Original Article
Using 3,4-dichloro benzoic acid as growth substrate by *Brevibacterium* spp: Effect of some growth conditions

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Abstract: *Brevibacterium* spp.that was already isolated from wastewater treatment plant of Petra City, Jordan. While 1 and 2 mM of 3,4-dichloro benzoic acid (3,4-DCBA) were used, the biodegradation rate was 14.5 and 21.25 µM/h, respectively, and the remaining was 58% for both. The remaining ratio to the quantity of 3,4-DCBA used was taken in consideration, as a result, 1 mM concentration of 3,4-DCBA substrate was applied for the rest of experiments because of the remaining amount is 0.58 mM 3,4-DCBA while 1.15 mM remained from 2mM concentration used. Thereafter, *Brevibacterium* spp was grown in batch cultures using M9 minimal media plus 1 mM concentrations of 3,4-DCBA as carbon and energy source. 3,4-DCBA degradation was optimally achieved at a 37 ºC incubation temperature, a pH of 7.0 and an agitation rate of 150 rpm. During carbon starvation, the increased biodegradation potential of *Brevibacterium* spp. was accompanied by a lessening in the acclimatization period besides enhancement of the biodegradation completion of 3,4-DCBA. The adaptation to 3,4-DCBA not only increased the degradation rate but also managed to reduce the time required for complete 3,4-DCBA degradation from 80 to nearly 40 hours. In conclusion, the acclimatized period, and carbon starvation and other optimal conditions were key factors for the enhancement of the degradation ability of 3,4-DCBA by *Brevibacterium* spp.

INTRODUCTION

The consumption of organic chemicals as carbon or energy sources by microbial cells is basic to all forms of life. Adaptation of microorganisms for long time to ingest the natural biochemical compounds found on earth is the commonly accepted story; however, the significant diverse produced organic species by man have caused environmental problems, due to resistance or complete recalcitrance to mineralization by any living species (Caplan, 1993; Leonard and Lindley, 1998; Ampe et al., 1998; Khleifat, 2006). Although bacteria are sensitive to some plant extracts, even those that produce antibiotics, they also have inverse biological relationship with heavy metals biosorption and biodegradation of toxic organic compounds such as phenol and derivatives (Reardon et al., 2002; Khleifat and Abboud, 2003; Khleifat et al., 2006a and b; Khleifat et al., 2009; Khleifat et al., 2010; Tarawneh et al., 2011; Zeidan et al., 2013; Khleifat et al., 2014; Majali et al., 2015; Khleifat et al., 2015; Althunibat et al., 2016). The substituted chlorobenzoic acid compounds are common precursors. These materials including phenol are waste by-products in the manufacture of industrial and agricultural products (Neumann et al., 2004). It can be toxic to some aquatic species at concentrations in the

low mg/mL range and causes taste and odour problems in drinking water at far lower concentrations (Rittmann and McCarty, 2001; Jiang et al., 2002). The high-volume use of these compounds in the United States and their potential toxicity has led the U.S. Environmental Protection Agency to include them on its list of priority pollutants (Khleifat, 2006). The environmental cleanup of such compounds by adsorption, solvent extraction, chemical oxidation, incineration and a biotic treatment procedure suffers from serious drawbacks such as economic issues and the production of hazardous byproducts (Loh et al., 2000). Biodegradation is generally preferred, due to lower costs and the complete mineralization. There is much controversy over whether to use natural or genetically engineered micro-organisms (GEM) in biodegradation. Government agencies are mostly unwilling to release GEMs into the environment due to the potential unforeseen ecological impact (Lal and Khanna, 1996; Beshay et al., 2002; Lob and Tar, 2000). There is considerable interest in the isolation of microbes, which are able to thrive on high concentrations of aromatic compounds (Loh et al., 2000; Bastos et al., 2000; Hill et al., 1996), such as the 3,4-DCBA compound studied here. In contrast, utilization by *Brevibacterium* spp of 3,4-DCBA

compound as the sole carbon and energy source has not been reported. It has been reported that the mode of catechol degradation, nutrient availability (C and N sources), the presence of toxins and physical parameters (i.e., temperature) could affect the growth of bacteria on phenol

Brevibacterium spp. are short coryneforms isolated from milk and dairy products and are known colonizers of human skin (Funke and Carlotti, 1994). They have been identified in environmental dust in schools, daycare centers, and animal sheds. Brevibacteria show biphasic morphology on culture, with young colonies demonstrating typical coryneform features (Eppert et al., 1997). As colonies age, the organisms mature into cocci or a coccobacillary appearance. Brevibacteria have been implicated in causing human foot odor when confining footwear results in a moist environment. Only a few species of *Brevibacterium* have been noted to cause infection (Gruner et al., 1993; Eppert et al., 1997). In this study, the degradation of phenol by *Brevibacterium* spp. was investigated for the first time through applying different growth conditions, including carbon starvation, pH, incubation temperature and aeration/agitation rate.

Materials and Methods
Bacterial Strains
The bacterium used in these experiments was *Brevibacterium* spp.that was already isolated from wastewater treatment plant of Petra City, which is located in southern Jordan. Its biochemical identity was re-verified using the REMEL kit (RapID™ ONE and RapID™ NF Plus systems) procedure as well as the morphological characteristics which were always microscopically checked.

Media and culture conditions
Cells were grown at 37 °C in nutrient broth (NB) for enzyme assay or in a minimal salt medium (MSM) for all 3,4-DCBA degradation experiments (Khleifat, 2007b; Khleifat et al., 2007). NB consisted of 1.0 g meat extract, 2.0 g yeast extract, 5.0 g peptone and 5.0 g sodium chloride per liter of solution. The MSM contained K_2HPO4 (10 mM), NaH_2PO4 (3 mM), $(NH_4)2SO4$ (10 mM), and $MgSO_4$ (1 mM). Trace elements were added to MSM to give the following final concentrations (in milligrams per liter): $CaSO_4$, 2; $FeSO_4$ 7H20, 2; $MnSO_4.H_2O$, 0.2; $CuSO_4$, 0.2; $ZnSO4.7H_2O$, 0.2; $CoSO4. 7H_2O$, 0.1; $NaMoO_4.2H20$, 0.1; H_3BO_3, 0.05.

Analytical procedures
Full wavelength scan (200-700 nm) was carried out by Perkin Elmer UV//Vis Spectrophotometer Lambda 25, USA. The λ max was detected at 235 nm (Marks et al., 1984; Yun et al., 2007). Thus, 3,4-DCBA concentration in culture medium was determined spectrophotometrically by monitoring absorbance at 235 nm. Cells were grown in MSM containing 2, 3

and 4 mM 3,4-DCBA. Samples (1ml) were removed at different time intervals for absorbance and inorganic chloride determinations. To check the growth kinetics by making growth curves at 2, 3 and 4 mM 3,4-DCBA concentrations, the same initial cell mass (starting inocula) for the three concentrations was taken into consideration, as mentioned above. Samples were then taken at time intervals and assayed for their cell mass OD_{600}.

The average initial degradation rates of 3,4-DCBA were measured by dividing the net amount of transformed 3,4-DCBA for 40 h, since within this time period many cells showed no further degradation, or it represented the corresponding elapsed time for all experiments conducted. The reason for calculating the average degradation by this method as suggested previously (Loh and Wang, 1997) to avoid any errors caused by different lengths of lag phases, and the difficulty in ascertaining the time required to achieve complete degradation or when the degradation had stopped.

Chloride determination.
Chloride release measurement was made as described previously (Hickey and Focht, 1990; Urgun-Demirtas et al., 2003). Inorganic chloride was determined turbidimetrically by measuring AgCl precipitation. Samples (1 ml) were acidified with 10 ,ll of 10 N H_2SO4 and centrifuged (5 min at 1,500 x g) to remove material that precipitated due to acidification alone. Each sample and standard was zeroed against itself at 525 nm on a Genesis 2 spectrophotometer to minimize background variation. Precipitation of AgCl was then measured by adding 10 ,ul of 0.1 M $AgNO_3$ (in 5 M H_3PO4) and immediately reading the sample A525. Chloride was quantified by reference to a standard curve that was linear from 0.1 to 3.2 mM. Blanks, consisting of MSM alone, were free of interferences due to precipitation of medium components.

Effect of 3,4-DCBA concentration, pH, incubation temperature and agitation rate on the 3,4-DCBA biodegradation ability
The effect of the different substrate concentrations (1, 2, and 3 mM) on the 3,4-DCBA degradation by *Brevibacterium* spp.cells was examined. The growth medium was an MSM medium incubated at 37 °C, under a 150 rpm shaking rate and a pH of 7.0. Different pHs (6, 6.5, 7, 7.5 and 8) of the growth media was used to assess the effect of variations in pH on the degradation ability of 3,4-DCBA by the same bacterium (Aljundi et al., 2010). The effect of different incubation temperatures (25, 30 and 37 °C) upon the percentage degradation of 3,4-DCBA by *Brevibacterium* spp.cells was investigated. The medium used was an MSM medium.

Enzyme assay
Brevibacterium spp.*cells* were grown on the nutrient broth medium (NB) plus 1 mM, as described above, to

the mid-log phase of their growth. The cells were harvested by centrifugation and kept at minus 20 ºC for next use. Then, the cells were suspended in a 5-ml potassium phosphate buffer, with a pH of 7.5, and discontinuously sonicated for 2 min (20 and 40 s in ice). The cell extract was centrifuged at 10,000 rpm for 20 min at 4 °C. The activity of catechol 1,2- and 2,3-dioxygenases was assayed as described by previous method (Banta and Kahlon, 2007). The concentration of the reaction products *cis-cis*-muconic acid and 2-hydroxymuconic semialdehyde was measured spectrophotometrically at 260 and 375 nm, respectively. The protein concentrations were estimated by the method developed by Lowry et al. (1951).

RESULTA AND DISCCUSSIONS

In this study, the bacterium *Brevibacterium Group B* was isolated from the wastewater treatment plant of Petra City, which is located in southern Jordan. Its identification was detected using morphological and biochemical characteristics as described previously (Gruner et al., 1993; Eppert et al., 1997). Four substituted chlorobenzoic acid compounds were tested for their biodegradability by this bacterium. These compounds include 2-chlorobenzoic acid (2-CBA), 3-chlorobenzoic acid (3-CBA), 4-chlorobenzoic acid (4-CBA) and 3,4-dichlorobenzoic acid compounds (3,4-DCBA). The degradation ability was checked through the release of chloride, disappearance of the substrate and finally the growth of bacterial cells on that substrate (Banta and Kahlon, 2007). *Brevibacterium* Group B was capable of using only 3,4-CBA compound as sole source of carbon and energy. To initiate the usability of these bacteria for organic compounds as carbon sources, a minimal salt medium was practiced that had this organic compound as sole carbon sources. Thus, the character of breakdown of

these substrates is the direct rise in cell growth measured as biomass (Khleifat 2006a-d). A negative controls was used to test the biodegradability of CBAs, the uninoculated culture, there was no biodegradation activity shown validating the biodegrading activity made by *Brevibacterium* Group B cells.

Effect of Substrate Degradation

To study the 3,4-CBA degradation, bacterial growth and Cl release as a function of time, two concentrations were used; 1 and 2 mM (Figure 1). When 1 and 2 mM were used, the biodegradation rate was 14.5 and 21.25 µM/h, respectively, and the remaining 58% for both. If the remaining ratio to the quantity used was taken into consideration, then 1 mM is better because of the remaining amount is 0.58 mM while 1.15 mM was remained from 2mM concentration used. Thefore, 1 mM concentration of 3,4-DCBA substrate was applied for the rest of experiments. In contrast, the growth reached the stationary phase after 40 h time of incubation. Also, the chlorine release was increased in parallel with the extent of 3,4-CBA reduction up to the end of experiment (data not shown). The degradation rates of 3,4-CBAs during the study were measured by dividing the remaining amount of converted 3,4-CBAs for 40 h, since in this time age many cells exhibited no more degradation, or it exemplified the matching elapsed time for all experiments made. The reason for computing the average of degradation via this procedure as suggested before (Loh and Wang, 1997; Khleifat, 2006a-d) to escape any faults produced by various extents of lag stages, and the exertion in determining the essential period for achieving complete degradation or after the degradation had stopped.
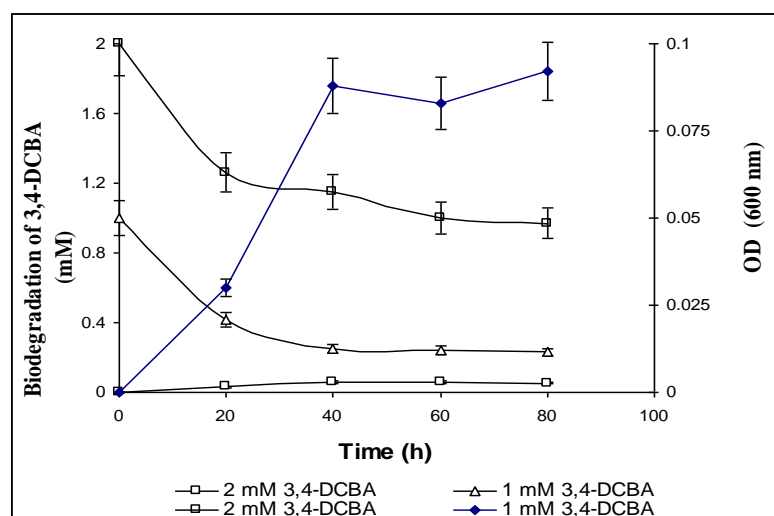

Figure 1. Biodegradation of 3,4-DCB by Brevibacterium Group B cells and simultaneous of their growth using 3,4-DCBA as substrate. Error bars indicate standard deviations; where not visible they are smaller than the diameters of the point.
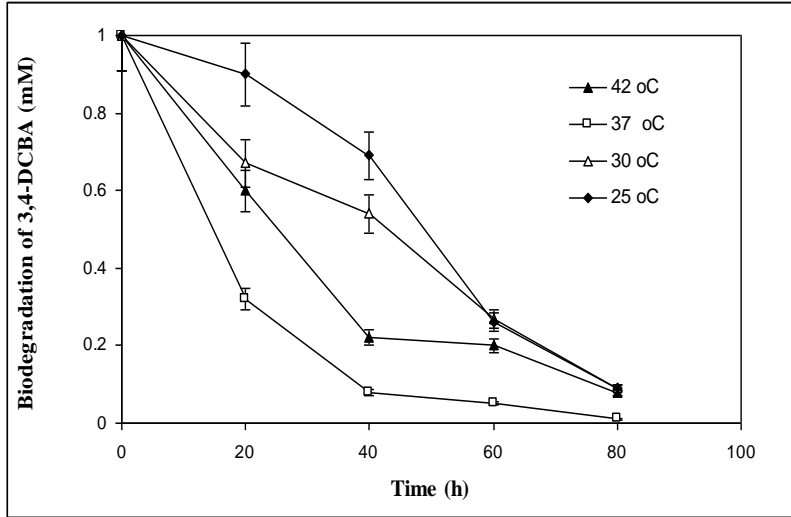
Figure 2. Effect of incubation temperature on the degradation rates of 3,4-CBA by *Brevibacterium* Group B cells. Error bars indicate standard deviations; where not visible they are smaller than the diameters of the points.
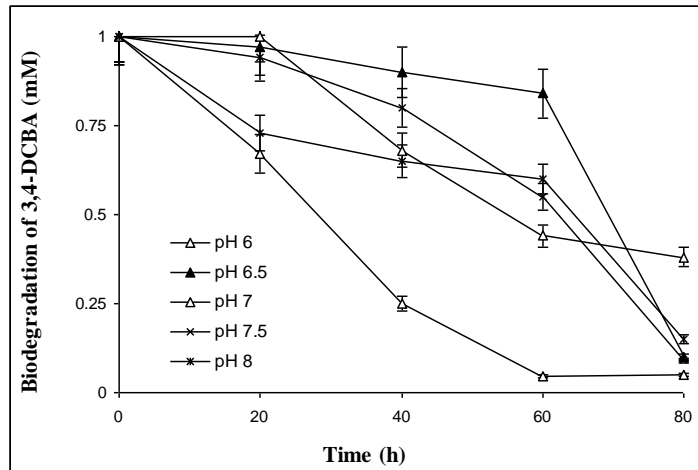

Figure 3. Effect of pH on the degradation rates of 3,4-CBA by *Brevibacterium* Group B cells. Error bars indicate standard deviations; where not visible they are smaller than the diameters of the points.
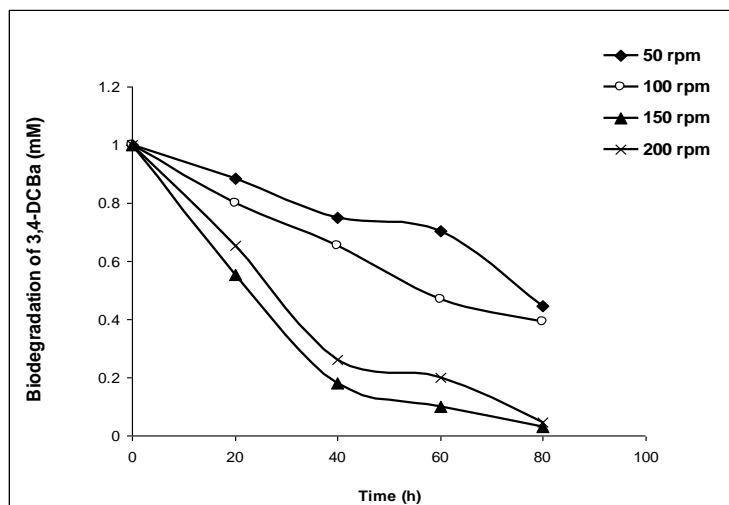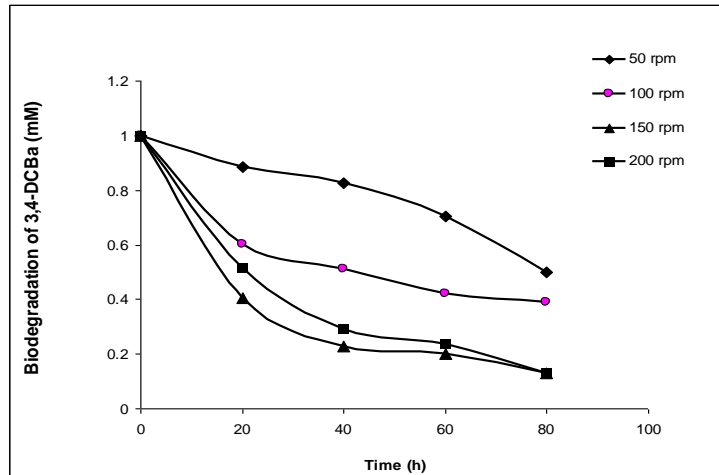

Figure 4. The effect of the agitation rate on the Biodegradation of 3,4-DCBA CBA by *Brevibacterium* Group B cells.

Table 1. Biodegradation rate (µM/h) for 3,4-CBA by carbon starved *Brevibacterium* Group B cells and 3,4-CBA pre-adapted cells.

Control		14.5 µM/h
Starvation	24 h	12 µM/h
	48 h	22.5 µM/h
Adaptation	24 h	16.5 µM/h
	48 h	21.0 µM/h

Figure 5. The effect of the agitation rate on the biodegradation of 3,4-DCBA By *Brevibacterium* Group B cells (with glucose).

Effect of Incubation Temperature

The experimental data on the degradation percentage at different incubation temperature; 25, 30, 37 and 42 °C showed almost similar results when using glucose-free (Figure 2) and glucose added media (data not shown). The incubation temperature of 37 ᵒC was the optimum for achieving cell biomass, chloride production and the initial degradation rate. For example, when culture had no glucose-added, the degradation rates for 3,4-CBA were 18, 6, 33 and 23 µM/h for those grown at 25, 30, 37 and 42°C incubation temperatures, respectively. When cells had glucose in the media (data not shown) the degradation rates for 3,4-CBA were 8, 6.5, 30 and 20 µM/h for those grown at 25, 30, 37 and 42°C incubation temperatures, respectively. However, the glucose caused the growth to be raised by a bout 7 fold (data not shown) more than those grown in glucose-free media (Figure 1)   As a result of glucose being included, the chlorine release started late (after 20 h) of incubation as compared with that of glucose free media.

The temperature turn out to be crucial above 25 °C. The effect of temperature was similarly occurred on the initial degradation rate and its percentage (Figures 2), chlorine production as well as the cell mass. Thus, it seems that biodegradation of 3,4-CBA could occur optimally at room temperature for *Brevibacterium* Group B cells. Temperature visibly had a physiologically prevailing effect on the result the 3,4-CBA biodegradation, as the 37 °C generated the best conditions for their consuming, or this possibly, the outcome of the incubation temperature could control the activities of 3,4-CBA degrading enzyme(s) (Leven

and Schnürer, 2005). It has been reported that the temperature could take part in an equivalent or better role than nutrient accessibility in the degradation of benzoic acid and phenol (Margesin and Schinner, 1997).

Effect of pH of Culture Media on Biodegradation of 3,4-CBA

The percentages of 3,4-CBA degradation brought about by *Brevibacterium* Group B cells under different pH levels are shown for glucose-free (Figure 3) and glucose-containing *Brevibacterium* Group B cultures (data not shown). It is shown that *Brevibacterium* Group B with higher favorability degrades the 3,4-CBA at a pH of 7.0. This favorability was same for the glucose-free and glucose containing culture media. The pH 7 was considered as a control experiment. However, the pH effect on the other two cases; growth biomass and chlorine was different in being the best pH was 8. It is possible that the enzymes for 3,4-CBA degradation have their optimum enzymatic activities at pH 7.0. It was reported that optimum pH for the biodegradation of different aromatic compounds was different from one bacterium to another, for example, pH varies between 8 and 11 for phenol and catechol biodegradation by *Halomonas* campisalis (Alva and Peyton, 2003) and the pH of 6.8 for phenol biodegradation by *Klebsiella oxytoca,* (Shawabkeh et al., 2007; Khleifat et al., 2008).

Effect of Shaking Rate

To test the effect of agitation rates on the ability of *Brevibacterium* Group B cells to degrade 3,4-CBA, the MSM medium was used, as mentioned previously, to grow bacterial cells at 37 °C under four different agitation rates: 50, 100, 150 and 200 rpm. Figures 4 show the effect of the aeration/agitation rate on the 3,4-CBA degradation by *Brevibacterium* Group B in glucose-free (Figures 4) and glucose-containing (Figure 5) MSM media. An increase in agitation rate up to 150 rpm resulted in a gradual elevation in the degradation rate. Further increasing the agitation rate above 150 rpm did not result in further improvement of the degrading ability. Therefore, a rate of 150 rpm was selected for the experiments. In addition the 3,4-CBA biodegradation and Cl production were better for cells grown in the glucose-free MSM media than those in glucose-containing MSM media.

In general, the biodegradation of 3,4-CBA under optimum conditions was better when the MSM media had no glucose added (Figure 4) than that when cells grown in glucose containing MSM media (Figure 5).

Effect of Carbon Starvation on 3,4-CBA degradation

After the optimization of growth conditions, effect of carbon-starvation on the 3,4-CBA degradation was investigated. One mM of 3,4-CBA degradation by carbon-starved *Brevibacterium* Group B cells for 48 h exhibited significantly higher initial degradation rate (22.5 µM/h) than that of 24 h-carbon-starved (12 µM/h) and non-starved cells (14.5 µM/h). This possibly, consequences from an initial expression of the 3,4-CBA catabolic genes (Matin et al., 1999; Reardon et al., 2002; Khleifat, 2006c). This hypothesis, probably is true as long as the growth of this bacterium in the log phase was clearly faster than that of the non-starved cells, based on the OD_{600nm} (data not shown). Carbon starvation in *E. coli* particularly, induces the expression of peptide transporter protein (carbon starvation protein A, *CstA*), even in the nonexistence of their relevant inducers or as a result of the induction of required degradative enzymes (Matin et al., 1999; Reardon et al., 2002). Previously, it was reported that carbon starvation response of non-differentiating Gram-negative bacteria is an increase in their ability to catabolize and scavenge nutrients from the environment (Matin et al., 1999).

Effect of Substrate Adaptation

To keep away from the substrate inhibition, substrate adaptation to high 3,4-CBA concentration by *Brevibacterium* Group B cells is one of the practices which were used to take care of aromatic compounds in wastewater (Lob and Tar, 2000). To compare the biodegradation of 3,4-CBA by both 3,4-CBA pre-adapted and non-adapted *Brevibacterium* Group B cells, the two experiments were done under similar conditions (Table 1). The 48h-adapted cells degraded the 3,4-CBA quicker (21µM/h) than the 24 h-adapted (16.5µM/h) as compared with the non-adapted cells (14.5µM/h). The time taken to achieve the initial degradation rates (21µM/h, 16.5µM/h and 14.5µM/h) for 48h-adapted, 24h-adapted and non-adapted, respectively was unchanged. Probably, this was due to the higher cell density resulting from simultaneous utilization of 3,4-CBA. It was reported that acclimatization treatment would increase the degradation efficiency (Zilli et al., 1993). The microorganisms are able to adapt to the presence of toxic organic compounds by using a series of metabolic modifications in the approaches such as adjustment in lipid composition of cell membranes (Neumann, et al., 2004; Khleifat et al., 2019; Qaralleh et al., 2019) to compensate for the CBA induced increase in membrane fluidity (Yap et al., 1999; Khleifat, 2007a).

Detection of Dioxygenase Activity

Chloride ions release was because of the oxygenase enzyme activities including more or less specific dioxygenases, for the halogenated compounds (Yun et al., 2007). In the course of our experiments, the chloride concentrations, was released as chloride ions. There was no delayed time (lag) was shown between 3,4-CBA compound degradation and chloride ion release**.** During the biodegradation of such compounds, two possible metabolic pathways were reported (Yun et al., 2007, Dorn and Knackmuss, 1978; Häggblom, 1992). The 1st one is catechol 2,3-dioxygenase activity, which was responded for extradiol cleavage of the meta-pathway in microorganisms that was detected in this experiments, but under low specific activity as compared with that of 1,2-dioxygenase . However the 2nd one is ortho (intradiol) cleavage catechol 1,2-dioxygenase activities which was detected here for 3,4-CBA investigated with higher extents than the 2,3-dioxygenase activity. 3,4-DCBA compound was added to the nutrient broth grown-cells in 0.5 mM concentration to induce the production of enzymes.

The time required to detect the maximal catechol 1,2-dioxygenase activities was shown to be 6-8 minutes (Figure 6). It is clearly shown that the 1,2-dioxygenase activity for 3,4--DCBA substrate is the choice. Meantime, the molar ratio of chlorine to the concentration of 3,4-DCBA is less than one. Ratios less than 1.0 are likely due to catabolism of 3,4-CBA by both *meta*–and ortho-cleavage pathway, (Schmidt and Knackmuss, 1980; Häggblom, 1992; Romanov and Hausinger, 1994; Arensdorf and Focht, 1994).
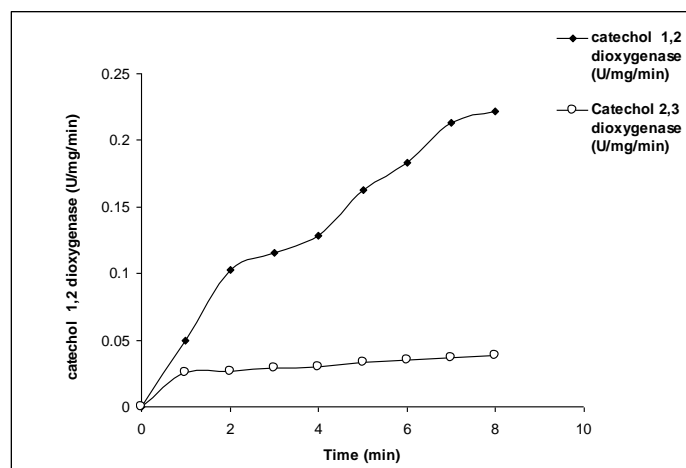
Fig. 6. Measurement of C230 (2,3 catechol dioxygenase) and C23O (2,3 catechol dioxygenase) for the meta pathway activities for the cell extracts of *Brevibacterium* Group B cells grown in nutrient broth plus 1 mM 3,4-DCBA . All data are average of three trials with error bars indicating STDEVs (σ_{n-1}).

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