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The characteristics and mechanisms of phenol biodegradation by *Fusarium* sp.

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Abstract

Fusarium sp. HJ01 can grow using phenol as only carbon resource and has strong ability of phenol degradation. The effect of pH, temperature and sucrose addition on biodegradative capacity of *Fusarium* sp. HJ01 was examined. The main metabolism pathways and mechanism of phenol degradation by HJ01 strain is described. This strain exhibited both cathecol 1,2-dioxygenase (C12) and cathecol 2,3-dioxygenase (C23) in free cell extracts obtained from cells grown exclusively on phenol or with sucrose added, suggesting that the intermediate cathecol can be oxidized in the catabolic pathway of ortho and meta fission. Mineral salts added in culture have an inhibition on both C12 and C23. These two enzymes can act and retain its catalytic ability over wide ranges of temperature and pH. C12 activity was optimal at pH 6.8 and 40 °C, with significant activity observed in the range from pH 3 to pH 8.8, and in the temperature range from 30 to 50 °C. In comparison with C12, the activity of C23 was slightly more sensitive to pH, C23 had a higher activity in alkalescence condition from pH 7.4 to pH 10.6 and was more stable at higher temperatures from 30 to 75 °C.
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Keywords: Phenol degradation; Catechol 1,2-dioxygenase; Catechol 2,3-dioxygenase; *Fusarium* sp. HJ01

1. Introduction

Phenol and its derivatives are widely distributed as environmental pollutants due to their common presence in the effluents of many industrial processes, including oil refineries, petrochemical plants and phenolic resin industries [1]. Phenol removal has been the subject of numerous investigations. There are several references about phenol biodegradation in both anoxic [2] and aerobic conditions [3]. Although microbial metabolism of phenols has been widely studied, most of the knowledge on metabolic pathways of aromatic degradation comes from studies with the bacteria. Santos and Linardi [4] and Garcia et al. [5] discovered that the mycelial fungi can show a strong strength of phenols degradation. It is important to study the fungi *Fusarium* sp. in the detoxification of phenol in the effluents.

The aerobic catabolism of aromatic compounds has been extensively investigated for a variety of microorganisms and for different natural and xenobiotic compounds [6]. In particular many microorganisms use a catabolic sequence for the degradation of aromatic compounds called β-ketoadipate pathway in which ring cleaving dioxygenases such as the catechol dioxygenases play a central role [7]. Usually the phenol degradation occurs by converting them into the corresponding catechol when the ring cleavage can occurs in two different orientations relative to vicinal diols and this different in cleavage site is used to classify catechol dioxygenases in two groups the intradiol and extradiol cleaving enzymes. The intradiol dioxygenases such as catechol 1,2-dioxygenases catalyze the intradiol cleavage of catechols to *cis*,*cis*-muconic acids with the incorporation of molecular oxygen in the first step of this pathway, and the extradiol dioxygenases catechol 2,3-dioxygenases catalyzes the extradiol ring-cleavage of catechol to form 2-hydroxymuconate semialdehyde with the insertion of two atoms of dioxygen. However, there are little reports about *Fusarium* sp. in degrading phenol and the catabolism mechanism of the intermediate catechol.

The purpose of this investigation was to characterize and screen *Fusarium* sp. fungi with potential for phenol degradation in the effluents and report the isolation, the characteristics of catechol 1,2-dioxygenase and 2,3-dioxyganase from a novel strain of *Fusarium* sp. grown on phenol as sole carbon and energy source. The implications of these findings are discussed.

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2. Materials and methods

2.1. Microorganism and cultivation condition

The selected *Fusarium* sp. strain was isolated from bentonite and identified on physiological and biochemical tests and genospecies as *Fusarium* sp. proliferatum called HJ01.

The strain was grown in the liquid minimal salt medium with 200 mg/l phenol at room temperature on a rotary shaker (60 rpm). Liquid mineral salt medium (LMS) contained: deionized water 1000 ml, $MgSO_4{\cdot}7H_2O$ 0.5 g, KH_2PO_4 11.8 g, K_2HPO_4 2.3 g, $CuSO_4$ 0.05 g, NaCl 0.05 g, NH_4Cl 0.25 g, $MnSO_4$ 0.01 g, $FeSO_4$ 0.1 g, $ZnSO_4$ 0.01 g, pH is adjusted to 6.0. Phenol was added from sterile stock solution to the autoclaves medium prior to inoculation.

2.2. Phenol degradation experiment

The startup of the experiments was obtained by inoculating 25 ml mineral cultures with 1 ml *Fusarium* sp. suspension which OD is about 1.62, this cell suspension is available by inoculating *Fusarium* sp. for 10 days in solid medium and then transfer into distilled water. This cell culture was added to 25 ml fresh mineral medium with 420 mg/l phenol. The effects of adding carbon, pH and temperature on phenol degradation are investigated. The values of initial pH are 2, 4, 6, 7, 8, 10, the adding carbon are 0 g/l, 3 g/l, 5 g/l sucrose, the temperature are 25, 30, 35 °C. During the period of batch culture, all samples were periodically taken for the biomass and the concentration of phenol. All experiments were carried out in duplicate.

2.3. Biomass and phenol determinations

The cell density and phenol was monitored spectrophotometrically by measuring the absorbances at wavelength 600 and 510 nm according to Ref. [8], respectively. To measure the concentration of phenol, the undegraded samples of suspended culture were centrifuged at 3000 rpm for 10 min. The free supernatants were used to determine the concentration of phenol by 4-amino-antipyrine spectrophotometric method [9].

2.4. Enzyme extraction

Approximately 2 g (wet weight) freshly harvested mycelium was washed twice in 50 mM acetone/potassium phosphate buffer (pH 7.5) at 4 °C and re-suspended into 5 ml of acetone/potassium phosphate buffer. The mycelium were disrupted by pestle on ice water, and the resulting crude extracts were centrifuged (11,000 × *g* for 10 min at 4 °C) to obtain soluble protein extracts, which were used to assay the enzyme activities [10].

2.5. Enzyme optimal conditions

In the experiments for the determination of the pH optimum, the following buffers were used: 50 mM citric acid (pH 3.0–7.0), and 0.1 M Tris–HCl (pH 7.0–9.0) and 50 mM glycin–NaOH (pH 9.0–10.6). The optimum pH was determined by measuring the activity at 25 °C and the pH range from 3.0 to 10.6. The optimum temperature was determined by assaying the enzyme activity at various temperatures (10–75 °C) in 50 mM Tris/HCl buffer solution (pH 7.5).

2.6. Enzyme assay

The catechol 1,2-dioxygenase (EC1.13.11.1) and catechol 2,3-dioxygenase (EC1.13.1.2) activities were measured spectrophotometrically by following the formation of *cis*,*cis*-muconic acid at 260 nm ($\varepsilon_{260\,nm} = 16{,}000\,(mol\,cm)^{-1}$ at 25 °C) and 2-hydroxymuconic semialdehyde ($\varepsilon_{375\,nm} = 12{,}000\,(mol\,cm)^{-1}$ at 25 °C) [11,12]. The assay mixture contained in 3 ml total: 1 μM catechol, 130 μM potassium phosphate buffer solution and 200 μl enzyme solution. The catechol 1,2-dioxygenase activity and the catechol 2,3-dioxygenase were determined under the same conditions reported for activity. One unit of enzymatic activity is defined as the amount of enzyme producing 1 μM of *cis*,*cis*-muconic acid or 2-hydroxymuconic semialdehyde per minute at 25 °C. The kinetic parameters were determined by fitting the data with a non-linear least squares fitting program to the typical Michaelis–Menten equation with phenol LMS substrates at pH 7.5, 25 °C [13].

The specific activities of enzymes which was defined as units milligram per protein measured in the cell extracts were expressed relative to protein content as determined by the method of Lowry et al. by using bovine serum albumin as the protein standard [14].

3. Results and discussion

3.1. Phenol degradation

3.1.1. The effect of sucrose addition

Fig. 1 was shown the effect of adding carbon concentration on the phenol degradation. The cells inoculated with 3 g/l additional carbon underwent a short lag phase, 420 mg/l of phenol was entirely degraded within 6 days, with the time consumed much shorter than that of the other two additional carbons (0 g/l and 5 g/l sucrose). It's because the overloaded sucrose inhibit

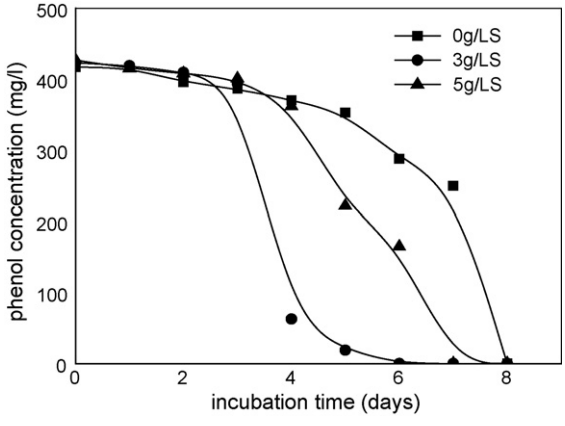

Fig. 1. The effects of sucrose addition on phenol degradation by *Fusarium* sp. HJ01.

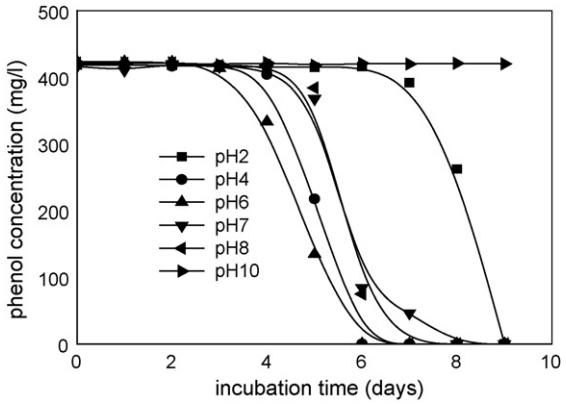

Fig. 2. The effects of pH on phenol degradation by *Fusarium* sp. HJ01.

the phenol degradation. The HJ01 can grow without any other supplement additive and can use phenol as the sole carbon source and energy source. This indicated that phenol consumed in medium was utilized to synthesize new cells.

3.1.2. The effect of pH

Fig. 2 was shown the phenol degradation at the different pH range from 2 to 10. It can be seen that 420 mg/l phenol could be completely degraded by *Fusarium* sp. HJ01 in 8 days with the pH from 4 to 8. Compared with the time consumed in the sample of pH 4 to pH 8, more time was spent in the sample of pH 2 for that matter and *Fusarium* sp. HJ01 even cannot grow in pH 10.

3.1.3. The effect of temperature

Fig. 3 was shown the effect of temperature on the phenol degradation using HJ01. This strain grew in LMS substrate at the temperature range from 25 to 35 °C. It can be seen that the optimal values of temperature was observed as 30 °C.

3.2. Catechol dioxygenase activities

3.2.1. Catechol dioxygenase activities for different substrate

In Figs. 4 and 5, the substrate specificity of C12 and C23 is reported. Catechols are converted by both C12 and C23 with

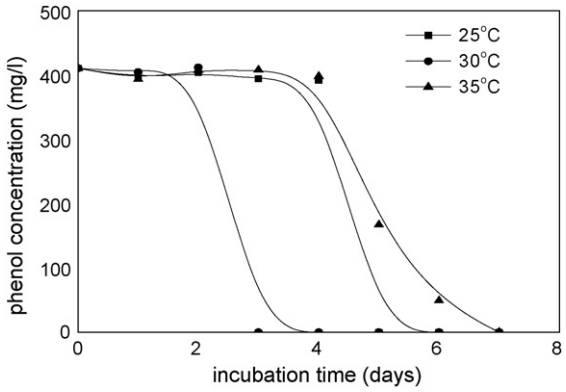

Fig. 3. The effects of temperature on phenol degradation by *Fusarium* sp. HJ01.

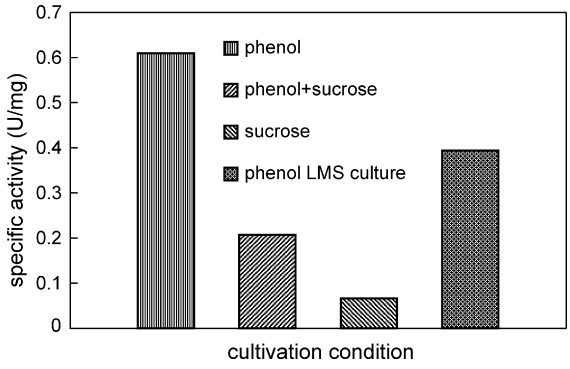

Fig. 4. The specific activity of C12 in different cultivation condition.

the four substrates. Different carbon sources have significantly influenced the activity of C12 and C23 production by *Fusarium* sp. It is shown that all of them catalyze the reaction of catechol with O_2 to afford mainly the oxidative intradiol but very small amounts of extradiol cleavage products. This is very much in contrast to the nearly quantitative yield of the oxidative intradiol cleavage products.

The activities of C12 and C23 reach highest value 0.609 and 0.125 U/mg when use phenol as single carbon resource, respectively. Added some mineral salts can decrease the activity, this is because some chemicals will inhibit the dioxygenase activities. Chuan et al. [15] has reported that when minor Cu^{2+} and Mn^{2+} existed, the C12 and C23 relative activity decline to 36% and 19%, respectively. Except above, other chemicals such as Zn^{2+} and Ca^{2+} will affect C23 activity evidently. Very weak or no activity was observed for sucrose as substrate, adding some phenol can increase activity of both C12 and C23, but is not very markedly. It is point out that the C12 activity for phenol liquid substrate in the present study is slightly more than these articles reported [15–17].

3.2.2. Enzyme activities in different cultivation time

Fig. 6 was shown the phenol degradation curve by *Fusarium* sp. HJ01, and Fig. 7 was shown the specific activity of the catechol dioxygenase in different incubation time. The catechol production activity by *Fusarium* sp. HJ01 reached a maximal level in a shorter period about 6 days when the phenol is degraded totally. As intermediate catechol converted from phenol exhausted, the catechol dioxygenase activities reduced.

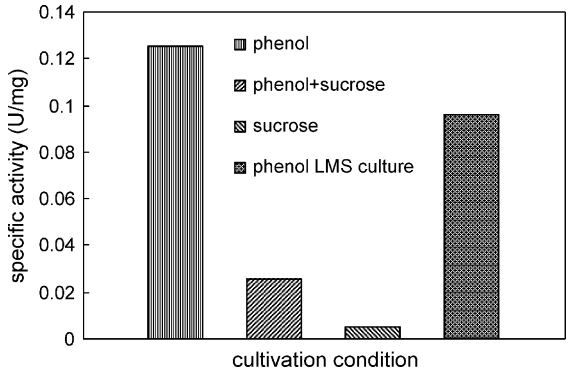

Fig. 5. The specific activity of C23 in different cultivation condition.

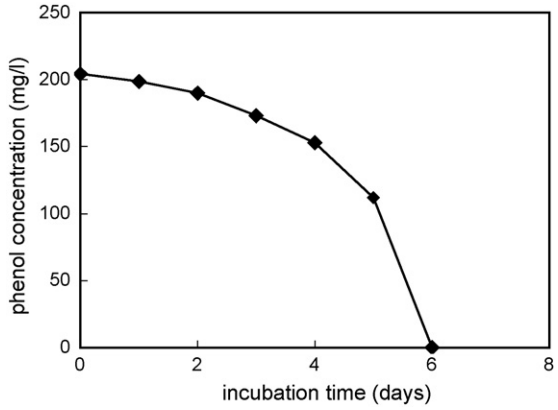

Fig. 6. The curve of phenol degradation by *Fusarium* sp. HJ01.

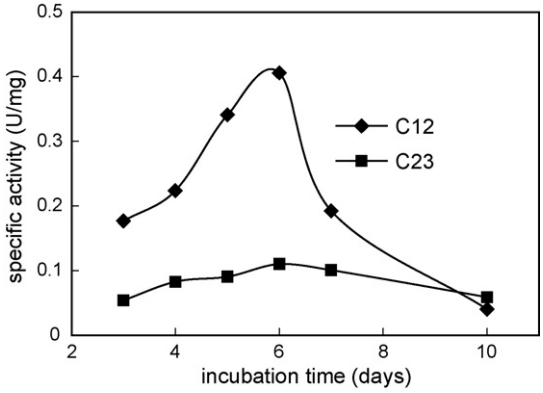

Fig. 7. The enzyme activities production curve.

These obtained data showed that the activities of catechol 1,2-dioxygenase is higher than that of catechol 2,3-dioxygenase, the maximum value of activities of C12 and C23 is 0.406 and 0.101 U/mg, respectively.

3.2.3. Effect of pH and temperature on C12 and C23 activities

The pH of the medium and temperature affected the catechol production by mycelium. The pH-activity (Fig. 8) curves showed that the maximum activity for C12 and C23 was at pH 6.8 and pH 9.4, respectively. The temperature–activity (Fig. 9) curves

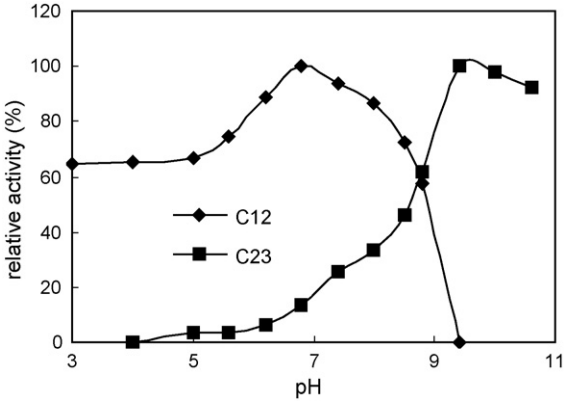

Fig. 8. The effect of pH on the C12 and C23 activities.

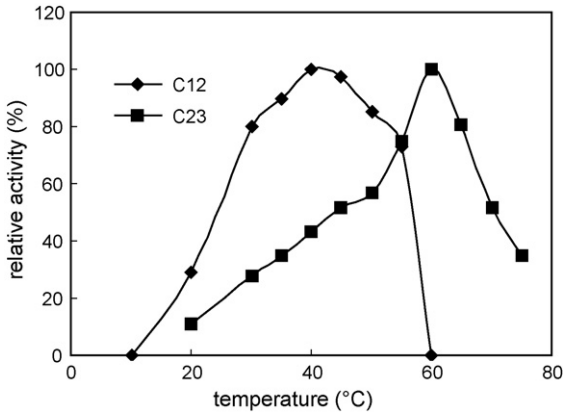

Fig. 9. The effect of temperature on the C12 and C23 activities.

showed that the maximum activity for C12 and C23 was at 40 and 60 °C, respectively. It is in accordance with Ref. [15]. The active of C23 was slightly more sensitive to pH, it has a higher activity in alkalescence condition from pH 7.4 to pH 10.6 and was more stable at elevated temperatures from 30 to 75 °C. There were Dong [18] reported that some thermophilic microorganism can produce the heat-resistant C23, but the mechanism of the particular characteristic has not been clear yet.

3.3. Catalytic activity of model

In this study, the Michaelis–Menten equation has been used as the model substrate for investigating the catechol dioxygenase activity as the main cleavage products are relatively stable. It is follows as the reports [19]:

$$v = \frac{V_{\max}[S]}{K_m + [S]} \quad (1)$$

Eq. (1) can be transformed into

$$\frac{1}{v} = \frac{K_m}{V_{\max}}\frac{1}{[S]} + \frac{1}{V_{\max}} \quad (2)$$

where v is the initial velocity of reaction (μmol/min); K_m is the Michaelis constant (mol/l); V_m is the maximum velocity (μmol/min); [S] is the substrate concentration (mol/l).

The kinetics of oxygenation of the adducts were followed by monitoring the production of *cis*,*cis*-muconic and 2-hydroxymuconic semialdehyde. Fig. 10 shows the relationship between $1/v$ and 1/[S] for C12 and C23. The data obtained are well described by the Michaelis–Menten equation when plotted according to Eq. (2). The values of K_m and $V_{\max}$ (Table 1) are determined from the intercept and the slope using the non-linear least-squares regression method.

Table 1
The kinetic parameters K_m and $V_{\max}$ for C12 and C23

The catechol dioxygenase	K_m (mmol/l)	$V_{\max}$ ($\times 10^{-3}$ U/min)	R^2
C12	0.0015	3.788	0.9981
C23	0.005	3.574	0.9986

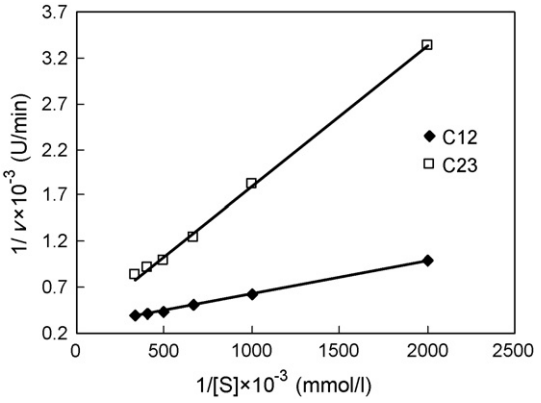

Fig. 10. The relationship between $1/v$ and 1/[S] for C12 and C23.

4. Conclusions

From the data presented in this study, it can be concluded that the investigated strain *Fusarium* sp. are considered to have good prospects for its application in the remediation of phenol contaminated environment and improvement of phenol removing treatment of the industrial wastewater. A general comparison of the major pathways for catabolism of phenol in fungi has revealed that the initial conversion steps are carried out similar to bacteria, which phenol is transformed into the intermediate catechol. This dihydroxylated intermediates are channelled into the ortho cleavage pathway (also termed β-ketoadipate pathway) or meta cleavage pathway by catechol 1,2-dioxygenase or catechol 2,3-dioxygenase. Both types of pathways lead to intermediates of central metabolic routes, such as the tricarboxylic acid cycle. Furthermore, the enzymes catechol 1,2-dioxygenase and catechol 2,3-dioxygenase of *Fusarium* sp. HJ01 exhibited certain similarities and differences compared to similar enzymes isolated from other microorganisms. This mechanism may contribute to metabolic adoption of ubiquitous fungus and some of its mutants found in nature, commonly isolated from soil, plant debris, and indoor air environment, such as *Fusarium* sp., exposed to the aromatic compounds. Besides, other fungus will be further studied for the possible utilization on the industrial effluent treatment and decontamination in the natural areas.

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References

[1] P.M. Schie, L.Y. Young, Biodegradation of phenol: mechanisms and applications, Bioremed. J. 4 (2000) 1–18.

[2] M. Eiroa, A. Vilar, L. Amor, Biodegradation and effect of formaldehyde and phenol on the denitrification process, Water Res. 39 (2005) 449–455.

[3] D. Leonard, N. Lindley, Growth of *Ralstonia eutropha* on inhibitory concentrations of phenol: diminished growth can be attributed to hydrophobic perturbation of phenol hydroxylase activity, Enzyme Microb. Technol. 25 (1999) 271–277.

[4] V.L. Santos, V.R. Linardi, Biodegradation of phenol by a filamentous fungi isolated from industrial effluents-identification and degradation potential, Process Biochem. 39 (2004) 1001–1006.

[5] G.I. Garcia, P.R. Jimenez Pena, J.L. Bonilla, M.A. Martin, S.A.A. Martin, C.E. Ramos, Removal of phenol compounds from olive mill wastewater using *Phanerochaete chrysosporium*, *Aspergillus niger*, *Aspergillus terreus* and *Geotrichum candidum*, Process Biochem. 35 (2000) 751–758.

[6] A. D'Annibale, R. Casa, F. Pieruccetti, *Lentinula edodes* removes phenols from olive-mill wastewater: impact on durum wheat (*Triticum durum* Desf) germinability, Chemosphere 54 (2004) 887–894.

[7] S. Harayama, M. Rekik, Bacterial aromatic ring cleavage enzymes are classified into two different gene families, Biol. Chem. 264 (1989) 15328–15333.

[8] D.I. Metelitza, A.V. Litvinchuk, M.I. Sevenkova, Peroxidase catalyzed co-oxidation of halogen-substituted phenols and 4-aminoantipyrine, J. Mol. Catal. 67 (1991) 401–411.

[9] Xi Danli, The Handbook of Environmental Engineering: Environmental Monitor Volume, Higher Education Press of China, Beijing, 1998, p. 421–425.

[10] T. Liu, C.K. Zhang, Y. Xue, Z.X. Liu, Purification and proterties of catechol 2,3-dioxygenase from strain *L68*, J. Food Sci. Biotechnol. 21 (2002) 53–57.

[11] Z. Liu, H. Yang, Z. Huang, Degradation of aniline by newly isolated, extremely aniline-tolerant *Delftia sp*. AN3, Appl. Microbiol. Biotechnol. 58 (2002) 679–682.

[12] P.D. Strachan, A.A. Freer, C.A. Fewson, Purification and characterization of catechol 1,2-dioxygenase from *Rhodococcus rhodochrous* NCIMB 13259 and cloning and sequencing of its catA gene lJj, Biochem. J. 333 (1998) 741–747.

[13] A. Jaafar, A. Musa, Y.H. Lee, K. Nadarajah, S. Hamidah, Chitosan-based tyrosinase optical phenol biosensor employing hybrid nafion/sol–gel silicate for MBTH immobilization, Talanta 70 (2006) 527–532.

[14] O.H. Lowry, N.J. Rosebrough, A.L. Farret, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.

[15] L.W. Chuan, L.Y. Su, L.W. San, Purification and characterization of a novel catechol 1,2-dioxygenase from *Pseudomonas aeruginosa* with benzoic acid as a carbon source, Process Biochem. 41 (2006) 1594–1601.

[16] A. Zlatka, G. Maria, Z. Plamena, N. Peneva, Comparison of growth kinetics and phenol metabolizing enzymes of *Trichosporon cutaneum* R57 and mutants with modified degradation abilities, Enzyme Microb. Technol. 34 (2004) 242–247.

[17] E. Kalogeris, J. Sanakis, D. Mammac, P. Christakopoulos, D. KeKOS, H. Stamatis, Properties of catechol 1,2-dioxygenase from *Pseudomonas putida* immobilized in calcium alginate hydrogels, Enzyme Microb. Technol. 39 (2006) 1113–1121.

[18] F.M. Dong, Molecular cloning and mapping of phenol degradation genes from *Bacillus stearotherm ophilus* FDTP-3 and their expression in *Escherichia coli*, Appl. Environ. Microb. 58 (1992) 2531–2535.

[19] M.J. Frouws, K. Vellenga, H.G.J. De Wilt, Combined external and internal mass transfer effects in heterogeneous (enzyme) catalysis, Biotechnol. Bioeng. 18 (2004) 53–62.