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NON-MEDIATOR SUPPORTED NOVEL NATURAL OXIDATIVE BIODEGRADATION OF BISPHENOL A (BPA) IN CONTAMINATED INDUSTRIAL WASTEWATER BY *PSEUDOMONAS AERUGINOSA* NR.22

Nik Him NIK RAIKHAN*, Othman NUR HIDAYATI, Zainuddin MOHD FIQRI

Bioprocess Engineering Department, Faculty of Chemical Engineering,
Universiti Teknologi MARA (UiTM), 40450 Shah Alam, Selangor, Malaysia

Abstract

The strain Pseudomonas aeruginosa NR.22 *(Ps.NR.22); an extracellular laccase enzyme producer isolated from a lake in Malaysia has been proved to grow very well in more than 50ppm BPA in the industrial wastewater collected from Bukit Minyak Industrial Park, Pulau Pinang, Malaysia. The 50ppm BPA degradation was catalyzed by 1.0U/mL of extracellular crude laccase without addition of types any mediator and was completely removed within 24 hours. Components of β-hydroxybutyric acid ($C_4H_8O_3$), pyroglutamic acid ($C_5H_7NO_3$), hydrocinnamic acid ($C_9H_{10}O_2$) and tartaric acid ($C_4H_6O_6$) were detected as direct oxidative degradation products from the BPA; identified by the GC-MS. The GC-MS didn't detect a formation of any intermediate compound, which was believed to be caused by the high laccase activity in the product interchange; therefore the intermediate compound has appeared in a very short time and was hardly detectable by GC-MS. Correlation between laccase activities with the BPA concentration was studied in the kinetic of BPA as a substrate for laccase. At 1000ppm BPA concentration, the rate of BPA used was recorded as $0.94 g \cdot L^{-1} \cdot h^{-1}$ or 940ppm. The ability to degrade 94% of 50ppm BPA has been catalyzed by 27.42±0.01U/mL laccase in 18 hours. In conclusion, laccase Ps.NR.22 has played a critical role in BPA biodegradation and catalyzed a cross-coupling reaction with four major acid group compounds as the products which has been listed above. This strain is highly potential for industrial application in maintaining water quality and land conservation from BPA contamination.*

Keywords*: Bacterial growth; kinetic model; substrate utilization; Pseudomonas aeruginosa* NR.22*; BPA.*

Introduction

Bisphenol A (BPA) is an important monomer in the manufacturing of polycarbonate plastics, food and drink packaging applications, medical sample bottles, and huge numbers of daily needs containers and surfaces including PVC water pipelines. BPA has recently received particular attention from the public and the scientific community. Several studies have shown

* Corresponding author: raikhan7952@salam.uitm.edu.my

that BPA may be associated with a number of health problems and diseases. Although BPA is quickly metabolized to BPA monoglucuronide and rapidly excreted in urine in adults, some residual free BPA is still available for receptor binding that directly affects the nucleotide replication. Post-consumer releases are primarily via effluent discharge from municipal wastewater treatment plants, leaching from landfills, combustion of domestic waste, and the natural breakdown of plastics in the environment [1]. Level of BPA in landfill leachate and river stream can be very high according to report from Canada, with concentration up to 360 mg/kg dry weight and >100mg/L. However, these findings were based on 6–12 days of BPA exposure and therefore did not take into account the effects of chronic BPA exposure. Much lower concentrations range between 1–10µg/L have been shown to be acutely toxic to freshwater and marine species [2]. Recent study from China and United State on commercial canned food and school meal has evidently shown positive results of 2.85µg/L [3] and 1.19µg/kg - body weight/day of BPA, respectively. US EPA Oral Reference Dose (RFD) affirmed that this result was too high to be exposed to children since the limit is only 3.65µg/day. Apart from that, BPA is also being used as chemicals in beginning monomer to form polymer especially plastic materials.

Production of polymers such as epoxy resins or polycarbonate plastics for example have been using these monomers; making it constantly being released and distributed into water bodies, sediments and soils in high concentration. This contamination has earned great attention and responses from huge numbers of scientists and respective bodies on scaling down the BPA production as well as to support the treatment in those contaminated areas. Methods to efficiently remove BPA from the environment are urgently introduced unfortunately treating BPA chemically has brought the environment another huge environmental issue. Lately, in reduction amount BPA are still low by affinity absorption treatment with stacking combination of sand, granulated activated carbon and moss peat with efficiency reduction only 50% to 80% [4]. Thus, BPA degradation has been proposed using microbial enzymatic catalysis which is mainly depended on the metabolism of bacteria itself. Metabolic pathways of BPA degradation in specific bacterial strains commonly is proposed based on the metabolic intermediates detected during the degradation process. In this paper, the BPA-degrading bacteria are expressed with potential BPA degradation pathway mediated by *Pseudomonas aeruginosa* NR.22 is referred with introduction to its potential enzyme, laccase.

Materials and Methods

Reagent, chemicals and pH. All material and chemicals were collected from various sources and quality as listed. Bisphenol A (BPA, R&M Chemicals, 98%), bacteriological peptone (Ultrapure, protein = N×6.38 ≥ 76.5%), ethyl acetate (anhydrous, 99.8%, Sigma-Aldrich), anhydrous sodium sulfate (CAS Number: 7757-82-6, Na_2SO_4), BSTFA + TMCS reagent (99:1, Sigma-Aldrich), ammonium phosphate monobasic (CAS Number 7722-76-1, ≥ 98%, $NH_4H_2PO_4$, Sigma-Aldrich), glycerol (Merck), Pseudomonas agar (Microbiology), nutrient agar (Merck), nutrient broth (Merck), potassium dihydrogen phosphate (KH_2PO_4, V90004, Vetec™ reagent grade, 99%, Vetec), ethanol (CH_3CH_2OH analytical grade, Sigma-Aldrich), sodium hydroxide (NaOH, Fluka), meat extract (Merck Milipore, granulated, for microbiology), 2,2′-azino-di(3-ethylbenzothiazoline-6-sulfonic acid (Sigma-Aldrich) and sodium chloride (NaCl, Sigma).

BPA Contaminated Water Source. The contaminated industrial wastewater was collected from Bukit Minyak Industrial Park, Pulau Pinang, Malaysia. The BPA content was

detected to be 1100ppm. The waste water was diluted to form a 50ppm dilution. The water quality is listed in Table 1.

Table 1. Characteristics of contaminated wastewater Bukit Minyak Industrial Park, Pulau Pinang

Parameters	Concentration	Parameters	Concentration
COD	7.7 ± 0.01 g/L	Viscosity	1.85±0.01mm^2/s
Ammoniac	0.5 ± 0.01 g/L	Total polar material TPM	6.22±0.01%
Phosphate	2.1 ± 0.01 g/L	Iodine value (IV)	3.18±0.03 gI^2/100g
BPA content	1100 ppm	Peroxide value (PV)	2.11±0.01meq/kg
pH	6.1	Temperature	40°C

Pseudomonas Species, Enrichment Medium, Fermentation Broth for BPA Degradation and Inoculum Preparation. *Pseudomonas aeruginosa* NR.22 (Ps.NR.22); a Gram negative-rod shaped bacteria was isolated from a lake in Shah Alam, Selangor, Malaysia [5], and has been confirmed to carry a 16S rRNA gene. The strain was maintained at -20ºC in Pseudomonas agar with 5% (w/v) glycerol. The enrichment medium was consisted of 0.80% (w/v) of nutrient broth powder (NB), 1.0% (w/v) of meat extract, 1.0% (w/v) peptone and 0.05% (w/v) NaCl. Into this medium, 80% (v/v) of contaminated industrial wastewater has been added to a final volume of 100mL (fermentation medium). Then, the pH was adjusted to pH 7.0. A volume of 100mL fermentation volume was sterile in a 250mL Erlenmeyer flask using steam at a pressure of 15 psi with the attaining temperature at 121°C (30 minutes). The inoculum of *Pseudomonas aeruginosa* NR.22 was prepared in 10mL sterile nutrient broth (pH 7.0, 150rpm) and was used at 24 hours of growth. The amount of 8.0 (v/v) of 5.0×10^6 cells/mL of the inoculum was transferred into the sterile fermentation medium, followed by an incubation at 37°C, rotary shaker at 150 rpm, 24 hours with triplicate flasks for each run [6]. Figure 1 shows the green pigmentation produced by of *Pseudomonas aeruginosa* NR.22 after 24 hours incubation on Pseudomonas agar and good growth in BPA contaminated industrial wastewater.

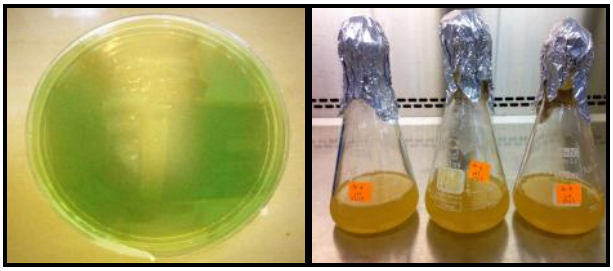


Fig. 1. Green pigmentation produced by of *Pseudomonas aeruginosa* NR.22 after 24 hours incubation on Pseudomonas agar [5] (A). Growth in BPA contaminated water (B).

Estimation of Bacterial Cell Mass. The Optical Density (OD) of the culture broth at 540nm was measured periodically using Spectrophotometer and about 2.0mL sample of the culture was withdrawn from the flask and centrifuged at 10000rpm for 10 minutes for every 3 hours of sampling. The supernatant was poured out and the remaining cell is suspended in distilled water and the cell suspension was centrifuged again at the same parameter. The supernatant was again poured out and the remaining cell was washing slowly using of distilled water on the suspension of cells. Then, it was transferred to a falcon tube with small opening into a desiccator at room temperature overnight. After 24 hours, the falcon tube containing dry cell was weight. The difference between the final weight of falcon tube and pre-weight falcon tube were used to estimate Cell Dry Weight (CDW). This method was adapted and modified using *N.H. Nik Raikhan* [7].

Crude Laccase Enzyme and Laccase Assay (Oxidation Test). The method was modified from *Y.J. Xuan et al*. [8]. An amount of 100mL of 24 hours fermentation broth was centrifuged at 6,000×g for 30 min at 4°C to precipitate the cellular debris and to obtain clear supernatants which is the source of the extracellular laccase. Laccase activity in 24 hours fermentation broth was determined spectrophotometrically at 436 nm ($\varepsilon 436 = 29{,}300 M^{-1} \cdot cm^{-1}$) as described by *M.L. Niku-Paavola et al.* [9]. Laccase activity in the oxidation assay was determined spectrophotometrically at 465 nm with 5ppm BPA as a substrate in a reaction mixture containing 50mM phosphate buffer (pH 6.5) ($\varepsilon 465 = 48{,}000 M^{-1} \cdot cm^{-1}$). One unit of enzyme activity was defined as the amount of enzyme that increased the absorbance by 0.001 units per min at 37°C; the activities were expressed in U/mL. All assays are repeated thrice to gain the mean ± sd of three applications. All spectrophotometric measurements were carried out using a UV–Vis spectrophotometer (SPEKOL 2000, Germany).

Extraction and derivation for GC Analysis. All samples were extracted twice with 2 volumes of ethyl acetate. The extracted solution was dehydrated with anhydrous sodium sulphate and concentrated by rotary evaporation. Triplicate aliquots of this solution were withdrawn and 5mL of 0.01% isotopically labelled BPA in the same solvent were added as internal standard, drying with a gentle nitrogen stream. Before GC-MS analysis, samples were trimethylsilylated (TMS) at 50°C for 30 min using 0.2mL of the BSTFA reagent [5, 10].

Gas Chromatography Analysis and HPLC. A gas chromatograph equipped with an HP-5MS column (30μM x 0.25 mm internal diameter; 0.25mM film thickness) coupled to a quadruple mass detector (GC-MS system 7980A-5975C, Agilent Technologies). Helium was the carrier gas at a flow rate of 1.2mL/min^{-1}. Injector and detector were programmed at 320°C. This method has been adapted from *D. Daassi et al* [11]. HPLC analysis on the BPA for standard and the contaminated industrial wastewater is summarised in Table 2 below.

Table 2. Parameters for HPLC analysis

Parameters	Description
Column	Perkin Elmer C18
Mobile Phase	Solvent A: Acetonitrile (40%) Solvent B: Water (60%)
Analysis Time	5.0min
Flow Rate	0.5mL/min
Oven Temperature	40°C
Detection	230nm
Injection Volume	1μL

Kinetics Study of Pseudomonas aeruginosa* NR.22 *towards BPA Degradation. The *Pseudomonas aeruginosa* NR.22 colonies were inoculated into the nutrient broth (NB) containing BPA at different concentrations (50, 200, 400, 600, 800 ppm). *Pseudomonas aeruginosa* NR.22 strain were cultivated in 250mL Erlenmeyer flasks containing 100mL of working volume of NB at 37°C with agitation speed of 150 rpm in incubator shaker. Growth of the bacterial species was monitored by measuring optical density at 540 nm (OD_{540}) using a Shimadzu UV-Visible double-beam Spectrophotometer at every 3 hours gaps for about 3 days. The blank used for absorbance reading of cultivated *Pseudomonas* sp. was nutrient broth (NB). The kinetics study will be specified different concentrations of BPA. In growth kinetics, the maximum specified growth rate of *Pseudomonas aeruginosa* NR.22 can be calculated based on the increment of the cell mass over a period of time. Equation 1 was used to calculate maximum specific growth rate, μ_{max} of the *Pseudomonas aeruginosa* NR.22 growth [5].

$$\mu_{max} = K = \ln(m_{t2}/m_{t1})/(t2-t1), \qquad (1)$$

where: μ_{max} - maximum specific growth rate; K - growth rate constant; m_t - biomass at different time at the exponential phase.

Results and Discussion

The BPA in contaminated wastewater was confirmed using HPLC. Figure 2 shows the peaks of BPA appearance at 2.55min for both standard and contaminated industrial wastewater from Bukit Minyak Industrial Park, Pulau Pinang.

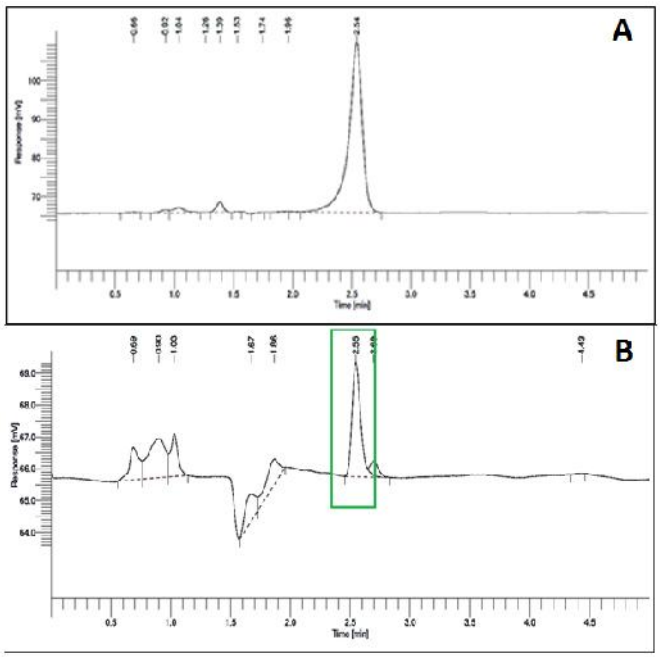


Fig. 2. HPLC results for BPA standard (A), and contaminated industrial wastewater from Bukit Minyak Industrial Park, Pulau Pinang (B) with peaks appeared both at 2.55min.

Degradation of BPA. Laccase enzyme or benzenediol oxygen oxidoreductases (EC 1.10.3.2) is a copper-containing enzymes that is responsible for the oxidation of a assorted phenolic compounds and aromatic amines with the reduction of molecular oxygen to form water [12]. Laccase oxidizes ABTS (2,2′-azino-di(3-ethylbenzothiazoline-6-sulfonic acid) as a redox indicator to form a blue-green cation radical, a method which can be adapted for quantitative estimation using spectrophotometry [11]. Laccase is superior because it uses molecular oxygen as a co-substrate and is frequently called blue copper oxidases. Whereas the substrate BPA [$(CH_3)_2C(C_6H_4OH)_2$] is grouped into diphenylmethane derivatives and bisphenols, contains two hydroxyphenyl groups, a colorless solid, very soluble in organic solvents, but very poorly soluble in water. Figure 3 depicted the chromatograms of the degradation of 50ppm of BPA by laccase enzyme from *Pseudomonas aeruginosa* NR.22. The major peak of BPA before the addition of the crude laccase was detected at Rt = 13.5min (see Figure 3A) indicating BPA has not been oxidized. The products of BPA degradation by laccase enzyme from *Pseudomonas aeruginosa* NR.22 were β-hydroxybutyric acid, pyroglutamic acid, hydrocinnamic acid and tartaric acid. In a research on effect of temperature on leaking of BPA

from plastic drinking bottles and its self-degradation by *N.H. Nik Raikhan 2016* (unpublished, in press), sour smell was recorded to be a sign of the drinking water contamination suggested to be caused by at least few acidic groups formed from the BPA degradation. In Figure 3B, the four peaks of *a* (β-hydroxybutyric acid, Rt = 8.0 min), *b* (pyroglutamic acid, Rt = 9.75), *c* (hydrocinnamic acid, Rt = 10.16min) and *d* (tartaric acid, Rt = 10.45 min) were clearly seen but with poor peaks presentation. This is believed to be caused by the GC's poor sensitivity towards any analysis of low volatility polar compounds such as phenolic and acidic compounds [13] but since GC-MS is always the easiest way to analyse data of acidic oxidation intermediates, the accumulated products is still the choice of GC-MS to be revealed (*N.H. Nik Raikhan*, 2016). In a recent review of the BPA degradation products by *Q. Husain and S. Qayyum* [14], the report stated that the treatment of BPA by biological and enzymatic methods will produce several of polycarboxylic acids and other transformation products which were determined mostly using the GC-MS. When comparing the chromatograms of the extracted compounds (see Figure 3B), a similar profile was observed in the reaction catalyzed by laccase from the *Bjerkandera adusta* (11B) [KU904462] in the research of *D. Daasii et al* [11]. β-hydroxybutyric produced in this study is a common precursor to polyesters which are one kind of biodegradable plastic. This statement is supported by report from *R. Rameshwari and M. Meenakshisundaram* [15]. Had registered any formation of known intermediates which are more toxic than the BPA itself, but we believed stages of intermediates formation and the products from the BPA degradation is very much depending on the sensitivity of the enzyme, enzyme concentrations, various other physical parameters (pH, temperatures, shaking rates, types of buffers, incubation rate, suppressor, mediator) and finally the enzyme purity in which we are working with crude laccase. Our finding on the four acid products is the novelty of this research since there was no optimization of such was done to support the oxidation process, because we chose to let the crude laccase do the job without even with the addition of a mediator to support the biodegradation. Study on laccase by *Bjerkandera adusta* (11B) [KU904462] by *D. Daasii et al* [11] with an addition of a redox mediator; 1.0mM hydroxybenzotriazole (HBT) has not produced such toxic intermediates as well, not like what the study by *N. Lu et al* [16] has reported; with the detection of p-hydroxyacetophenone, p-hydroxybenzaldehyde, p-hydroxybenzoic acid, hydroquinone and phenol. We might need to repeat this study using purified laccase from *Pseudomonas aeruginosa* NR.22 in order to confirm the toxic intermediates since enzymatic system rely so much on other factors that has not been focused in this study.

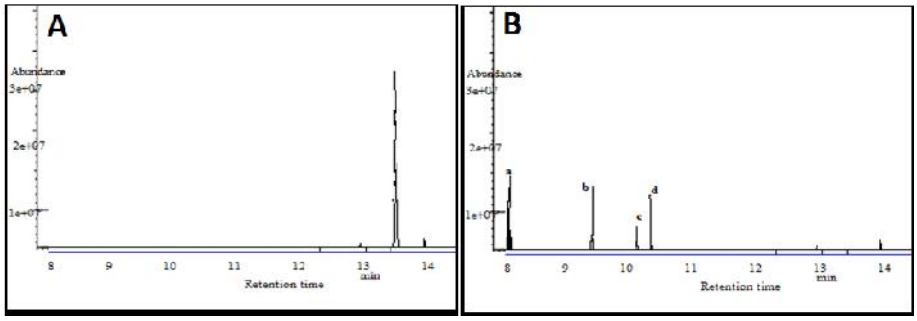
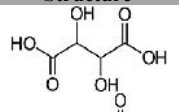
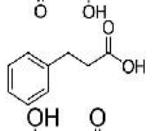
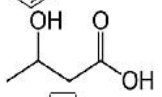
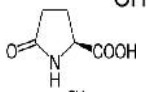
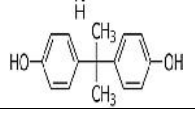


Fig. 3. Chromatograms showing the degradation of 50 ppm of BPA.

Table 3 represents the GC-MS identification of the BPA and the products detected in BPA degradation catalyzed by crude laccase from *Pseudomonas aeruginosa* NR.22. The identification was then designed to sort the possible pathway of the BPA degradation (see Figure 4). There was no formation of any intermediate compound detected, which was believed

to be contributed by high laccase activity that give arise to a very fast product interchange, therefore the intermediate molecules has appeared only in a very short time and hardly detectable by the GC-MS machine.

Table 3: GC-MS identification of the BPA and products detected in BPA degradation catalysed by crude *Pseudomonas aeruginosa* NR.22 laccase enzyme.

Metabolites	Molecular Formula	Retention Time (R_t, min)	Molecular Structure
Tartaric acid	$C_4H_6O_6$	10.45	
Hydrocinnamic acid	$C_9H_{10}O_2$	10.16	
β-hydroxybutyric acid	$C_4H_8O_3$	8.0	
Pyroglutamic acid	$C_5H_7NO_3$	9.75	
2,2-bis[(4'-hydroxy) phenyl] propane (BPA)	$C_{15}H_{16}O_2$	13.5	

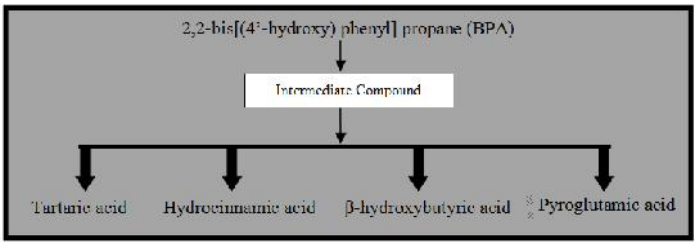


Fig. 4. Products from the BPA degradation by *Pseudomonas aeruginosa* NR.22 using laccase enzyme pathway without detection of any intermediate compound.

Kinetic Study Using BPA as a Substrate for Crude Laccase. In order to calculate the *Pseudomonas aeruginosa* NR.22's potential and ability to degrade BPA in the industrial wastewater, we have run a study on the kinetics of BPA degradation and the results were surprisingly excellent. As expected, this strain has performed exclusive kinetic characteristics; the values are listed in Table 4. The rate of BPA degradation has been showing a significant correlation with the maximum growth rate. By taking for example BPA at 400ppm BPA; the rate of BPA used by the *Pseudomonas aeruginosa* NR.22 to grow was calculated to be $0.39g \cdot L^{-1} \cdot h^{-1}$, which mean the amount of degraded BPA was 390ppm. Therefore, at 400ppm, using the shaking rate at 150rpm, this novel species has biodegraded 97.5% BPA as a sole carbon. On the other hand, when using 1000ppm of BPA, the rate of BPA used was recorded as $0.94g \cdot L^{-1} \cdot h^{-1}$ or 940ppm degradation achieved (94% BPA was used). These values and all other values in the Table 4 has strongly shown the ability of this novel activities of BPA degradation. Upon the use of true industrial wastewater, there are few factors have to be considered regarding the effect of wastewater content to the crude laccase, enzyme deactivation or maybe fluctuate specific growth rate. Fortunately, the content of Bukit Minyak Industrial Park's

wastewater was supporting the bacterial growth due to low ammonia and phosphate content. But if we are running other samples at large, wastewater characterization is vital to avoid lysis cells.

Table 4. Kinetic parameters of BPA degradation by *Pseudomonas aeruginosa* NR.22 using different BPA concentrations.

BPA	KINETIC PARAMETERS	VALUES
50 ppm	-Laccase maximum activity (U/mL)	18.35 ± 0.04
	-Time to reach maximum laccase activity (h)	9
	-Maximum growth (g/L)	0.485 ± 0.01
	-Time for maximum growth to achieve (h)	12
	-Rate of BPA used ($g \cdot L^{-1} \cdot h^{-1}$)	0.041
200 ppm	-Laccase maximum activity (U/mL)	29.89 ± 0.12
	-Time to reach maximum laccase activity (h)	9
	-Maximum growth (g/L)	2.30 ± 0.01
	-Time for maximum growth to achieve (h)	12
	-Rate of BPA used ($g \cdot L^{-1} \cdot h^{-1}$)	0.19
400 ppm	-Laccase maximum activity (U/mL)	34.70 ± 0.72
	-Time to reach maximum laccase activity (h)	12
	-Maximum growth (g/L)	4.75 ± 0.04
	-Time for maximum growth to achieve (h)	12
	-Rate of BPA used ($g \cdot L^{-1} \cdot h^{-1}$)	0.39
600 ppm	-Laccase maximum activity (U/mL)	34.81 ± 0.12
	-Time to reach maximum laccase activity (h)	18
	-Maximum growth (g/L)	10.80 ± 0.005
	-Time for maximum growth to achieve (h)	18
	-Rate of BPA used ($g \cdot L^{-1} \cdot h^{-1}$)	0.60
800 ppm	-Laccase maximum activity (U/mL)	37.47 ± 0.05
	-Time to reach maximum laccase activity (h)	18
	-Maximum growth (g/L)	13.89 ± 0.01
	-Time for maximum growth to achieve (h)	18
	-Rate of BPA used ($g \cdot L^{-1} \cdot h^{-1}$)	0.77
1000 ppm	-Laccase maximum activity (U/mL)	27.42 ± 0.01
	-Time to reach maximum laccase activity (h)	18
	-Maximum growth (g/L)	18.89 ± 0.01
	-Time for maximum growth to achieve (h)	18
	-Rate of BPA used ($g \cdot L^{-1} \cdot h^{-1}$)	0.94

Conclusion

The result from the study has proved the ability of *Pseudomonas aeruginosa* NR.22 as a highly potential species for the treatment of BPA contamination in any industrial wastewater. The rate of the BPA degradation has been correletated significantly to the maximum growth rate which applied the catalyzation of a cross-coupling reaction with four major acid group compounds as the products. This strain is highly potential for industrial application in maintaining water quality and land conservation from BPA contamination.

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