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Formation of *ortho*-benzoquinone from sodium benzoate by *Pseudomonas mendocina* P_2d

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Pseudomonas mendocina P_2d grew in sodium benzoate at as high as 1% concentration and formed a quinonoid compound, identified as *ortho*-benzoquinone, that rendered the medium orange to wine-red in colour. The quinone was not metabilised further by the organism. Sodium benzoate was converted to catechol, which was a central metabolite forming *ortho*-benzoquinone and 2- hydroxymuconic semialdehyde (2-HMS) via. *meta* ring cleavage pathway.

Keywords: Catechol, 2-HMS, *Ortho*-benzoquinone, *Pseudomonas mendocina*, Sodium benzoate

Quinones in nature range in colour from pale yellow, orange, red to black and find applications in various industries especially in food as natural colourants and also in photographic developments[1,2].

Dopaquinone formed from tyrosine is an important intermediate in the formation of melanin, which is considered to be the cause for melanosis, the black spots, in prawns[3]. *Para*-quinones are often formed as intermediates during metabolism of various aromatic compounds such as *p*-nitrophenol and 2,4,5-trichlorophenoxy-acetic acid giving 1,4-benzoquinone and 2-hydroxy-1,4-benzoquinone [4-7].

Formation of *ortho*-benzoquinone has not been reported widely. The present paper reports the formation of a red colour *ortho*-benzoquinone from sodium benzoate by *Pseudomonas mendocina* P_2d, which remains unutilized in the growth medium and renders red colour to the medium.

Materials and Methods

Chemicals and media—All the chemicals used were of analytical grade. 2-Hydroxymuconic semialdehyde (2-HMS) was prepared[8] in the laboratory using standard culture *Pseudomonas cepacia* AC1100 by the method described by Bayley and Dagley (1969)[9]. *ortho*-Benzoquinone was prepared by auto-oxidation of aqueous catechol solution (50 m*M*) by incubating on shaker for overnight till pink colour appeared. Excess of catechol was removed by repeated extraction with diethyl ether.

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Bacterial strain and maintenance—*Pseudomonas mendocina* P_2d[10], used in the present study was maintained routinely on mineral salts medium (MSM)[11] agar with 0.3% sodium benzoate as sole carbon source and grown in liquid MSM medium at 28°C at 150 rpm for 24 hr. This culture forms exopolysaccharide that gels under cold conditions[10] and can utilize various aromatic compounds.

Culture pellet and suspension—*Pseudomonas mendocina* P_2d cells, grown in 0.3% sodium benzoate MSM, were centrifuged at 6000 rpm for 10 min. The pelleted cells were vortexed and suspended in 0.05 *M* phosphate buffer (*p*H 7) and centrifuged again. The pellet, thus obtained was resuspended in buffer to a desired absorbance. Also, the wet weight of the washed pelleted cells and the dry weight was noted on drying the cells at 80°C till constant weight.

Analysis of culture supernatant—The culture supernatant of strain P_2d grown in sodium benzoate (0.3%) was scanned using Shimadzu UV-1601 spectrophotometer. Unacidified and acidified (*p*H 2) culture supernatants were extracted with diethyl ether. Thin layer chromatogram (TLC) on silica gel plates were run in benzene : methanol : ethyl-acetate (40:60:40) and spots were visualized on exposure to iodine vapours. Silica gel from the area of spots on TLC plate was scrapped off and suspended in phosphate buffer (0.05 *M*). The silica was allowed to settle. The UV-visible spectra of the filtrates were determined.

High Pressure Liquid Chromatography (HPLC) analysis of the aqueous culture supernatants was

carried out using RP-C18 Nucleosil column and an isocratic solvent system of acetonitrile : water (20:80) with a flow-rate of 1 ml/min with UV-visible detector (Spectra-Physics SP800) at 270 nm.

Catechol assay [12]—The culture supernatant (5 ml) acidified to *p*H 2 with 6 N HCl was extracted with diethyl ether. The ether layer was dried. To the residue dissolved in 1 ml distilled water, 1 ml of 10% sodium molybdate and 0.5 ml of 0.5 N HCl were added followed by 1 ml of 0.5% sodium nitrite solution and 1 ml of 0.5 N NaOH. The absorbance of cherry-red colour reaction mixture was read at 510 nm.

Identification of quinone—Presence of quinonoid compounds in the red culture supernatant was detected qualitatively by various tests[13]. The structural identity of the quinone was confirmed by formation of 4,5-dianilino-derivative[12]. Cell pellet from 50 ml of 24 hr old culture was suspended in 50 ml catechol (25 m*M*) in 0.05 *M* of phosphate buffer (*p*H 7) with aniline (1%, v/v) and incubated on shaker at 150 rpm at 28°C. At various predetermined intervals, the red precipitate of the derivative was filtered using Whatman filter paper no. 1 and weighed. The precipitate was extracted in diethyl ether. The derivative was recrystallized from light petroleum (40°-60°C) dried and weighed. The derivative was then dissolved in methanol and absorbance read at 490 nm. Control without aniline was kept. Melting point of the crystals was determined using Theil's tube method. The infra-red spectrum (IR) of the crystals was scanned using KBr pellets on FT_1R-8101A Shimadzu spectrophotometer.

Effect of copper-chelators on catechol-oxidase—Cell pellet obtained from 50 ml of culture grown in 0.3% sodium benzoate medium for 24 hr was suspended in 10 ml of catechol (25 m*M*) in 0.05 *M* phosphate buffer (*p*H 7), incubated at 35°C for 10 min and the colour change was noted. Enzyme inhibitors namely, mercaptoethanol, cysteine, sodium-bisulphide and ascorbic acid, were added individually at a concentration of 1 m*M*, to the above reaction mixture and their effects on the colour and formation of various transformation products were noted.

Oxygen uptake analysis—Metabolic response of benzoate grown cells to different metabolic intermediates was studied using Gibson 5/6 oxygraph by oxygen uptake analysis, expressed as nmole/min/mg dry weight of cells.

Results and Discussion

Pseudomonas mendocina P_2d formed cream colour, smooth colonies on nutrient agar, while it formed pinpoint cream colonies with yellow halo on 0.1% sodium benzoate agar and similar colonies with orange halo on 0.3% sodium benzoate agar within 24 hr. At 0.7 and 1% sodium benzoate concentrations, there was an extended lag and colonies appeared after 36 to 48 hr, turning the entire agar medium red. Growth in 0.3% liquid sodium benzoate medium displayed a similar array of colours. It turned yellow after 8 hr of growth, which intensified with growth. After 12-14 hr, medium started turning orange and finally red in colour.

TLC of ether extracts of unacidified 9 and 15 hr old culture supernatant showed a distinct spot of Rf value 0.91, which corresponded with authentic catechol. A steady increase in catechol concentration as detected colorimetrically, was observed from 3 hr reaching a maximum concentration of 0.73 mg% at 9 hr of growth, followed by a decline (Fig. 1). Oxygen uptake rate of 189.4×10^{-6} nmole/min/mg shown by P_2d cells for catechol was found to be much higher than that for other substrates including sodium benzoate (40.15×10^{-6} nmole/min/mg).

HPLC profile of 24 hr old culture supernatant showed peak at 5.62 min (Fig. 2a), which was accentuated on addition of standard catechol (Fig. 2b). These observations indicated catechol to be the intermediate in the breakdown of sodium benzoate by *Pseudomonas mendocina* P_2d, as has been reported for other bacterial strains[14-16]. Hydroxylation of ring structure of aromatic compounds is an essential step in its cleavage. Catechol is one of the central

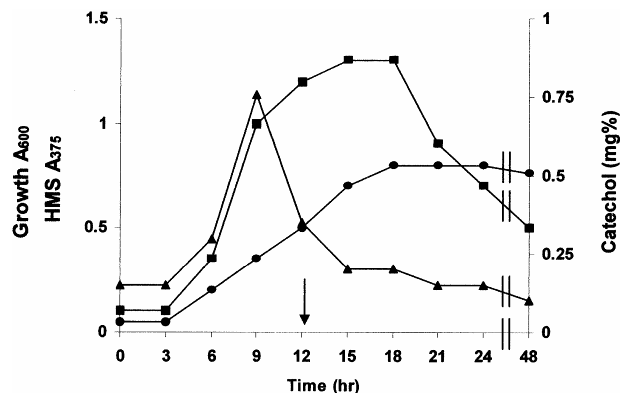

Fig. 1—Formation of catechol (▲) and 2-HMS (■) during the growth of *Pseudomonas mendocina* P_2d in 0.3% of sodium benzoate medium (●); (↓) indicates appearance of red colour in the growth medium.

intermediates formed on hydroxylation of aromatic compounds [17,18].

TLC of the ether extracts of acidified 12, 15 and 24 hr old culture supernatants showed yellow spot of Rf value 0.165 and a pink spot at Rf 0.02, before exposure to iodine. On exposure to iodine yet another spot was visible at Rf value 0.699, corresponding to authentic benzoate. The yellow spot of TLC, on elution showed absorption peak at 375 nm, the characteristic of 2-HMS, the *meta* ring cleavage product of catechol [8,15,19]. UV-visible spectrum of the culture supernatant during growth in sodium benzoate medium (0.3%) also showed a peak at 375 nm (Fig. 3). Its intensity increasing steadily with incubation time to a maximum at 15 hr of growth (Fig. 1) with concomitant deepening of yellow colour. Addition of standard 2-HMS to the red supernatant obtained from P_2d cells accentuated the HPLC peak at retention time 2.32 min confirming the identity of 2-HMS (Fig. 2c). Thus, UV, TLC and HPLC data confirmed the yellow product to be 2-HMS.

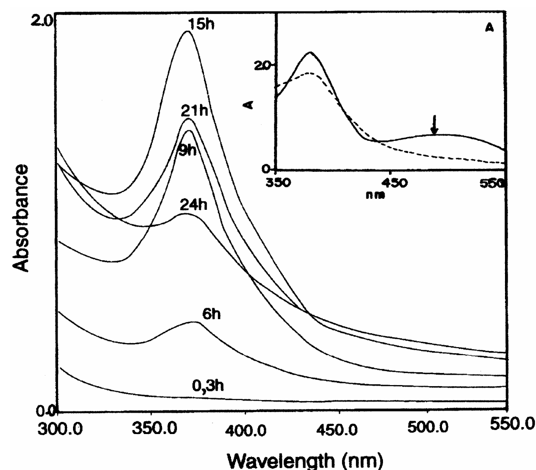

Fig. 3—UV-visible spectrum of culture supernatant (10^{-1}) of *Pseudomonas mendocina* P_2d during growth in sodium benzoate (0.3%) at intervals of 0, 3, 6, 9, 15, 21, 24hr. Inset A-UV-visible spectrum of culture supernatant of *Pseudomonas mendocina* P_2d grown in 0.3% of sodium benzoate for 48 hr, with (——) and without (------) 2,2′bipyridyll (2 m*M*), latter was grown for 24 hr and diluted ten – fold. (↓), absorption peak at 490 nm.

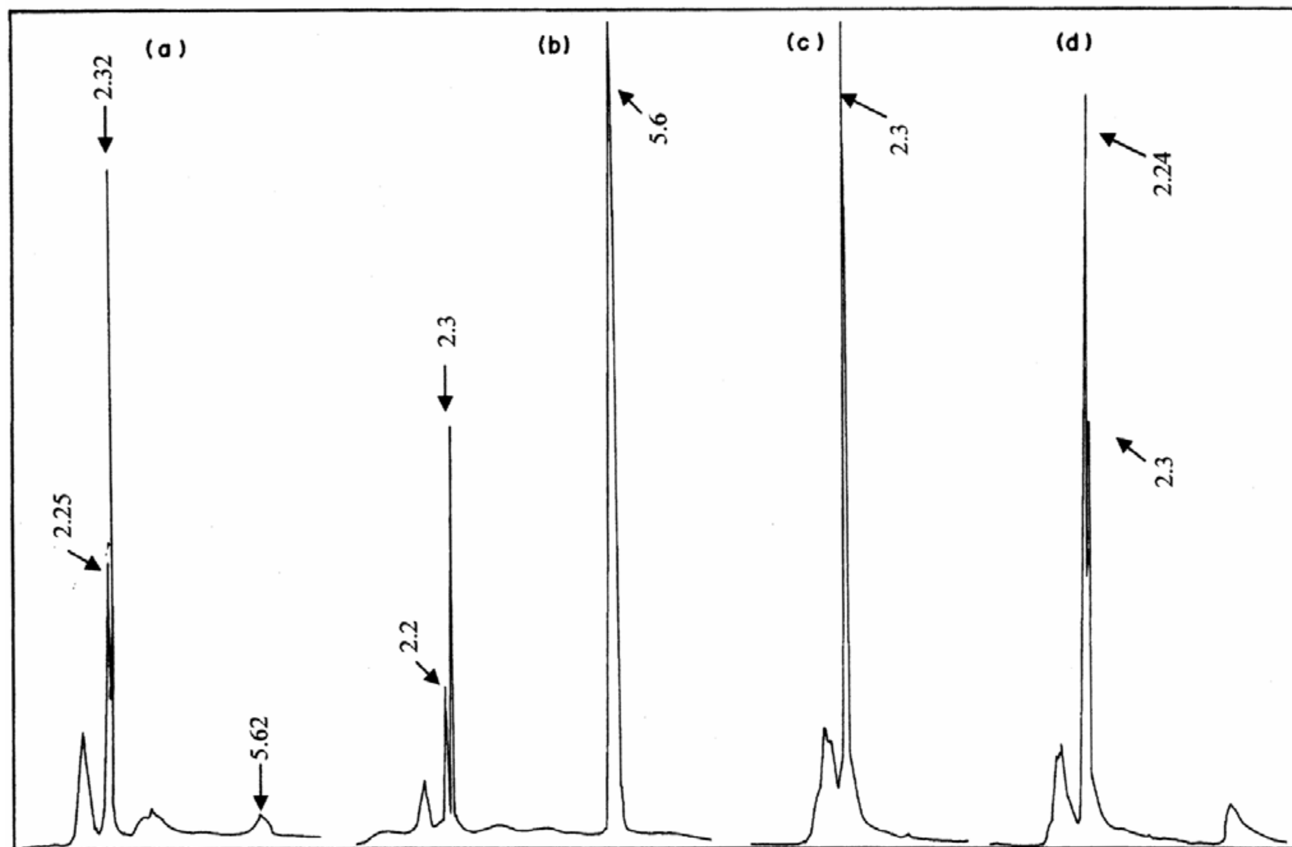

Fig. 2—HPLC of (a) Culture supernatant of strain P_2d grown in 0.3% of sodium benzoate medium for 24 hr, red in colour, mixed with standard (b) Catechol; (c) 2-HMS; (d) *ortho*-Benzoquinone, to detect peak accentuation, if any; (↓) in Fig. 2(a) indicates the peaks corresponding to the standard *ortho*-benzoquinone (2.25 min), 2-HMS (2.32 min) and catechol (5.62 min).

Cells incubated in the presence of 2-HMS as sole carbon source, showed growth and an oxygen uptake of 47.0×10^{-6} nmole/min/mg. However, the cells failed to form red colour indicating that 2-HMS was formed either subsequent to the red metabolite or by a separate pathway. During growth in sodium benzoate medium, red colour appeared after 12-14 hr of growth turning the medium orange, followed by deepening of red colour as the culture entered in stationary phase and 2-HMS formation declined (Fig. 1).

Strain P_2d cells incubated for 3 hr with 1 m*M* catechol yielded no colour change, possibly due to the low concentration of the product. With 5 m*M* of catechol, however, yellow colour appeared instantly, whereas at 10 m*M* of concentration, yellow colour was followed by red colour formation in about 50-60 min. Increasing the catechol concentration to 25 m*M* resulted in deepening of red colour to wine-red. Further increase in concentration to 50 m*M* and above resulted in browning of the solution due to autooxidation of catechol. Control catchol solution (10-25 m*M*), incubated without the bacterial cells took a considerably long period of 2-3 hr to show the faint red colouration of quinone, indicating aerial oxidation. Accelerated and significantly higher concentration of quinone in the presence of cells was also confirmed by turbidometric and gravimetric assay of quinone.

Decolourization with acid/reducing agents followed by reappearance of colour on addition of alkali/aeration, confirmed the red product in the supernatant to be quinonoid compound[13]. The quinone was derivatized as 4,5-dianilino-*ortho*-benzoquinone by incubating cells of strain P_2d in 25 m*M* of catechol and 1% (v/v) of aniline. Melting point at 190°-193°C[20] and IR (Fig. 4A), identified the aniline derivative to be 4,5-dianilino-*ortho*-benzoquinone, confirming the red product in the supernatant to be *ortho*-benzoquinone[20,21]. The pink spot of Rf value 0.02 on TLC appeared at 12 hr of growth, coincided with the appearance of orange red colour in the medium. A peak of retention time 2.25 min on HPLC of red supernatant got accentuated on addition of standard *ortho*-benzoquinone (Fig. 2a,d).

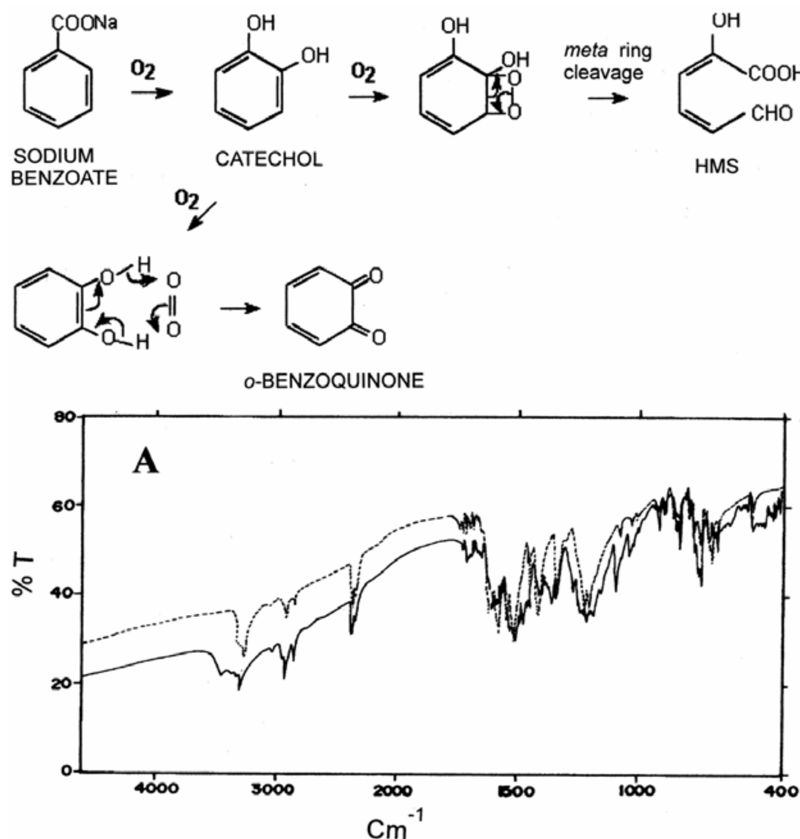

Fig. 4—Proposed pathway for biodegradation of sodium benzoate by *Pseudomonas mendocina* P_2d; inset A- (——) IR of 4,5-dianilino-derivative of quinone formed by strain P_2d in sodium benzoate medium; (------), standard *ortho*-benzoquinone.

The red colour remained unchanged in the culture medium without any decline in intensity during further incubation. *ortho*-Benzoquinone used as sole carbon source did not support the growth of the organism. Also, the cells did not show any oxygen uptake with this substrate. Unlike other reported quinones that are formed as intermediates[4–7,22], *ortho*-benzoquinone is apparently formed as a by-product, not metabolized further by the cells.

During growth on benzoate, in the presence of 2,2′-bipyridyl (2 m*M*), a known inhibitor of *meta* cleavage pathway[23], a lag of 24 hr was noted, followed by growth as in the control flask (data not shown). Although the absorption peak at 375 nm was detected, no yellow colour was visible. However, after about 48 hr, wine-red colour was intensified as reflected by increase in absorbance at 490 nm (Fig. 3A). During growth, the surplus catechol may be channeled into the formation of quinone resulting thereby in steep decline in catechol coinciding with appearance of orange red colour in the growth medium (Fig. 1). This may be the reason that the strain P_2d cells were not affected by sodium benzoate toxicity and could grow even at high concentration (1%) of sodium benzoate.

Benzoate grown cells incubated with catechol formed red colour *ortho*-benzoquinone. The presence of copper chelators such as mercaptoethanol, cysteine, sodium-bisulfide and ascorbic acid in such a reaction mixture did not affect the formation of 2-HMS however they inhibited the quinone formation, indicating involvement of the enzyme catechol-oxidase in the formation of quinone from catechol[24].

Strain P_2d harbours multiple pathways enabling it to grow on range of aromatic compounds. From the observations reported here, it is proposed that catechol to be the central metabolite in sodium benzoate biodegradation linking the two pathways, namely the *meta* cleavage resulting in formation of 2-HMS and *ortho* cleavage leading to benzoquinone formation, both of which operate simultaneously during sodium benzoate biodegradation (Fig. 4).

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