PNP which completely degraded through the growth of *R. eutropha* was 13 mg/l. The adaptation protocol thus was provided the growth medium without any source of carbon and nitrogen was supplied mainly in the form of yeast extract while no peptone was used in this medium formulation (actually the level of yeast extract was reduced by more than 80 %) (Table 1). A summary of these findings including efficiency of the PNP removal (in percent), is shown in Figure 1.Growth of the *R. eutropha* with the specifications given above, was confirmed by obtaining trend of the cells' growth (Fig. 2). PNP degraded completely in < 10 hrs when the growth medium was supplied with yeast extract only. Advantages of these results were shown to be used for the biodegradation study in the next step.

Thereafter, capability of *R. eutropha* for PNP degradation was examined. The results showed that when PNP was present in the growth medium with either yeast extract or glucose degradation of PNP occurred within rather short time (9 and 14 hrs, respectively). Absence of traditional costly glucose and presence of both were indicative of efficient consumption of PNP by *R. eutropha*. The complete degradation of PNP thus occurred between 20 hrs.

IV. Conclusions

A. Capability of the adapted *Ralstonia eutropha* for performing of this oxidative reaction was studied with use of simple protocol considering changes of concentration of growth medium compositions. Presence versus absence of three common microbial nutrients namely glucose, yeast extract and peptone was tested to define the adaptation process for *R. eutropha* at different PNP concentration.

The results showed pollutant removal issues are easier to handle with use of adapted microbes and complete decomposition of PNP (13 mg/l) by *R. eurtopha* was occurred in 20 hrs at 30°C and in absence of glucose, peptone while the level of yeast extract was decreased to 80%.

Table 1: Results obtained from the bacterial adaptive study (see the text for details).

Initial PNP concentration (mg/l)	Treatment specification (g/l)			Time for complete degradation of PNP(hr)
	Glucose	Peptone	Yeast extract	
15	3	2	2	16
41	2	1	1	44
63	2	1	1	90
52	2	1	1	42
37	1	1	1	48
39	0.5	1	1	72
24	0.25	1	1	48
15	0	1	1	48
41	0	1	1	36
13	0	0	0.5	14
13	0	0	0.25	20

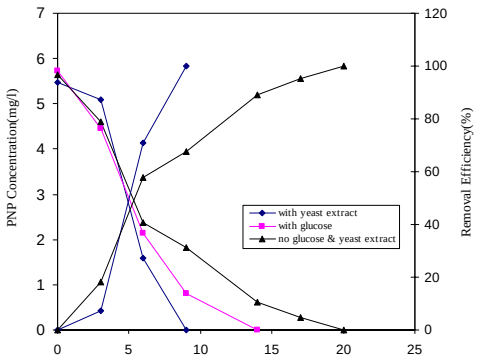

Fig.1. PNP biodegradation by R. eutropha (concentration of PNP was set at 6 mg/l).The efficiency of PNP was also shown.

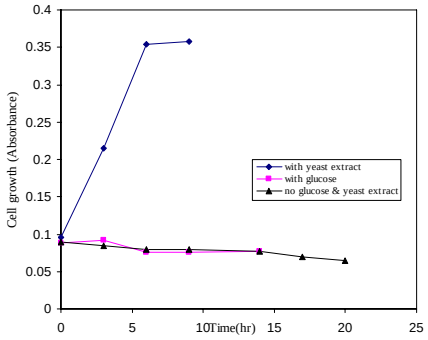

Fig. 2. Time course of *R. eutropha* growth in the standard medium containing PNP at the level of 6 mg/l.

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