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Statistical optimization of the enzymatic breakdown of 2-Nitrophloroglucinol using thermo tolerant mixed Intracellular enzymes from *Serratia marcescens*

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ABSTRACT

Serratia marcescens, capable of degrading 2-Nitrophloroglucinol (NPG), an endocrine disruptor, was isolated from the soil contaminated with tannery wastewater. The mixed intracellular enzymes (MICE) from *S. marcescens* were extracted and characterized. The activity of MICE was found to be the maximum at pH, 7 and at temperature, 40 °C. The optimum conditions for degradation of NPG by thermo tolerant MICE were determined using Plackett–Burman design and response surface methodology. The maximum formation of pyruvic acid, the degradation end product was obtained at time, 3 h; pH, 7; temperature, 40 °C; concentration of MICE, 4 mg and agitation speed, 75 rpm. The amount of pyruvic acid formed upon degradation (94.65%) of NPG from its initial value of 75 μg was 31.25 μg. The degradation of NPG by thermo tolerant MICE was found to be enhanced by the addition of metal ions such as Zn^{2+}, Cu^{2+}, Ca^{2+} and V^{3+} ions with complete conversion of NPG into pyruvic acid. The degradation of NPG by MICE was evaluated through UV–visible, fluorescence and Fourier Transform-Infra Red spectroscopy. The NPG degradation by MICE was confirmed using high pressure liquid chromatography, nuclear magnetic resonance and GC–MS spectroscopy.

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1. Introduction

Endocrine disrupting chemicals (EDCs) present in wastewater at low concentrations could affect the environment as they consist of phenolic moiety that is thought to mimic the natural steroid hormones and enable EDCs to interact with steroid hormone receptors as analogues or antagonists [1–3]. EDCs were designed to have longer half-lives which benefitted their industrial use, but it has turned out to be quite detrimental to wildlife and humans [1]. EDCs do not decay completely, may not be metabolized, may be metabolized or broken down into more toxic compounds than the parent molecule [1].

Nitroaromatic compounds are among the largest and most important groups of industrial chemicals in use. The nitro group is strongly deactivating toward electrophilic aromatic substitution of the benzene ring which also contributes to the recalcitrance of nitro compounds to biodegradation [4]. The electron withdrawing nature of the nitro group, along with the stability of the benzene ring, makes nitroaromatic compounds resistant to oxidative degradation [4].

Nitroaromatic compounds are acutely toxic and mutagenic, and many are suspected or established carcinogens [5–8]. Oxidation and reduction products of nitroaromatic compounds can damage DNA directly or to cause the formation of adducts that induce mutagenesis by misincorporation of nucleotides during DNA synthesis. Structural and spectroscopic studies have observed that the nitro group as the substituent on the aromatic ring and the presence of other functional groups could develop the mutagenicity and carcinogenicity of these chemicals [7]. In wastewater treatment processes, EDCs such as natural and synthetic estrogens present in wastewater were removed by adsorption onto common particulates and by membrane processes [9].

2-nitrophloroglucinol (NPG) is a synthetic intermediate, used to prepare benzoxazoles and benzothiazoles as selective ligands for human β-estrogen receptor (ER-β) [10] which is capable of interfering with estrogenic activities. In tannery, wattle tannin is composed of catechin units, which upon biodegradation yields protocatechuic acid and phloroglucinol carboxylic acid [11]. Also the soluble sulphur dyes such as sulphur black 1, sulphur red 6 and sulphur brown 14 are commonly used for dyeing process in tanneries and NPG is released as the by-product during this process.

Many bacteria behave distinctly while facing the new molecules that they have not encountered before during their evolution. Bacteria develop new degradative pathway or enzymes for the

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degradation of recalcitrant compounds by means of proteins which display a binding ability to such recalcitrant compounds and bring about the specific transcriptional responses (different transcriptional and post-transcriptional mechanisms) [12,13].

The toxic pollutants were carried out either through intracellular accumulation or via enzymatic transformation to less or non-toxic compounds in biodegradation [14]. This technique is limited to biodegradable compounds. Sometimes the end products of biodegradation are more toxic and persistent than the parent compound. Also it takes longer time than other methods and it has been cost effective [15]. The removal of aromatic nitro groups by dioxygenase was first reported by Ecker et al. [16]. Additional support for the reaction mechanism came from the observation that the enzyme used by *Pseudomonas sp. for* the initial oxidation of chlorobenzenes could catalyse the elimination of the nitro group from 2, 4, 5-trichloronitrobenzene [17].

Thus, an alternate treatment method where EDCs could be degraded into nontoxic product using intracellular enzymes was attempted and it formed the first report on enzymatic degradation of NPG. In the present investigation, the mixed intracellular enzymes (MICE) from *S. marcescens* were extracted and characterized. The MICE were used for the degradation of NPG, and statistical optimization studies for the formation of end product, pyruvic acid was studied.

2. Materials and methods

2.1. Material

2-nitrophloroglucinol of 98% high purity was obtained from Alfa Aesar (India).

2.2. Culture enrichment and isolation

The NPG-degrading bacterium was first enriched from the terrestrial soil, Chennai, India. Enrichment was carried out with M9 Minimal medium salts (5X) (HiMedia) [Composition: Na_2HPO_4, 33.90 g/L; KH_2PO_4, 15.00 g/L; NaCl, 2.5 g/L and NH_4Cl, 5 g/L] of volume 100 ml with samples containing 2.5 mg of NPG as the sole carbon source. NPG concentration in minimal media was increased from 0.5 to 2.5 mg with an incremental increase of 0.5 mg. A red pigmented bacterium isolated from acclimatized soil was observed to degrade NPG in the solution.

2.3. Identification of the strains and phylogenetic analysis

The red pigmented bacterium was identified by 16S ribosomal DNA (16S rDNA) sequencing and phylogenetic analysis. The genomic DNA was isolated as follows: Gram negative cells were heated to facilitate cell lysis in the presence of sodium dodecyl sulphate (SDS) and to inactivate nucleases. After the cell disruption, DNA was isolated by inhibiting nucleases using chelating agents such as citrate or EDTA that bind with divalent ions. Perchlorate (1M) was added to dissociate the DNA from protein and then 0.54 volumes of isopropanol was added to differentially precipitate DNA from RNA [18]. Phylogenetic analysis was performed by subjecting the deduced sequence to the 16S rDNA database to obtain the similar sequences and the phylogenetic tree was constructed using the Phylip package [19]. The guanine–cytosine content in the bacterial genome was obtained using the online oligonucleotide properties calculator of Bioinformatics software and tools.

2.4. Culture conditions for intracellular enzyme production

The bacterium was aseptically inoculated into Erlenmeyer flasks of volume 1000 ml containing 12.5 mg NPG as the substrate in 500 ml of minimal media for intracellular enzyme production. Culture flasks were incubated on the shaker at 150 rpm, pH 7 and 35 °C for 72 h. The bacterial cells were separated from the culture flasks by centrifugation at 6000 rpm for 20 min. The bacterial biomass was washed twice with the phosphate buffer (pH 7.0) and stored at –20 °C.

2.5. Extraction of intracellular enzymes

The method for intracellular protein extraction was adapted from Naiem and Jai [20] with some modifications. The bacterial cells were sonicated with 1 ml of 0.1 M phosphate buffer. The extracts were centrifuged at 14,000×g for 30 min. The cell-free supernatant was collected and dialysed against 0.1 M phosphate buffer (pH 7.0) for overnight. The purified MICE were lyophilized and used for further analysis and for the degradation of NPG.

2.6. Assays of intracellular enzymes

The lyophilized enzyme extract was analysed for various intracellular enzymes present. The enzymes involved in the degradation of NPG were determined using the respective assay procedures: catechol dioxygenase [21], dehydrogenase [22], phenol hydroxylase [23], alkaline phosphatase [24], pyruvate decarboxylase [25], nitrate reductase [26] and pyruvate kinase [27]. Protein concentration was determined by Lowry method [28], employing bovine serum albumin as the standard. The intracellular enzymes were quantified and used as a mixture of intracellular enzymes for the degradation of NPG.

2.7. Molecular weight determination of Purified MICE

The molecular weight of enzymes was determined by using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) according to the method followed by Laemmli [29], on a 5% stacking gel and 12% resolving gel. The protein marker ranging from 14.3 to 94.7 KDa was used as a standard marker for the determination of molecular weight.

2.8. Amino acid composition of MICE by HPLC

The amino acid composition of MICE was determined by HPLC. The MICE was hydrolysed at 100 °C with 6 N HCl for 20 h and neutralized with 1 M NaOH. The amino acid composition was analysed using Agilent 1100 HPLC amino acid analyser (Agilent Technologies, Middleburg, Netherlands) and the data analysis was performed by using HP chem station [30].

2.9. Statistical optimization for degradation of NPG by MICE

The various parameters such as time, (0.5–4 h); pH, (1–10); temperature, (20–70 °C), MICE concentration, (1–5 mg) and agitation speed (30–150 rpm) for the degradation of NPG by MICE were optimized using one parameter at a time for the degradation of NPG (75 μg) in buffered solution of volume15 ml.

2.9.1. Plackett–Burman design

The preliminary experiments revealed that five factors, including time, temperature, concentration of MICE, pH and agitation speed, were supposed to have effect on pyruvic acid formation from enzymatic breakdown of NPG. It was known that the Plackett–Burman design could evaluate the main effects of factors. The factors having significant effects on NPG degradation were identified using this experimental design. As shown in the Table 1, the design matrix covered five factors with two dummy variables to evaluate their effects and amount of pyruvic acid as response value. Each factor was investigated at a high (+1) and a low (–1) level. The factors, which were significant at 95% of confidence level ($P < 0.05$) were considered to have

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