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One-step production of picolinic acids from 2-aminophenols catalyzed by 2-aminophenol 1,6-dioxygenase

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Picolinic acids have been synthesized previously from catechols by the action of catechol 2,3-dioxygenase and a subsequent chemical reaction in the presence of ammonia. 2-Aminophenol 1,6-dioxygenase catalyzes ring cleavage of several *ortho*-aminophenols. The ring fission products spontaneously convert to picolinic acids. Resting cells of *Escherichia coli* DH5α/pNBZ14 harboring the genes for 2-aminophenol 1,6-dioxygenase converted 2-aminophenol and 6-amino-*m*-cresol to picolinic acid and 5-methylpicolinic acid with yields greater than 90%. The results provide a convenient strategy for the synthesis of substituted picolinic acids from the corresponding aminophenols. *Journal of Industrial Microbiology & Biotechnology* (2000) **25,** 25–28.

Keywords: biocatalysis; picolinic acids; 2-aminophenol; aminophenol dioxygenase; ring cleavage

Aromatic *N*-heterocyclic compounds are important building blocks for synthesis of a wide variety of chemical products and biologically active compounds. Picolinic acids can be starting materials for organic syntheses of pharmaceuticals, herbicides and dyes [1]. It has long been known that catechol 2,3-dioxygenase catalyzes the cleavage of catechol and that the resultant product, 2-hydroxymuconic semialdehyde, forms picolinic acid when reacted with ammonium hydroxide (Figure 1) [2–4,14]. Previous work [1,6] has established that the strategy can be used for the preparation of picolinic acids in good yields. To obtain an appropriate biocatalyst, Hagedorn [6] constructed a *Pseudomonas putida* mutant strain that expresses catechol 2,3-dioxygenase and not 2-hydroxymuconic semialdehyde dehydrogenase and the corresponding hydrolase. More recently, Asano *et al.* [1] constructed an *Escherichia coli* transformant expressing catechol 2,3-dioxygenase from *P. putida* mt-2 and demonstrated the conversion of several substituted catechols to the corresponding picolinic acids.

Pseudomonas pseudoalcaligenes JS45 degrades nitrobenzene via 2-aminophenol [8,10,13]. Nishino and Spain [13] have shown that the ring of 2-aminophenol is cleaved to 2-aminomuconic semialdehyde in a reaction catalyzed by 2-aminophenol 1,6-dioxygenase in strain JS45. The semialdehyde is enzymatically converted *in vivo* to 2-aminomuconate in an NAD^+-dependent reaction [7,10]. The semialdehyde rapidly and spontaneously converts to picolinic acid in the absence of the dehydrogenase enzyme (Figure 1). Lendenmann and Spain [11] purified and characterized the 2-aminophenol 1,6-dioxygenase from strain JS45. The enzyme is also able to cleave 6-amino-*m*-cresol, 2-amino-*m*-cresol, and 2-amino-4-chlorophenol with relative activities of 25, 1, and 12%, compared with the activity on 2-aminophenol. The purified enzyme, however, was not very stable. The previous investigations [11,13] suggested that the enzyme might provide an alternative one-step method to synthesize picolinic acids. Recently, the genes encoding the 2-aminophenol 1,6-dioxygenase from strain JS45 have been cloned in *E. coli* and sequenced [5]. In the present paper, we explore the possibility of using resting cells of the *E. coli* transformant expressing 2-aminophenol 1,6-dioxygenase to produce picolinic acid, and 5-methyl picolinic acid from the corresponding aminophenols.

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Materials and methods

Bacterial strains and growth conditions

Plasmid pNBZ14 contains the 2-aminophenol 1,6-dioxygenase genes of *P. pseudoalcaligenes* JS45 inserted into pUC18 [5,9]. *E. coli* DH5α/pNBZ14 was grown in Luria broth (Difco, Detroit, MI) containing 50 μg ampicillin/ml at 37°C. The cells were harvested by centrifugation when the OD_{595} of the culture reached 1.8.

Source of the enzyme

Resting cells suspended in phosphate buffer (25 mM, pH 7.0) were used as the source of dioxygenase, or they were broken by two passages through a French pressure cell at 20,000 lb/in.^{2}. The suspension of broken cells was centrifuged at 20,000×*g* for 30 min and the pellets were discarded. The crude extracts were stored at −70°C. Enzyme activity in crude extracts was measured by the disappearance of substrates, which was monitored spectrophotometrically [11].

Measurement of substrates and products

2-Aminophenol, picolinic acid, 6-amino-*m*-cresol and 5-methylpicolinic acid, were analyzed by high performance liquid chromatography (HPLC) with a diode array detector (Hewlett-Packard, Wilmington, DE). The column was Supelcosil LC-ABZ (25 cm×4.6 mm ID, Supelco, Bellfonte, PA). The mobile phase was 2.4% acetonitrile in 80 mM potassium phosphate (pH 3.3, adjusted with HCl). The flow rate was 1 ml/min. Authentic 2-aminophenol, picolinic acid and 6-amino-*m*-cresol

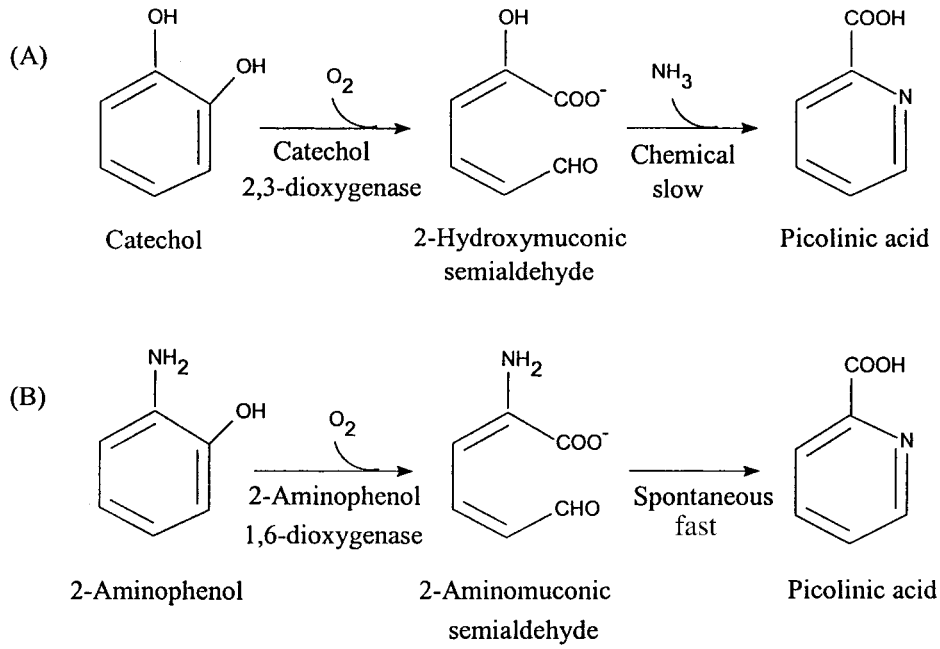

Figure 1 Synthesis of picolinic acid from catechol (4)(A) and from 2-aminophenol (B).

were from Aldrich Chemical Company (Milwauke, WI). 5-Methylpicolinic acid was generated from 6-amino-*m*-cresol with 2-aminophenol 1,6-dioxygenase in crude extracts [11]. Excess enzyme was used to assure that all of the substrate was converted.

Production of 5-methylpicolinic acid and picolinic acid

6-Amino-*m*-cresol (80 mg) in ethanol was added slowly over 1 h into a suspension of fourfold concentrated resting cells ($OD_{595}\approx7.2$) in 50 mM potassium phosphate (pH 8.0, 300 ml). The suspension was incubated with shaking until all of the 6-amino-*m*-cresol was consumed and the cells were removed by centrifugation. The supernatant fluid was evaporated to dryness in a rotary evaporator. The residue was dissolved in 300 ml of ethanol, and insoluble salts were removed by filtration. The ethanol was removed under vacuum. The extraction was repeated with 20 ml of ethanol. The dry product was then dissolved in 15 ml of 0.1 M HCl. After removal of undissolved solids, the solution was evaporated to dryness and residue was taken up in 10 ml ethanol. The isolated material was treated with excess HCl (0.1 M) to convert it into the hydrochloride. The final product obtained by evaporation of the solvent was stored in a desiccator. Identical conditions were used for the transformation of 2-aminophenol (130 mg) to picolinic acid hydrochloride.

Results and discussion

The effect of pH on enzyme activity

2-Aminophenol or 6-amino-*m*-cresol were added to reaction mixtures containing crude extracts (0.15 mg protein per ml) in 25 mM potassium phosphate buffer at 22°C and consumption of the substrates was monitored. Potassium phosphate buffer was used for pH 6.5 to 8.0, and Tris–HCl buffer was used for pH 7.5 to 8.5. The activity was not affected significantly by the pH change in the medium and was slightly higher at pH 8.0 in phosphate buffer with both substrates.

The effect of temperature on enzyme activity

The rate of transformation of 2-aminophenol by crude extracts and resting cells was measured between 15°C and 40°C. The results indicate that the enzyme is less active at lower

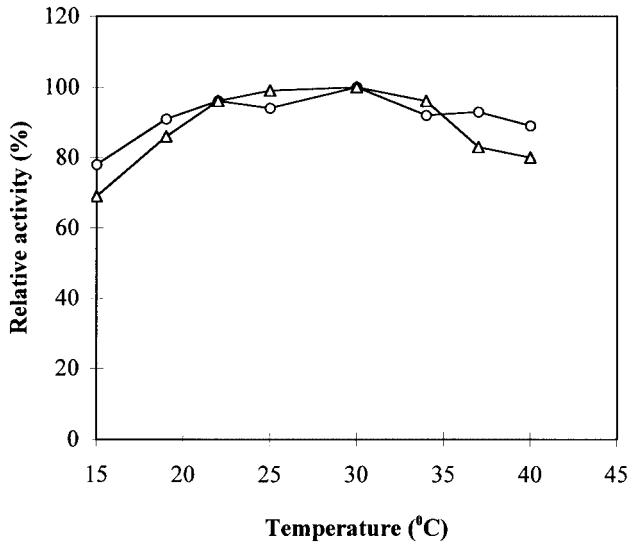

Figure 2 The effect of temperature on the transformation 2-aminophenol. The reaction mixtures containing the crude extracts (– ○ –) or the resting cells (-△-) were preincubated at the specific temperature for 10 min, then incubated 10 more min after the addition of 2-aminophenol (1.5 mM final concentration). The reaction was terminated by addition of 20% 1 M HCl. One hundred percent of activity is equal to production of 0.19 μmol picolinic acid/min/mg protein for crude extracts, and of 0.01 μmol picolinic acid/min/OD_{595} for resting cells.

temperatures, and it is less stable at higher temperatures. The activity did not change significantly between 22°C and 34°C (Figure 2).

Effect of the concentration of substrate on activity in resting cells

Under optimum conditions (pH 8.0, potassium phosphate buffer, 22°C), resting cells ($OD_{595}\approx7.2$) were used to convert 2-aminophenol to picolinic acid. All 2-aminophenol was transformed when the concentration was 1.6 or 3.2 mM. The product reached maximum concentrations of 3.7 and 3.8 mM in 80 min from 7 mM 2-aminophenol (Figure 3). Incubation for 60 more minutes did not increase yields of the product significantly. These observations suggest that high substrate concentration is harmful to resting cells. The fact that the K_m for 2-aminophenol is 4.2 uM [11] suggests that the best strategy for substrate addition would be continuous slow addition. In a practical application the substrate could be pumped into the reactor continuously or provided in a biphasic system.

Respike experiments

To further test the capacity for conversion by resting cells, respike experiments were carried out. 2-Aminophenol was added six times (1.1 mM final concentration each) to a suspension of resting cells ($OD_{595}\approx7.2$) in 50 mM potassium phosphate (pH 8.0) at 22°C (Figure 4). The rate of conversion of 2-aminophenol to picolinic acid was significantly reduced, starting with the fifth addition. The final concentration of picolinic acid was 6.2 mM. The inclusion of glucose (0.5%) in the reaction mixture did not prevent the loss of activity. In two experiments 96.5±3.5% of the 2-aminophenol transformed was converted to picolinic acid. Under the same conditions, 6-amino-*m*-cresol was converted more slowly (2.5 mM total), (data not shown) apparently due to lower activity of the dioxygenase towards the substrate [11].

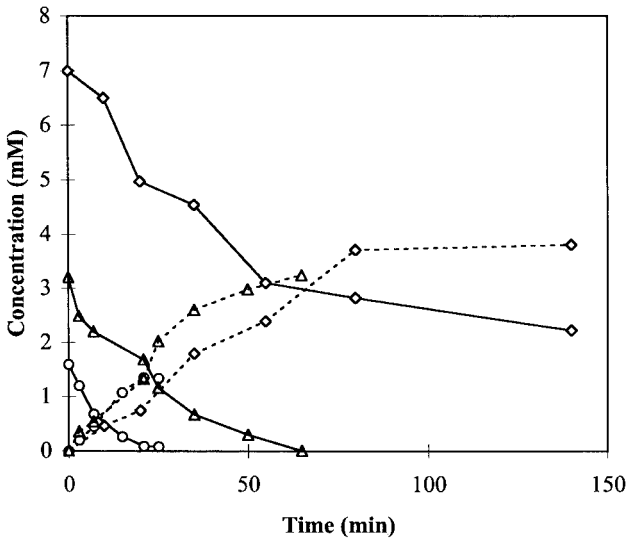

Figure 3 Conversion of 2-aminophenol to picolinic acid. Cells ($OD_{595}\approx7.2$) were incubated in 50 mM potassium phosphate buffer (pH 8.0) at 22°C with 2-aminophenol at starting concentrations of 1.6 mM (– ○ –), 3.2 mM (-△-), and 7.0 mM (-◇-). Solid curves, 2-aminophenol; dashed curves, picolinic acid.

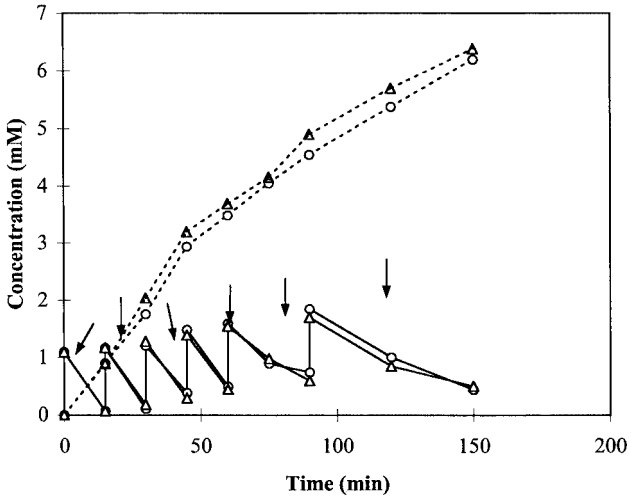

Figure 4 Production of picolinic acid by resting cells of *E. coli* DH5α/pNBZ14. Cells ($OD_{595}\approx7.2$) were incubated with 2-aminophenol in 50 mM potassium phosphate buffer (pH 8.0) at 22°C. (– ○ –), no additives; (-△-), in the presence of 0.5% glucose which was added 30 min prior to addition of the first batch of 2-aminophenol. Arrows indicate the addition of 2-aminophenol (1.1 mM final concentration). Solid curves, 2-aminophenol; dashed curves, picolinic acid.

Preparation and isolation of 5-methylpicolinic acid and picolinic acid

An optimized protocol was used to prepare and isolate 5-methylpicolinic acid and picolinic acid as described in Materials and methods. During the reaction 2.2 mM 6-amino-*m*-cresol yielded 2.0 mM 5-methylpicolinic acid and 4.0 mM 2-aminophenol yielded 3.6 mM picolinic acid. In each transformation, conversion of the aminophenol to the corresponding picolinic acid was above 90%. When the products were isolated and converted to the hydrochlorides, 77.6 and 150 mg of products were obtained from 80 mg of 6-amino-*m*-cresol and 130 mg of 2-aminophenol. Thus the final yields after extraction and recovery were 70% for the preparation of 5-methylpicolinic acid from 6-amino-*m*-cresol, and 78% for the preparation of picolinic acid from 2-aminophenol. No attempt was made to optimize the extraction and recovery procedure. The 5-methylpicolinic acid and picolinic acid prepared from 6-amino-*m*-cresol and 2-aminophenol by the 2-aminophenol 1,6-dioxygenase in this work were identical by HPLC analysis to those previously identified by GC-MS analysis of their methyl ester derivatives [11]. HPLC chromatograms of the two products showed that picolinic acid eluted at 4.2 min with no evidence of impurities and 5-methylpicolinic acid eluted at 5.2 min as the major component, but a minor impurity eluted at 3.8 min. The melting point of the product obtained from 6-amino-*m*-cresol was 204–206°C, which is close to the previously reported 203–204°C for 5-methylpicolinic acid hydrochloride [2]. The product obtained from 2-aminophenol sublimed at 208–216°C. When authentic picolinic acid (m.p. 139–142°C) was treated with HCl and evaporated to dryness, it also sublimed at 208–216°C. The results clearly indicated that the final products were 5-methylpicolinic acid hydrochloride and picolinic acid hydrochloride.

The commercially available free acid form of picolinic acid is white crystals. 5-Methylpicolinic acid is not commercially available. The picolinic acid hydrochloride and 5-methylpicolinic acid hydrochloride prepared by our method were yellowish or

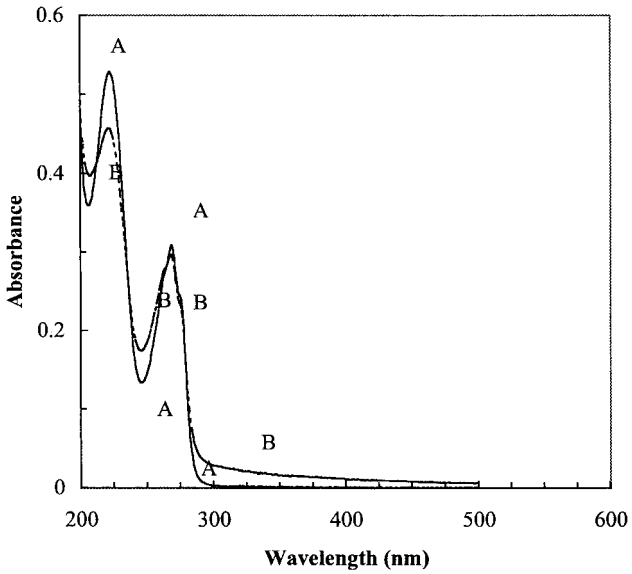

Figure 5 The spectra of 5-methylpicolinic acid. (A) Sublimed white material (15 μg/ml); (B) brownish material (9.6 μg/ml) in 25 mM potassium phosphate buffer (pH 7.0).

brown. During measurement of the melting point of 5-methylpicolinic acid hydrochloride, some sublimation took place and produced white crystals. The spectrum of the 5-methylpicolinic acid hydrochloride we obtained (Figure 5) was similar to that reported previously [2]. The absorbance maximum was at 269 nm. Small shoulder peaks and some baseline absorbance above 300 nm were due to the abovementioned impurities. The sublimed white substance showed an almost identical spectrum, but no baseline absorbance above 300 nm (Figure 5). This observation indicated that the white substance was the more pure free acid form of 5-methylpicolinic acid.

In the preparation of picolinic acids [1,6], catechol 2,3-dioxygenase catalyzes the oxidation of catechol to 2-hydroxymuconic semialdehyde. Although the ring fission product is relatively unstable, it must be accumulated to the highest possible concentration prior to the addition of ammonia to the reaction mixture because ammonia inhibits the action of the catechol 2,3-dioxygenase [1]. The efficiency of conversion from the two-step process ranges from 68% to 47% depending on the substituted catechol used as starting material [1]. We show here that the resting cells of *E. coli* DH5α/pNBZ14 harboring the genes for 2-aminophenol 1,6-dioxygenase were able to catalyze the ring cleavage of 2-aminophenol and 6-amino-*m*-cresol efficiently. The instability of the ring cleavage intermediates is a disadvantage in the syntheses of picolinic acids from catechols, but an advantage in syntheses starting with aminophenols because the spontaneous conversion of 2-aminomuconic semialdehydes not only eliminates the subsequent chemical treatment required in the catechol method, it also increases the efficiency of the conversion to 90–100%. The strategy provides a simple alternative approach to the production of substituted picolinic acids where the corresponding aminophenols are able to serve as the substrates of the 2-aminophenol 1,6-dioxygenase [11]. We have also developed a strategy, using the enzymes in strain JS45, for the biotransformation of nitroaromatic compounds to the corresponding *ortho* aminophenols [12]. It may be possible to combine the two strategies to produce substituted picolinic acids from the corresponding nitrobenzenes in a single reactor.

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References

1 Asano Y, Y Yamamoto and H Yamada. 1994. Catechol 2,3-dioxygenase-catalyzed synthesis of picolinic acids from catechols. *Biosci Biotech Biochem* 58: 2054–2056.
2 Cain RB and DR Farr. 1968. Metabolism of arylsulphonates by micro-organisms. *Biochem J* 106: 859–877.
3 Dagley S, WC Evans and DW Ribbons. 1960. New pathways in the oxidative metabolism of aromatic compounds by micro-organisms. *Nature* 188: 560–566.
4 Dagley S and DT Gibson. 1965. The bacterial degradation of catechol. *Biochem J* 95: 466–474.
5 Davis JK, Z He, CC Somerville and JC Spain. 1999. Genetic and biochemical comparison of 2-aminophenol 1,6-dioxygenase of *Pseudomonas pseudoalcaligenes* JS45 to *meta* cleavage dioxygenases: divergent evolution of 2-aminophenol *meta* cleavage pathway. *Arch Microbiol* 172: 330–339.
6 Hagedorn S. 1987. U.S. patent 4,666,841.
7 He Z, JK Davis and JC Spain. 1998. Purification, characterization, and sequence analysis of 2-aminomuconic 6-semialdehyde dehydrogenase from *Pseudomonas pseudoalcaligenes* JS45. *J Bacteriol* 180: 4591–4595.
8 He Z and JC Spain. 1999. Comparison of the downstream pathways for degradation of nitrobenzene by *Pseudomonas pseudoalcaligenes* JS45 (2-aminophenol pathway) and by *Comamonas* sp. JS765 (catechol pathway). *Arch Microbiol* 171: 309–316.
9 He Z and JC Spain. 1999. Preparation of 2-aminomuconate from 2-aminophenol by coupled enzymatic dioxygenation and dehydrogenation reactions. *J Ind Microbiol Biotechnol* 23: 138–142.
10 He Z and JC Spain. 1997. Studies of the catabolic pathway of degradation of nitrobenzene by *Pseudomonas pseudoalcaligenes* JS45: removal of the amino group from 2-aminomuconic semialdehyde. *Appl Environ Microbiol* 63: 4839–4843.
11 Lendenmann U and JC Spain. 1996. 2-Aminophenol 1,6-dioxygenase: a novel aromatic ring-cleavage enzyme purified from *Pseudomonas pseudoalcaligenes* JS45. *J Bacteriol* 178: 6227–6232.
12 Nadeau LJ, Z He and JC Spain. 2000. Production of 2-amino-5-phenoxyphenol from 4-nitrobiphenyl ether using nitrobenzene nitroreductase and hydroxylaminobenzene mutase from *Pseudomonas pseudoalcaligenes* strain JS45. *J Ind Microbiol Biotechnol* In press.
13 Nishino SF and JC Spain. 1993. Degradation of nitrobenzene by a *Pseudomonas pseudoalcaligenes*. *Appl Environ Microbiol* 59: 2520–2525.
14 Ribbons DW and PJ Senior. 1970. 2,3-Dihydroxybenzoate 3,4-oxygenase from *Pseudomonas fluorescens*-oxidation of a substrate analog. *Arch Biochem Biophys* 138: 557–565.