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Bioremediation of 3,5-dinitrobenzoic acid and aniline by a *Corynebacterium* sp.

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***Corynebacterium* sp was isolated from the soil by using 3-5 dinitro benzoic acid (DNB) as a sole carbon source. The highest rate of degradation of aniline (AN) or DNB was found in the exponential phase of the growth of the bacterium. After 24 h, about 50% of DNB and 30% of AN were degraded by *Corynebacterium* sp. At a concentration of 0.5 to 1 g/L of AN or DNB, good growth was obtained and the protocatechuic acid was detected. The optimum concentration of yeast extract was 2 g/l. Catechol 1-2 dioxygenase was induced in the cells grown on a medium containing AN or DNB. A significant activity of this enzyme was detected, which means that *ortho* cleavage pathway may be present in *Corynebacterium* sp.**

Key words: *Corynebacterium* sp., degradation, dinitrobenzoic acid (DNB), aniline (AN), protocatechuic acid, catechol, catechol 1-2 dioxygenase, *Ortho* cleavage, high performance liquid chromatography (HPLC).

INTRODUCTION

Majority of organic compound residues cause environmental hazards. Nitroaromatics and aniline are toxic chemicals present in the effluent of many industries as they are widely used as raw materials in the manufacturing of a number of products such as dyes, plastics, resins, pharmaceuticals, petro-chemicals, herbicides, pesticides among others. (Nishino and Spain, 2004, 2006; Peres and Agathos, 2000).

Many nitroaromatics have been shown to be toxic or mutagenic to many bacteria, yeasts, fungi, unicellular algae, todepool copepods and oyster larvae (Won et al. 1976; Marvin-Sikkema and de Bont, 1994). However, some bacteria such as *Pseudomonas* sp. strain JS42 (Haigler et al., 1994), *Pseudomonas putida* OU83 (Walia et al., 2003) and *Micrococcus* sp. (Mulla et al., 2011) could use 2-nitrotoluene as a sole source of carbon. Samanta et al. (2000) isolated *Ralstonias* sp. SL 98 by chemotactic enrichment technique. This organism is capable of utilizing different nitroaromatic compounds. Bengtsson and Carlsson (2001) isolated bacteria from pristine groundwater that degraded aniline in a slow rate. Liu et al. (2002) isolated a bacterial strain, AN3, which was able to use aniline or acotanilide as sole carbon and nitrogen sources from activated sludge and identified it as *Delftia*sp. AN3. This strain was capable of growing in concentrations of aniline up to 5000 mg/l.

Corynebacterium glutamicum has been used as a model bacterium for fermentative production of various amino acids and vitamins. The knowledge of aromatic degradation and assimilation by this bacterium had been rarely explored until recently (Shen et al., 2012). *C. glutamicum* grows on the following aromatic compounds: benzoate, phenol (Shen et al., 2004), 3-hydrobenzoate, gentisate (Shen et al., 2005), resorcinol (Huang et al., 2006) and naphthalene (Lee et al., 2010).

This paper focuses on the characterization of

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C*orynebacterium* sp isolated from the Egyptian soil which is able to grow on dinitrobenzene and aniline and also detects the cleavage pathway of these compounds.

MATERIALS AND METHODS

Microorganism

Isolation and characterization

A strain was isolated from soil of Tahwhay-Monifia (Egypt), using 3-5 dinitro benzoic acid (DNB) as a sole carbon source. The soil sample (10 g/l) was suspended in 100 ml MSM media supplemented with 4 g/l (DNB) in 250 ml Erlenmeyer flasks on shaker at 120 rpm and 30°C. It was sub-cultured for 7 days until the growth was evidenced by increasing turbidity. Bacterial strains in the degrading culture were purified by repetitive streaking onto minimal salt medium (MSM); 1 g/l DNB was added as sole carbon source and pure colony was obtained after 6 days.

Diagnostic tests for the morphological and physiological studies were carried out according to the "Bergery's Manual of Determinative Bacteriology," 7 th Ed. (1957) and Lechevaller et al. (1980).

Media

Minimal salt medium (MSM)

MSM medium with 20 g/l agar was used to test the growth of bacterium on each phenol (Ph), bromobenzene (BrB), salicylate (Sal), benzoate (Ben) or gallic acid (GaA) as sole carbon source. Phenol (Ph), bromobezene (BrB), salicylic (Sal), benzoate (Ben) and galic acid (GaA) were sterilized by filtration and were added as sole carbon source (1 g/l).

The minimal salt medium (MSM) consisted of the following component: g/l tap water, 1; KH_2PO_4, 0.6; K_2 HPO_4, 1; $(NH)_2$ SO_4, 0.5, $MgSO_4$ .7 H_2O. The medium pH was adjusted to pH 7 - 7.5 by NaOH (1 N).

Maintenance medium

The same MSM medium containing 20 g/l agar was used in case of plate technique for bacterial isolation. To maintain the bacterial strain 2 g/l yeast extract was added to this medium.

Fermentation medium

The growth medium contained the following: 1.5 g; KH_2PO_4, 1.0 g; K_2 HPO_4 , 2.0 g; $(NH)_2$ SO_4, 0.5 g; $MgSO_4.7H_2O$, 5 mg; $FeSO_4.4H_2O$ and 2 g, yeast extract in tap water (1 L). The medium pH was adjusted to pH 7-7.5 by NaOH (1N). DNB and AN were sterilized by filtration and were added as sole carbon sources at 1 g/L. Twenty-five milliliter (25 ml) of the growth medium was added to Erlenmeyer flasks (250 ml) on shaker at 120 rpm and 30°C for two days.

Analytical methods

Growth determination

The growth of cells was determined by measuring the absorbance at 600 nm spectrophotometrically (Shimadzu 24016), according to the methods of Puntus et al. (1997). Absorbency was converted to dry weight by using a standard curve.

Metabolite formation

Utilization of aromatic compounds by whole cells was monitored by spectrophotometrically scanning the culture medium in the UV range (Sutherland et al., 1981).

High performance liquid chromatography (HPLC)

HPLC analysis was carried out with Schimadzu HPLC LC10. The samples were analyzed by separation on Shim pack-CLCODSCM (4.6 x 150 nm) using methanol as the mobile phase, at a flow rate of 1 ml/min at room temperature. DNA, aniline and fermentation products eluted out were detected with a UV detector at 254 nm.

Extraction of enzyme

Exponentially, growing cells were harvested by centrifugation at 5000 rpm for 20 min under cooling at 10°C. The cells were washed in 20 ml of chilled 20 mM Tris-HCl buffer, pH 7.5 and centrifuged as usual. The pellets were re-suspended in 5 ml of the same buffer and frozen. The frozen cell suspensions were thawed and sonically disrupted for 7 min with Gallen Kamp (24 Amp) Germany. The probe was pre-chilled and samples cooled in water during sonic treatment. The extract was centrifuged at 5000 rpm for 20 min.

Total proteins in the clear dark yellow cell-free extracts were determined by the method of Lowry et al. (1951). Bovine serum albumin (Sigma, Fraction V) was used as a standard.

Enzyme assays

Catechol 2,3-dioxygenase activity

Reaction mixtures (3 ml) in 50 mm KH_2PO4:K_2HPO_4 buffer pH 7.2 containing 1 mmol catechol were equilibrated at 55°C before adding the cell extract (100 ml). The increase in absorbance at 375 nm caused by the formations of the reaction product 2-hydroxymuconic semialdehyde was monitored. (Asturias and Timmis, 1993).

Catechol 1,2-dioxygenase activity

Reaction conditions were identical to those of the catechol-2,3-dioxygenase assay except the formation of cis, cis-muconic acid which was monitored at 260 nm (Dorn and Knackmuss, 1978). One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of product per min in 1 ml reaction mixture. Specific activities were expressed as units per mg of protein.

RESULTS AND DISCUSSION

Isolation of the microorganism from the soil

Different carbon sources such as phenol (Ph), bromobenzene (BrB), salicylate (Sal), benzoate (Ben), gallic acid (GaA), aniline (AN) and DNB at concentration of 1 g/l each were added to agar MSM at 30°C. It was found that the isolated strains assimilate Ph, BrB, Sal,

Table 1. Taxonomical studies of strain.

No.	Cell morphology
A.	**Short rods-** occurring in pairs, also singly and irregular masses. Gram-positive. Non-motile . Non- sporulating, Not acid-fast.
B.	**Cultural profiles**
1	Nutrient agar colony: moderate growth, circular , smooth, slightly elevated
2	Nutrient agar slant : moderate growth , filiform , opaque
3	Nutrient broth: slightly to moderately turbid, flocculent sediment.
C.	**Physiological characteristics**
1	Optimum temperature, 25 to 37°C, faint growth at 45°C.
2	Optimum pH 7-8; range 6-9
3-	Not heat tolerant
4-	Aerobic to facultative anaerobic
5	Gelatin not liquefied.
6	Litmus milk: no change or slightly alkaline
7	Starch not hydrolyzed.
8	Fermentative glucose
9	Casein not dissimilated
10	Catalase: positive
11	Oxidase: positive
12	Phosphatase: negative
13	Methylene blue reduced

Ben, GaA, AN and DNB. Good growth was found in the case of AN and DNB as carbon source.

Taxonomical studies of the strain

The results of taxonomical studies are shown in Table 1. Based on the tests used in the 7th edition of " Bergery's Manual of Determinative bacteriology," and Lechevaller et al. (1980), the strain was identified as *Corynebacterium* sp.

Degradation of DNB or AN in shaking flasks

1 g/L of DNB or AN was added as sole carbon source to the growth medium; control flask with each carbon (DNB or AN) was uesd without inoculum. Both growth and degradation of DNB or AN were followed spectrophotometerically and HPLC respectively, in addition to pH change through the growth period (6 days), as shown in Figures 1 and 2. It is noticeable that, after a fermentation period of 48 h the color of medium of DNB became deep yellow. Both DNB and AN media became brown after 72 h; then it became dark brown after 6 days. No changes were remarked in control flask. Conversion of DNB and AN to brown coloured oxygenated products was reported by Parris (1980). Polymerization of highly reactive catechol or proto-catechute was reported also by Loidl et al. (1990) and Bachofer et al. (1975); it may give a reason for the accumulation of the brownish product.

From Figures 1 and 2, it was found that, in the exponential phase *Corynebacterium* sp. was active in the degradation of DNB and AN and it reached the stationary phase after 72 h. Within 24 h, about 50% of DNB and 30% of AN were degraded. Then they were decreased to a minimum concentration after 6 days. In case of DNB degradation by *Corynebacterium* sp, the pH values were decreased from 7.5 to 6.57. This may be due to the presence of acidic oxygenated products while the pH of aniline degradation was found to be between pH 7 and 7.4. Similar study was done by Yanase et al. (1992) and Liu et al. (2002) who mentioned that phenol was degraded optimally at pH 8.

Effect of yeast extract on the degradation of AN and DNB

Yeast extract contains important growth factors for the growth of the bacterium and nitrogen source. Different concentrations of yeast extract (0 to 5 g/l) were tested for studying their effects on DNB and AN degradation as shown in Figures 3 and 4. After 48 h, the cells growth was determined spectrophotomatrically, while the residual and products were determined by HPLC. At concentration of 2-5 g/l yeast extract, good growth of *Corynebacterium* sp. was determined, in addition to the degradation of DNB and AN; and the intermediate protocatechuic acid formation increased.

Optimum concentration of AN and DNB for their degradation

Different concentrations of aniline or DNB ranged from

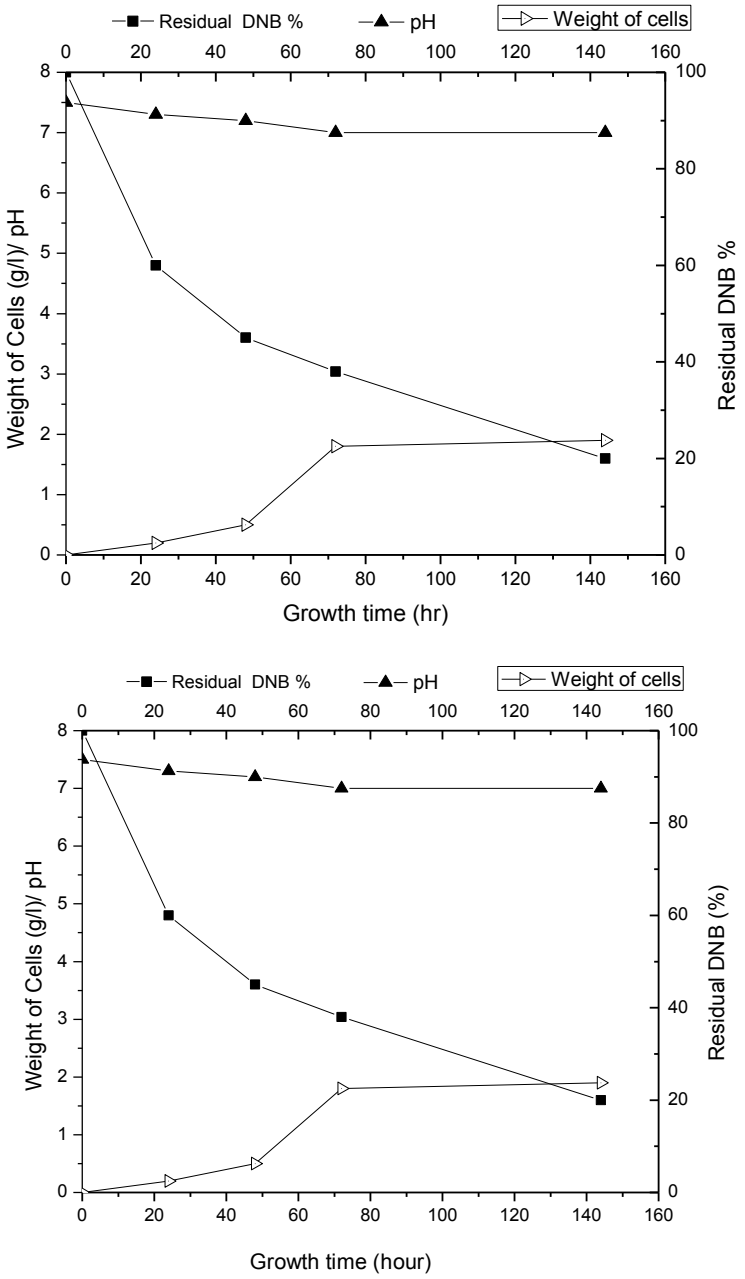

Figure 1. The relationship between the *Corynebacterium* sp. growth time and DNB degradation.

0.5 to 5 g/l as sole carbon sources were tested. The products were determined after 48 h by HPLC as shown in Figures 5 and 6. At concentration of 2.5-5 g/l of AN or DNB, no significance changes in their concentrations were detected in addition to their inhibition in the cells growth. Good growth was observed at concentrations from 0.5 to 1 g/l of AN and DNB as shown in Figures 5 and 6; maximum concentration of protocatechuic acid of 80 and 99% could be detected at concentration of 0.5 g/l of AN and DNB, respectively.

Ring cleavage enzyme

In order to find the initial pathway for degradation of DNB and AN by *Corynebacteria* sp. two key catalyzing enzymes, catechol 2,3 dioxygenase and catechol 1,2-dioxygenase, were assayed by using chatechol and protocatechuic acid as substrates.

Table 2 shows the specific activities of the enzymes extracted from the different kinds of cells grown with AN, DNB or glucose as the only carbon source in the medium.

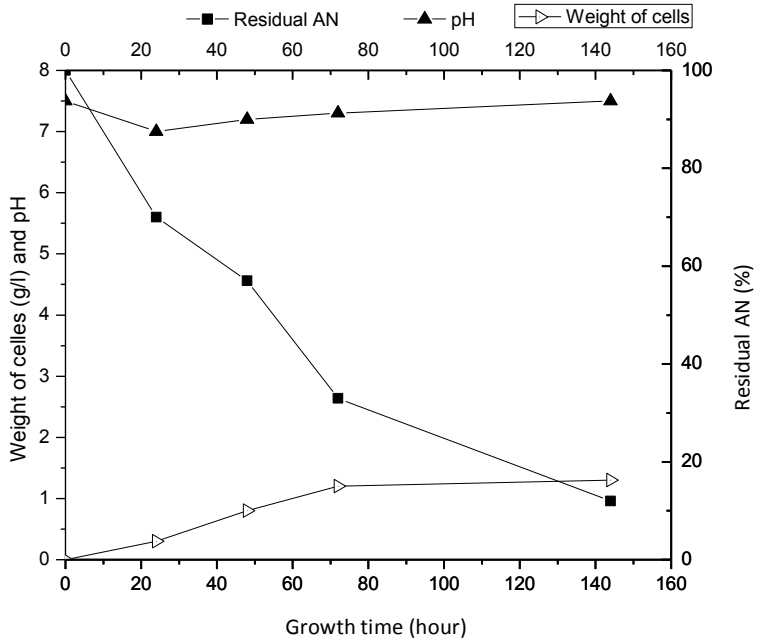

Figure 2. The relationship between the *Corynebacterium* sp. growth time and aniline degradation.

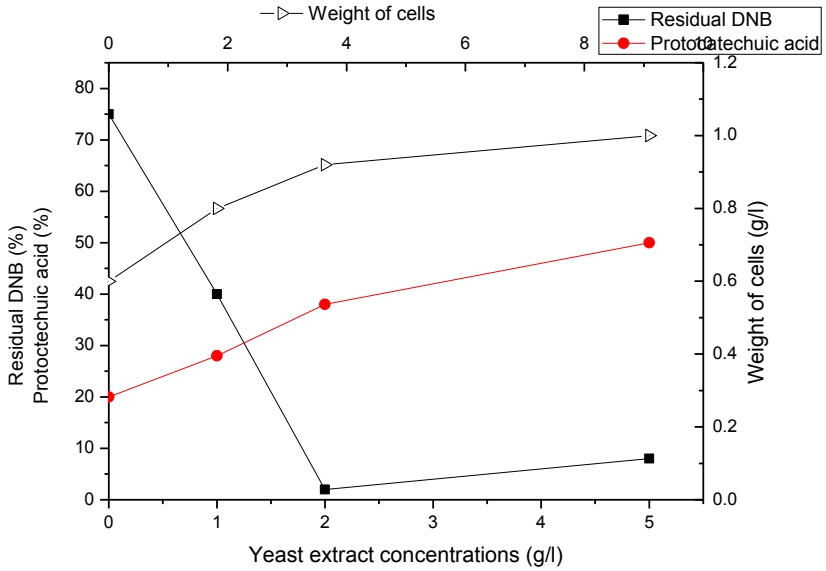

Figure 3. Effect of yeast extract concentration on the DNB degradation.

The cells grown on both AN and DNB showed high catechol 1,2 dioxygenase activity while no activity of catechol 2,3 dioxygenase was detected. Significant activity of catechol 1,2-dioxygenase was found only in the cells grown on medium containing AN or DNB. These results suggest that an *ortho* cleavage pathway may be present in this strain. Contrary to our results, Bae et al. (1996) and Liu et al. (2002) found that the degradation of aniline goes through *meta* cleavage; also, Mulla et al. (2011) found that *Micrococcus* sp. strain SMN-1 degraded 2-nitrotoluene through 3-methylcatechol by a meta-cleavage pathway, with release of nitrite. The results are in agreement with the results of Loidl et al. (1990) and Sutherland et al. (1981) who found that aniline

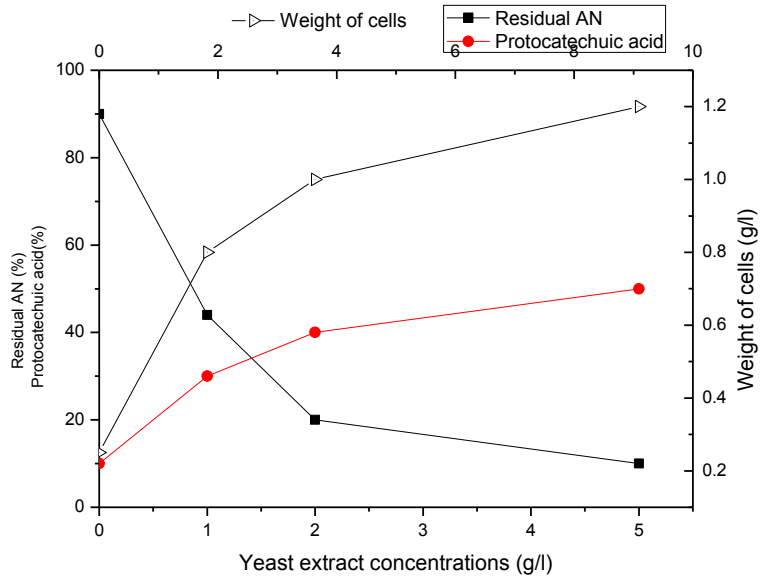

Figure 4. Effect of yeast extract concentration on the aniline degradation.

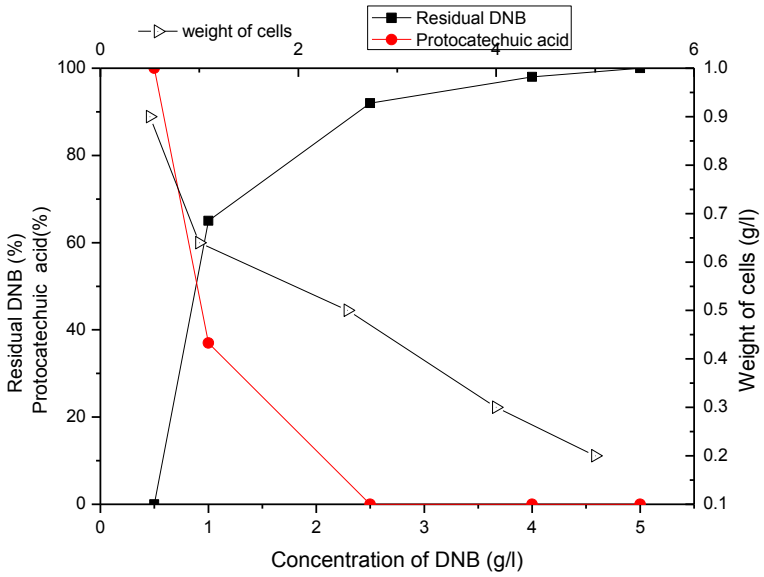

Figure 5. Effect of different DNB concentrations on the *Corynebacteium* sp. growth and protocatechuic acid production.

and benzoic were degraded via *ortho* pathway.

A new UV spectrum at 257 nm which may be β-carboxy -muconate was detected in enzyme reaction products of protocatechuic acid as substrate with catechol 1,2-dioxygenase enzyme (Ornston and Stanier, 1966). Then, the isolated strain *Corynebacterium* sp converted β-carboxy-muconate to β-ketoadipate which subsequently was cleaved to succinate and acetyl COA (Ornston and Stanier, 1966).

Conclusion

A *Corynebacterium* sp, which could utilize dinitrobenzoic acid and aniline as the sole carbon source, was isolated from the soil. The growth of this organism was optimum at a concentration of 0.5 to 1 g/l of either aniline or DNB where protocatechuic acid appeared in appreciable amounts in the media. The optimum concentration of yeast extract was 2 g/l. Catechol 1,2-dioxygenase was

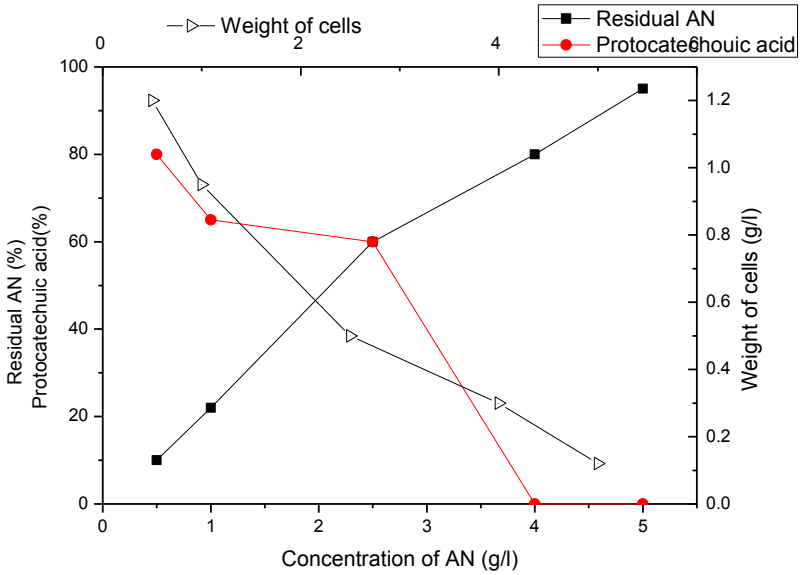

Figure 6. Effect of different aniline concentrations on the *Corynebacteium* sp. growth and protocatechuic acid production.

Table 2. Specific enzyme activities in cell extracts of *Corynebacterium* sp. growen in DNB or aniline.

Enzyme assayed	Substrate	Grown substrate		
		Glucose (U/mg)	DNB (U/mg)	Aniline (U/mg)
		Specific activities*		
Chatechol 2,3-dioxygenase	Chatechol	0	0	0.04
	Protocatechute	0.01	0	0.01
Chatechol 1,2-dioxygenase	Chatechol	0.02	67.0	48.0
	Protocatechute	0.0	50.0	37.0

Specific activities: Units per gram protein (U/mg).

found to be inducible enzyme, in cells grown aniline or DNB. These results indicate that the degradation of aniline or DNB was effected via *ortho* cleavage pathway.

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