

Pseucomonas aeruginosa TKU002所生產兒茶酚1,2-雙加氧?之純畫及定性

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ABSTRACT

A medium at pH5.5 containing sodium benzoate as the sole carbon source was used for screening of strains from soil. The selected strain was sent to Food Industrial Research and Development Institute for determination and named Pseudomonas aeruginosa TKU002. The strains used benzoate in medium and accumulated catechol as mid-product, which was further converted to cis,cis-mucomic acid by catechol 1,2-dioxygenase (C12O). The medium for optimal growing condition for strain TKU002 includes 0.05% urea for carbon source and 0.3% glycerol for nitrogen source. The C12O was purified by DEAE-Sepharose, Sephacryl S-200 and Sephacryl S-200 column chromatography . The molecular weight was about 22 kDa estimated by SDS-PAGE. The experiment determined C12O enzyme properties. The optimum pH and pH stability were 7.5 and 7~9, respectively. The optimum temperature was 40 and thermal stability was below 40 . About the effect of metal ions on the enzyme, Cu²⁺ reduced enzyme activity and Fe²⁺ increased the enzyme activity slightly.

Keywords : catechol

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REFERENCES

1. 王全祿, 2001, 微生物???安息香酸類?????類??變換???代謝?連酵素?特性解析, 神戶大?大?院自然科?研究科博士論文。
2. 林美鳳, 2000, 建立苯環化合物分解菌中苯環加氧與切割?-之基因偵測法, 國立中央大學生命科學研究所碩士論文。
3. 李幸慧, 1999, 偏鄰苯二酚雙加氧?活化中心位置的探討, 國立成功大學化學系碩士論文。
4. 陳霽芝, 1999, Corynebacterium pseudodiphtheriticum NTU-7C之苯甲酸降解研究, 國立台灣大學農業化學研究所碩士班論文。
5. Aoki, K., Nakanishi, Y., Murakami, S. and Shinke, R. 1990. Microbial metabolism of aniline through a meta-cleavage pathway: Isolation of strains and production of catechol 2,3-dioxygenase, Agric. Biol. Chem., 54:205-206
6. Aoki, K., Konohana, T., Shinke, R. and Nishira, H. 1984a. Purification and characterization of catechol 1,2-dioxygenase from aniline-assimilating Rhodococcus erythropolis AN-13, Agric. Biol. Chem., 48:2087-2095
7. Aoki, K., Konohana, T., Shinke, R., and Nishira, H. 1984b. Two catechol 1,2-dioxygenase from an aniline-assimilating bacterium, Frateuria species ANA-18. Agric. Biol. Chem., 48:2097-2104
8. Axcell, B. C. and Geary, P. J. 1975. Purification and some properties of a soluble benzene-oxidizing system from a strain of Pseudomonas, Biochem. J., 146:173-183
9. Batie, C. J., Lahaie, E. and Ballou, D. 1987. Purification and characterization of phthalate oxygenase and phthalate oxygenase reductase from Pseudomonas cepacia, J. Biochem. Chem., 262:1510-1518
10. Bernhardt, F. H., Erdin, N., Satudinger, H. and Ullrich, V. 1973. Interactions of substrates with a purified 4-methoxybenzoate monooxygenase system (o-demethylating) from Pseudomonas putida, Eur. J. Biochem., 35:126-134
11. Caposio, P., Pessione, E., Giuffrida, G., Conti, A., Landolfo, S., Giunta, C. and Gribaudo, G. 2002. Cloning and characterization of two catechol 1,2-dioxygenase genes from Acinetobacter radioresistens S13, Research in Microbiology., 153:69-74
12. Chio, C. Y., Choi, W. J., Lee, E. Y., and Cho, M. H. 1997. Enhanced production of cis,cis-muconate in cell-recycle bioreactor, J. Ferment. Bioeng., 84:70-76
13. Choi, C. Y. and Bang, S. 1995. DO-stat fed-batch production of cis,cis-muconic acid from benzoic acid by Pseudomonas putida BM014, J. Ferment. Bioeng., 79:381-383
14. Dagley, S. and Stopher, D. A. 1959. A new mode of fission of the benzene nucleus by bacteria, Biochem. J., 73:16-17
15. Fernandez, L. R., Guisan, J. M., Ali, S. and Cowan, D. 2000. Immobilization of functionally unstable

catechol-2,3-dioxygenase greatly improves operational stability, *Enzyme Microb. Technol.*, 26:568-573 16. Flamm, W. G., and Crandall, D. I. 1963. Purification of mammalian homogentisate oxidase and evidence for the existence of ferrous mercaptans in the active center, *J. Biol. Chem.*, 238: 389-396 17. Gibson, D. T. 1972. Degradation of synthetic organic molecules in the biosphere *Proc. Conference. NatL Acad Sci.*, Washington, D. C., p116 18. Horiguchi, S., and Yamada, K. 1968. Studies on the utilization of hydrocarbons by microorganisms, *Agr. Biol. Chem.*, 32:555-560 19. Irie, S., Shirai, K., Doi, S. and Yorifuji, T. 1987. Cloning of genes encoding oxidation of benzene in *Pseudomonas putida* and their expression in *Escherichia coli* and *P. putida* *Agric. Biol. Chem.*, 51:1489-1493 20. Jenkins, R. O., Stephens, G. M. and Dalton, H. 1987. Production of toluene cis-glycol by *Pseudomonas putida* in glucose fed-batch culture, *Biotech. and Bioeng.*, 29:873-883 21. Kimura, T., Zhang, Yan., Kodama, T. and Omori, T. 1996. Isolation and characterization of Tn-5-induced mutants deficient in carbazole catabolism, *FEMS Micro. Biol. Lett.*, 135:65-70 22. Kuwahara, M. and Tsuji, M. 1976. Accumulation of catechol from benzoic acid by a mutant induced from *Corynebacterium glutamicum*, *J. Ferment. Technol.*, 54:82-788 23. Loh, K. C. and Chua, S. S. 2002. Ortho Pathway of benzoate degradation in *Pseudomonas putida*: induction of meta pathway at high substrate concentrations, *Enzyme Microb. Technol.*, 30:620-626 24. Locher, H. H., Leisinger, T. and Cook, A. M. 1991. 4-Sulphobenzoate 3,4-dioxygenase. Purification and properties of a desulphonative two-component enzyme system from *Comamonas testosteroni* T-2, *Biochem. J.*, 274:833-842 25. Larue, T. A. and Blakley, E. R. 1964. Spectrophotometric determination of catechols with 4-aminoantipyrine, *Anal. Chim. Acta.*, 31:400-403 26. Murakami, S., Wang, C. L., Naito, A. R., Shinke, R. and Aoki, K. 1998. Purification and characterization of four 1,2-dioxygenase isozymes from the benzamide-assimilating bacterium *Arthrobacter* species BA-5-17. *Microbiol. Res.*, 153:163-171 27. Murakami, S., Kodama, N., Shinke, R. and Aoki K. 1997. Classification of catechol 1,2-dioxygenase family: sequence analysis of a gene for the catechol 1,2-dioxygenase showing high specificity for methylcatechols from gram+ aniline-assimilating *Rhodococcus erythropolis* AN-13, *Gene*, 185:9-54 28. Mizuno, S., Yoshikawa, N., Seki, M., Mikawa, T. and Imada, Y. 1988. Microbial production of cis,cis muconic acid from benzoic acid. *Appl. Microbiol. Biotechnol.*, 28:0-25 29. Neidle, E. L., Hartnett, C. L., Ornston, N., Bairoch, A., Reik, M. and Harayama, S. 1991. Nucleotide sequence of the *Acinetobacter calcoaceticus* benABC genes for benzoate 1,2-dioxygenase reveals evolutionary relationship among multicomponent oxygenase, *J. Bacteriol.*, 173:5385-5395 30. Nakai, C., Horiike, K., Kuramitsu, S., Kagamiyama, H. and Nozaki, M. 1990. Three isozymes of catechol 1,2-dioxygenase (pyrocatechase), , and , from *Pseudomonas arvilla* C-1. *J. Biol. Chem.*, 265:660-665 31. Neidle, E. L., Shapiro, M. K. and Ornston, L. N. 1987. Cloning and expression in *Escherichia coli* of *Acinetobacter calcoaceticus* genes for benzoate degradation, *J. Bacteriol.*, 169:5496-5503 32. Nozaki, M., Ono, K., Nakazawa, T., Kotani, S., and Hayaishi, O. 1986. Metapyrocatechase, II. The role of iron and sulfhydryl groups, *J. Biol. Chem.*, 243:682-2690 33. Nakai, C., Hori, K., Kagamiyama, H., Nakazawa, T., and Nozake, M. 1983. Purification, subunit structure, and partial amino acid sequence of metapyrocatechase, *J. Biol. Chem.*, 258:2916-2922 34. Nakai, C., Kagamiyama, H., Nazake, M., Nakawa, T., Inouye, S., Ebina, Y., and Nakazawa, A. 1983. Complete nucleotide sequence of the metapyrocatechase gene on the TOL plasmid of *Pseudomonas putida* mt-2, *J. Biol. Chem.*, 258:2923-2928 35. Nozaki, M., Nakazawa, T., Fujisawa, H., Kotani, S., Kojima, Y., and Hayaishi, O. 1968. Role of iron in dioxygenase reactions, *Adv. Chem. Ser.*, 79:242-251 36. Okuta, A., Ohnishi, K. and Harayama S. 1998. PCR isolation of catechol 2,3-dioxygenase gene fragments from environmental samples and their assembly into functional genes, *Gene*, 212:21-228 37. Patel, R. N., Hou, C. T., Felix, A. and Lillard, M. O. 1976. Catechol 1,2-dioxygenase from *Acinetobacter calcoaceticus*: Purification and properties, *J. Bacteriol.*, 127:536-544 38. Reiner, A. M. 1971. Metabolism of benzoate by bacteria: 3,5-cyclohexadiene-1,2-diol-1-carboxylic acid is an intermediate in the formation of catechol, *J. Biol.*, 108:89-94 39. Santos, P. M., Mignogna, G., Heipieper, H. J. and Zennaro, E. 2002. Occurrence and properties of glutathione s-transferases in phenol-degrading *Pseudomonas* strains, *Research in Microbiology*, 153:89-98 40. Strachan, P. D., Freer, A. A. and Fewson, C. A. 1998. Purification and characterization of catechol 1,2-dioxygenase from *Rhodococcus rhodochrous* NCIMB13259 and cloning and sequencing of its catA gene. *Biochem. J.*, 333:741-747 41. Shirai, K. 1987. Catechol production from benzene through reaction with resting and immobilized cell of a mutant strain of *Pseudomonas*, *Agric. Biol. Chem.*, 51:121-128 42. Shirai, K. 1986. Screening of microorganism for catechol production from benzene, *Agric. Biol. Chem.*, 50:2875-2880 43. Tweel, W. J. J., and Bont, J. A. M. 1988. Continuous production of cis-1,2-dihydroxycyclohexa-3,5-diene (cis-benzeneglycol) from benzene by a mutant of a benzene-degrading *Pseudomonas* sp., *Enzyme Microb. Technol.*, 10:136-142 44. Varga, J. M. and Neujahr, Y. 1970. Purification and properties of catechol 1,2-dioxygenase from *Trichosporon cutaneum*. *Eur. J. Biochem.*, 12:427-434 45. Patel, R. N., Hou, C. T., Felix, A., and Lillard, M. O. 1976. Catechol 1,2-dioxygenase from *Acinetobacter calcoaceticus*: purification and properties. *J. Bacteriol.*, 127:536-554 46. Wang, C. L., Takenaka, S., Murakami, S. and Aoki, K. 2001a. Isolation of a benzoate-utilizing *Pseudomonas* strain from soil and production of catechol from benzoate by transpositional mutants, *Microbiol. Res.*, 156:151-158 47. Wang, C. L., Takenaka, S., Murakami, S. and Aoki, K. 2001b. Production of catechol from benzoate by the wild strain *Ralstonia* species Ba-0323 and characterization of its catechol 1,2-dioxygenase, *Biosci. Biotechnol. Biochem.*, 65:1957-1964 48. Whited, G. M., McCombie, W. R., Kwart, L. D. and Gibson, D. T. 1986. Identification of cis-diols as intermediates in the oxidation of aromatic acid by a strain of *Pseudomonas putida* that contains s TOL plasmid, *J. Bacteriol.*, 166:1028-1035 49. Yarmoff, J. J., Kawalami, Y. and Yago, T. 1988. Cis-benzeneglycol production using a mutant *Pseudomonas* strain, *J. Ferment. Technol.*, 66:302-312 50. Yamaguchi, M. and Fujisawa, H. 1981. Reconstitution of iron-sulfur cluster of NADH-cytochrome c reductase, a component of benzoate 1,2-dioxygenase system from 1,2-dioxygenase system from *Pseudomonas arvilla* C-1, *J. of Biol. Chem.*, 256:6783-6787 51. Yamaguchi, M., Yamauchi, T. and Fujisawa, H. 1975. Studies on mechanism of double hydroxylation, *Biochem. and Biophys. Res. Commun.*, 67:264-271