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Original Article

3,4-Dichlorobenzoic acid biodegradation by the *Edwardsiella tarda*: Effect of Some Growth Conditions

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Abstract: The biodegradation of 3,4-DiChlorobenzoic acid was investigated by using *Edwardsiella tarda* and it used 3,4-DCBA as sole carbon and energy source. Several concentrations of 3,4-D CBAs (1mM, 2mM ,3mM ,4mM and 5mM) were used. The highest rate of degradation of 3,4-D CBAs was obtained at a concentration (2mM). The experiments were included substrate concentration, temperature, pH, starvation, adaptation, carbon and nitrogen sources. The degradation ability was monitored through the release of chloride disappearance of the substrate and finally the growth of bacterial cells on that substrate. The optimal temperature and pH for the bacteria were 42ºC and 7.5, respectively. Adaptation of the cells on 3,4-DCBA for 48 hours and cells starvation for 24 hours and 48 hours increasing the initial degradation rate. The carbon sources affected the 3,4 –DCBA degradation differently from that on chloride and cell mass production. Nitrogen sources supplied (yeast extract, L-proline, casein, NH4, K-Nitrate, arginine, urea and glycine). Urea and casine caused a repression in 3,4-DCBA degradation. Catechol 1,2 dioxygenase activity was found to be present in cell free extracts suggesting that 3,4-DCBA is catabolized by ortho-ring cleavage pathway.

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1. INTRODUCTION

Many synthetic organic compounds have been produced and released into the environment, especially in agriculture such as pesticides and herbicides, also in various industries, including paper, rubber, cosmetic, food and pharmaceutical industry, and in production of various preservatives paints, stabilizers, and cleaners. In many cases, their use was approved before an accurate testing of their toxicity was performed. Many of these compounds were declared harmful and their use either banned or restricted. However, some toxic chemicals have already been accumulated in many areas causing severe toxic pollution in soil and aquatic system, and this will threaten the health of humans and wildlife.(González-Gaya et al., 2019; Guo et al., 2019; Khleifat, 2006a).

A huge number of these chemicals are toxic themselves, and some are also toxic after partial degradation, as metabolic intermediates. In general, removal of these synthetic compounds from the environment is very difficult and costly. Chemical clean-up is often impossible due to the nature of the polluted site, and could introduce new polluting agents into the environment. In the last two decades, possibility to remove these undesirable compounds by biodegradation came to focus (Huang et al., 2019; Khleifat et al., 2006b; Khleifat et al., 2006c; Semprini, 1995). it was clearly observed that some toxic chemicals are rapidly degraded in certain areas. Based on the analyses of samples from these sites revealed the presence of microorganisms that were able to degrade these synthetic compounds, and even use them as sole sources of carbon and energy (Gallego et al., 2001; Jeon et al., 2003). Many field tests have been performed to determine the rate of degradation of aromatic compound from soil and water sample (Khleifat, 2006b, 2006c; Khleifat et al., 2015).

Microorganisms are challenged to develop new pathways by altering their own preexisting genetic information as a result of either mutation(s) in single structural and/or regulatory genes or perhaps recruitment of single silent genes when they encounter the foreign compounds (Benedetti et al., 2016; Díaz and Prieto, 2000). A huge number of bacterial and fungal genera possess the capability to degrade organic pollutants and they represent essential components of the global carbon cycle. In addition, it appears that most xenobiotic industrial chemicals can be degraded by microorganisms (Khleifat, 2010), either by a combination of co metabolic steps, often yielding partial degradation, or by serving as growth substrate which is

accompanied by mineralization of at least part of the molecule (Aljundi and Khleifat, 2010; Ghosal et al., 1985).

Furthermore, it has been shown repeatedly that degradation patterns of pollutants can differ from single-compound studies and this due to inhibiting effects on the aerobic biodegradation (Tiehm and Fritzsche, 1995; Shawabkeh et al., 2007; Al-Asoufi et al., 2017; Al-Limoun et al., 2019; Qaralleh et al., 2019; Zhao et al., 2010; Zeidan et al., 2013). However, co-metabolic degradation of poorly degradable compounds in the presence of growth substrates can improve overall degradation. Additionally, pH and temperature affect biodegradation , and high pollutant concentrations can result in toxic effects (Aljundi et al., 2010; Khleifat, 2007; Khleifat et al., 2019). It is worthy to be mentioned that continuously-growing culture conditions; are also an powerful factor in answering to many bacterial activities mainly physiological responses (Khleifat et al., 2003; Khleifat, 2007; Abboud et al., 2009; Qaralleh et al., 2009; Khleifat, 2006c; Khleifat, 2006d; Qaralleh et al., 2010).

Catechol is a key metabolite in the degradation of many aromatic compounds. Bacteria can degrade the aromatic ring of catechol by ortho or meta-fission. The nucleus of catechol in the case of ortho-fission is cleaved between the position C1 and C2 both bearing the hydroxyl groups by catechol 1,2-dioxygenase to yield cis-cis muconic acid (Dagley, 1977). In the other hand, The nucleus of the catechol in the case of meta-fissions cleaved between positions C2 and C3bythe action of catechol 2,3-dioxygenase to yield 2-hydroxymuconic semi aldehyde. (Aljundi and Khleifat, 2010; Sung et al., 1997; Veenagayathri and Vasudevan, 2011; Wu et al., 2018).

Edwardsiella tarda has been isolated from the wastewater treatment plant in Petra, Jordan. Petra was announced as one of the new Seven Wonders of the World and has become the perfect and best tourist destination. To best of our knowledge, no studies involving *E. tarda* have been published and this is the first study concerning the biodegradation of 3,4-DCBA compound by *E. tarda*. In fact, few studies have been conducted on microbial degradation of 3,4-DCBA compound. The objective of this study was to examine the ability of *E. tarda* bacteria in aerobic degradation of 3,4- Di Chlorobenzoic acid as single source of carbon. Biodegradation was studied under varying concentrations of 3,4-DCBA, temperatures, pH and aeration rates to obtain the optimum condition for the bacterial growth. In addition, we investigated the effect of substrate starvation and adaptation on biodegradation of 3,4-DCBA by *E. tarda* under aerobic conditions. Also, different carbon and nitrogen sources along with 3,4-DCBA were provided as the source of carbon, nitrogen and energy and its effect on the rate of degradation as a cometabolite was evaluated.

2. MATERIALS AND METHODS

2.1 Preparation of microbial culture

2.1.1 Bacterial Strains: *Edwardsiella tarda* was isolated from the wastewater treatment plant in Petra, Jordan. Its biochemical identity was verified using the REMEL kit (RapID ONE and RapID NF Plus systems) procedure as well as the morphological characteristics which were checked by microscope. for all 3,4-DCBA degradation experiments cells were grown in minimal salt media (MSM) reported previously by Aljundi et al., (2010). For enzyme assay, cells were grown at 37 °C in nutrient broth (NB) (Tarawneh et al., 2009; Al-Asoufi et al., 2017).

2.2 Analytical procedure

The concentration of 3,4-DCBA in culture medium was determined spectrophotometrically by monitoring absorbance at 235nm. Free chloride concentration was measured as described previously by (Alqudah et al., 2014).

2.3 Effect of Different Growth Conditions on the 3,4-DCBA Biodegradation Ability.

2.3.1 **Effect of 3,4-DCBA Concentration, pH, Incubation Temperature, and Agitation rate.**

Five different concentrations (1,2,3,4 and 5 mM) of 3,4-DCBA were examined. The growth medium was an MSM medium incubated at 37 °C, under a 150 rpm agitation rate and a pH of 7.0. The dependence on temperature of the rate at which the degradation process occurs is related to the metabolism of microorganisms responsible for degradation. The effect of different incubation temperatures (20,27,30,37 and 42˚ C) on 3,4-DCBA degradation by *E. tarda* was studied. Also, Different pH (6, 6.5, 7, 7.5 and 8) of the growth media were used to test the effect of variations in pH on the degradation ability of 3,4- DCBA by the same bacterium. To test the effect of agitation rates on the 3,4- DCBA degradation by *E. tarda*, five different agitation rates (50, 100, 150, 200,and 250rpm) was investigated.

2.3.2 **Effect of starvation on the 3,4-dichlorobenzoic acid biodegradation:**

Bacteria were grown on nutrient broth (NB) at 37 °C, 150 rpm and pH 7.5 to mid-log phase prior to starvation. Then, the cells were harvested by centrifugation (5000 rpm, 10 min, 4 °C), washed twice with equal volumes of sterile M9 minimal medium and suspended in the same medium. The cell suspension was immediately used as the non-starved experimental control to assess 3,4-DCBA

degradation by bacteria. A sub-sample of the cell suspension was C-starved in the M9 minimal medium at 42 °C with an agitation rate of 250 rpm and left for 24 hr and 48 hr. Then the carbon-starved bacteria in MSM were supplied with (2 mM) 3,4-DCBA and tested for their 3,4-DCBA degrading ability.

2.3.3 **Effect of adaptation on the 3,4-dichlorobenzoic acid biodegradation:**

Bacteria were inoculated into MSM minimal salt media containing (2mM) 3,4-DCBA as the sole carbon source at (250 rpm, 42°C, pH 7.5), after (48 hour) of incubation the cells were harvested by centrifugation and washed twice with equal volume of sterilized MSM minimal salt media. The same procedure was repeated twice and the harvested cells were suspended into (2mM) 3,4-DCBA containing MSM minimal salt media as adapted cells at (250 rpm, 42°C, pH 7.5). One control was used, the usual experiment (non - adapted cells). The degradation was analyzed as a function of time (Khleifat, 2007).

2.3.4 **Effects of different nitrogen source on 2-chlorobenzoic acid biodegradation:**

Several nitrogen sources were used with 0.2% of each of the following (glycine, glutamine, ammonium nitrate, potassium nitrate, arginin, casein, Lproline, urea and yeast extracts). The ammonium chloride was omitted from the M9 media as nitrogen source . The cells were then incubated at 42°C, pH 7.5 and 250 rpm .Biodegradation of 3. 4- DCBA, chloride production and growth were monitored as described previously.

2.3.5 **Effects of different carbon source on 3,4-Dchlorobenzoic acid biodegradation:**

Different carbon sources (fructose, mannitol, maltose, sucrose and succinic acid) supplied independently to MSM with 0.2% concentration. The cells were then incubated at 42°C, pH 7.5 and 250 rpm. biodegradation of 3,4-DCBA. chloride production and growth were monitored as described previously.

2.4 Enzyme Activity:

2.4.1 **Preparation of crude Extract:**

Cells were grown in 1.5 liters of nutrient broth for (24 hours), then they centrifuged at 6000 rpm for 15 min at 5˚C. The cells were disrupted by sonication at 0˚C using ice bath for 8- 10 min in 30 sec bursts. The sonicated samples were again centrifuged at 6000 rpm for 10 min at 5 ˚C. The supernatant was tested for enzyme assay and total protein estimation. The proteins in the crude extract were measured by the method described by (Lowry et al., 1951). Bovine serum albumin was used as standard (Banta and Kahlon, 2007). The procedure of pyrocatechase and metapyrocatechase experiment was described previously by (Alqudah et al., 2014).

3. RESULTS AND DISCUSSION

Edwardsiella tarda was grown in minimal medium that have 3,4- DCBA compound as a sole carbon sources. The biodegradation was tested using 5 different concentrations (1,2,3,4and5mM of 3,4-DCBA). The formation of any bacterial biomass will be a function of exhaustion of this substrates (Khleifat, 2006b). The degradation ability was monitored through the release of chloride, depletion of the substrate and finally the growth of bacterial cells on that substrate (Banta and Kahlon, 2007).

3.1 Biodegradation of 3,4-DCBA by *Edwardsiella tarda*:

Five different concentrations were used (1, 2, 3, 4 and 5mM). Using of 2 mM concentration of 3,4-DCBA more than 80% degradation was a achieved with initial degradation rate of (57 µM/h) (Fig. 1). The 87% was almost attained in 80 hours of incubation time. Using (1, 3, 4 and 5mM) concentration of 3,4-DCBA under the same conditions of previous experiments shown less degradation rate than 2Mm.

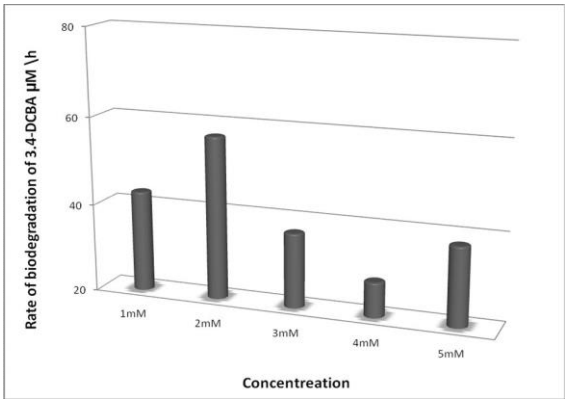

Figure 1 Initial rate of 3,4- DCBA degradation by *Edwardsiella tarda* (during 20 h) when it's grown in MSM supplemented with (1mM, 2mM, 3mM, 4mM and 5mM) of 3,4-DCBA, under 42 ºC incubation temperature, aeration rate 250 rpm and pH 7.5.

3.2 Effect of pH of Culture Media on Biodegradation of 3,4-DCBA.

The percentages of 3,4-DCBA degradation by *E. tarda* under different pH levels are shown highly percentage of degradation with 95% and this higher than the other levels of pH (Table 1). It is seen from that *E. tarda* optimally degrades the 3,4- DCBA at pH 7.5 (Fig. 2). This pH would be the optimum for maximum degradation of 3,4-DCBA by this organism. It was reported that optimum pH for the biodegradation of different aromatic compounds was different from one bacterium to another, for example, an optimum pH for biodegradation of 4-CBA by *Arthrobacter* is 6.8 (Marks et al., 1984), pH ranges between 8 and 11 for biodegradation of phenol and catechol by the bacterium *Halomonas campisalis* (Alva and Peyton, 2003) and biodegradation of phenol by *Klebsiella oxytoca*, the pH was 6.8 (Shawabkeh et al., 2007).

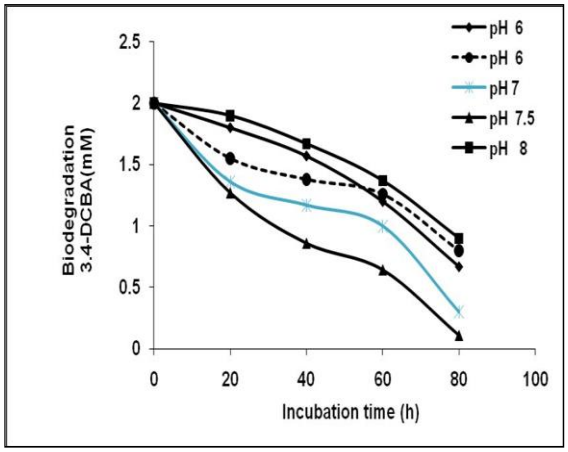

Figure 2 The effect of pH of culture media on biodegradation of 3,4- CBA of bacterial cells of *E. tarda*. The concentration of 3,4-DCBA used was (2mM) and its degradation was detected using OD230nm for 3,4-DCBA disappearance, as described in materials and methods. Cells were grown at 42 °C incubation temperature, aeration rate of 250 rpm and pH 6, 6.5, 7,7.5 and 8.

3.3 Effect of aeration rate

To assess the effect of aeration rate on 3,4-DCBA degradation ability by *E. tarda*, different aeration rate were used (50,100,150,200 and 250 rpm) (Fig.3). It is clear that the degradation process for 3,4- DCBA was achieved under all shaking rates used. When culture incubated without shaking rate no degradation ability shown at all. The interpretation for this phenomenon probably the degradation of 3,4- DCBA by this bacterium is an aerobic process even under hypoxic condition, since at zero aeration rates no degradation was observed and no significant different in the degradation ability between the higher (250 rpm) and the lower aeration (50 rpm). this slight difference in the degradation ability probably as result of increase the cell mass with further increasing the aeration rate.

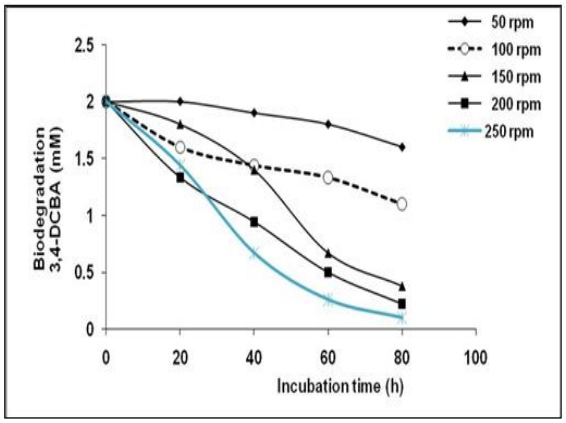

Figure 3 The Effect of aeration rate of culture media on biodegradation of 3,4- DCBA by bacterial *Edwardsiella tarda*. The concentration of 3,4-DCBA used was (2mM) and its degradation was detected using OD 230nm for 3,4- DCBA disappearance. Cells were grown at 42 °C incubation temperature, pH 7.5 and aeration rate 50, 100, 150, 200 and 250 rpm.

3.4 Effect of Substrate Starvation on biodegradation of 3,4-DCBA:

To prove that the starvation of the bacteria has the ability to stimulate the bacteria on the degradation of 3,4 –DCBA. The experiment on starving the bacteria for 24 hours and 48 hours on minimal salts medium. Then transferred
to minimal media with 3,4 – DCBA. The result show increase of degradation of 3,4 –DCBA, with greater the period of starvation (Fig.4). The percentage of degradation rate (Table 1) shown highly percentage degradation at 48h starvation than 24h starvation. Cells that were starved for 48 h degrade 3,4 –DCBA slightly quicker (58µM\h) than the cells that were starved for 24 h and the cell that not was starved (50µM\h)(49µM\h).respectively (Fig. 5).In general that the starvation of bacteria for periods of more than ability of the bacteria on the degradation of toxic compound as 3,4 –DCBA.

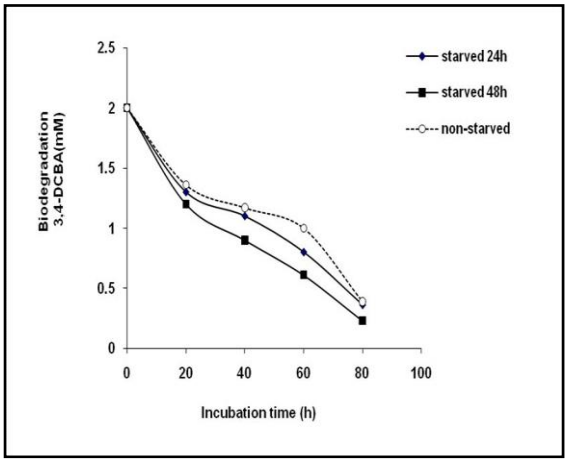

Figure 4 The effect starvation on the 3,4DCBA degradation by Edwardsiella tarda when it's grown on MSM for 24h and 48h,under 42 ºC incubation temperature, aeration rate of 250 rpm and pH 7.5 .

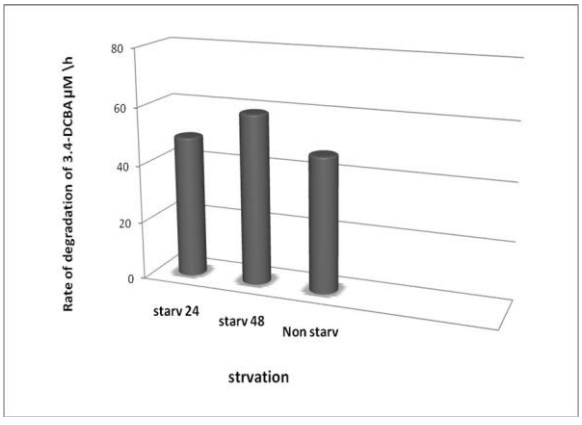

Figure 5 Initial rate of 3,4- DCBA degradation of starvation at 24h and 48h by *Edwardsiella tarda* when it's grown in MSM supplemented with (2mM) of 3,4- DCBA, under 42ºC incubation temperature, aeration rate 250 rpm and pH 7.5.

Table 1 Degradation percentage of 3,4 DCBA at Different Starvation Time

Starvation	20 h	40 h	60 h	80 h
24 h	10%	33%	68%	85%
48 h	17%	45%	72%	88%
Non starved	10%	30%	67%	81%

3.5 Effect of substrate adaptation on biodegradation of 3,4-DCBA:

This experiment was conducted to investigate if there was a relationship between the adaptation of bacteria and biodegradation of 3,4–DCBA. To compare the biodegradation of 3,4 –DCBA by both adaptation and Non adaptation of by *E. tarda* cells. the two experiments were done under similar conditions (Fig. 6). The adaptation cells degrade the 3,4 – DCBA slightly quicker (60µM\h) than the non-adapted cells (52µM/h) (Fig. 7). The microorganisms are able to adapt to the presence of toxic organic compounds by using a chain of alteration strategies such as modification in lipid composition of cell membranes (Neumann et al., 2004).

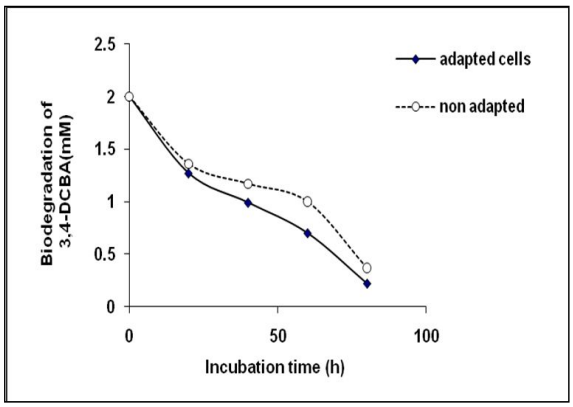

Figure 6 The effect adaptation on the 3,4DCBA degradation by *Edwardsiella tarda* when it's grown on MSM for ,under 42 ºC incubation temperature, aeration rate of 250 rpm and pH 7.5.

3.6 Effect of Nitrogen Sources on Biodegradation of 3,4-DCBA:

The effect of different nitrogen sources (yeast extract, L-proline, casein, glycine, arginine, urea, NH4 and glutamine) at 0.2% concentration on the biodegradation of 3,4-CBA was investigated (Fig. 8)., as well as the cell mass (Fig. 9), chlorine production (Fig. 10). The percentage of degradation rate shows highly degradation efficiency with (98%) at the media containing arginine whereas media with urea shows less degradation efficiency (45%) (Table 2). The rate of degradation shows the most nitrogen sources being tested caused an increase in the 3,4-DCBA degradation rate excluding ammonium, casine and urea. The inhibition of 3,4-DCBA degradation by (ammonium, casine and urea), might indicate that there is an optimal amount of this nitrogen source to be supplemented for optimal rate of 3,4-DCBA. Discussed the existence of an optimum amount of carbon to be supplemented for the biodegradation of pentachlorophenol. The reason for the enhanced degradation rate of 3,4- DCBA by *E. tarda* could be attributed to the attenuation of 3,4-DCBA toxicity by available nutrients and consequently the build-up of more cell mass (Khleifat et al., 2007; Abboud et al., 2010; Wang and Loh, 1999; Al-Limoun et al., 2019).

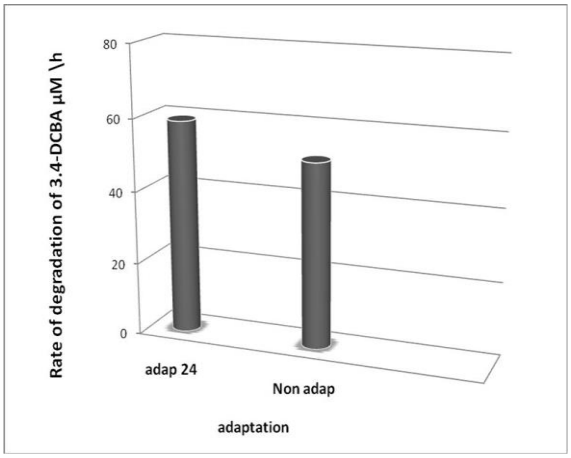

Figure 7 Initial rate of 3,4- DCBA degradation of adaptation at 24h,48h by *Edwardsiella tarda* when it's grown in MSM supplemented with 2mM of 3,4- DCBA, under 42 ºC incubation temperature, aeration rate 250 rpm and pH 7.5.

Table 2 Degradation percentage of 3,4 DCBA at Different N – source

N – source	20 h	40 h	60 h	80h
Glycine	65%	69%	83%	89%
Urea	20%	25%	34%	45%
yeast extract	28%	39%	78%	81%
Arginine	45%	81%	88%	98%
Casine	17%	25%	42%	61%
K nit	55%	77%	88%	89%
Proline	33%	73%	85%	90%
NH4	28%	30%	40%	59%
Glutamine	34%	67%	69%	82%
Control	32%	43%	50%	85%

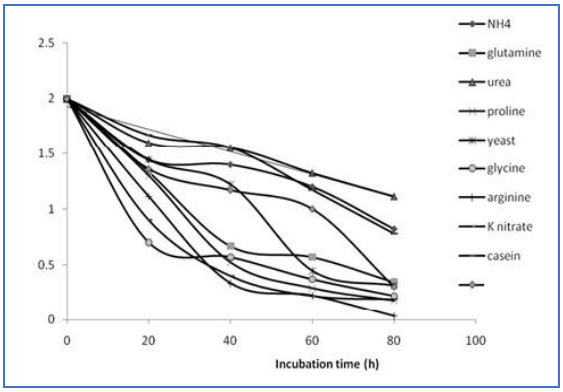

Figure 8 Effect of addition of different nitrogen source, on the biodegradation level of 3,4-DCBA(2mM) by *E. tarda* . The bacterial cells were grown on (2mM) 3,4-DCBA in minimal media (M9 media) plus each corrospondly nitrogen source .The temperature, aeration rates & pH were, 42 ºC, 250 rpm,7.5,respectively. Data are average of three independent experiments

3.7 Effect of Carbon Sources on Biodegradation of 3,4-DCBA:

A possible method of increasing the tolerance of the cells to substrate inhibition is to supplement the growth medium with different carbon sources including (maltose, mannitol, sucrose, fructose and succinic acid). Thus the experimental control used was only 3,4-DCBA as carbon and energy sources (Fig. 12). Generally, all carbon sources expect fructose and mannitol enhanced the 3,4-DCBA degradation by *E. tarda*. Nitrogen source as succinic acid increase percentage of degradation rate (90%) than fructose (45%) (Table 3). The inhibition of 3,4-DCBA degradation by fructose might indicate that there is an optimal amount of fructose and mannitol to be supplemented for optimal rate of 3,4-DCBA degradation.

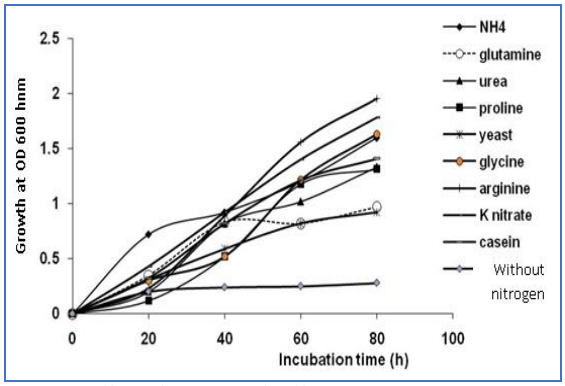

Figure 9 Effect of addition of different nitrogen source, on the growth of 3,4- DCBA(2mM) by *Edwardsiella tarda*. The bacterial cells were grown on (2mM) 3,4-DCBA in minimal media (M9 media) plus each corrospondly nitrogen source. The temperature, aeration rates & pH were, 42 ºC, 250 rpm,7.5,respectively. Data are average of three independent experiments

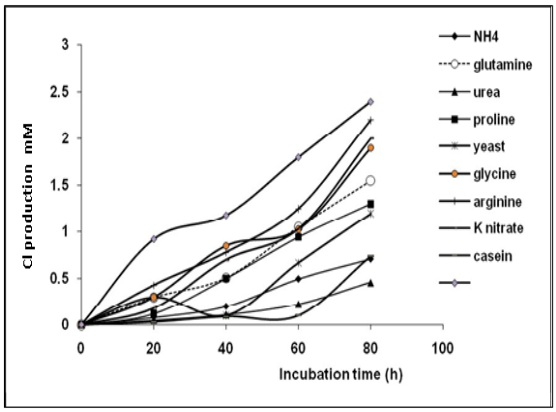

Figure 10 Effect of addition of different nitrogen source, on the Cl production of 3,4- DCBA(2mM) by *E. tarda* . The bacterial cells were grown on (2mM) 3,4-DCBA in minimal media (M9 media) plus each correspondingly nitrogen source. The temperature, aeration rates & pH were, 42ºC, 250rpm,7.5,respectively. Data are average of three independent experiments.

3.8 Detection of Dioxygenase Activity:

Chloride ions release was due to the oxygenase enzyme activities including more or less specific dioxygenases, for the halogenated compounds. In the course of our experiments, the chloride concentrations, was released as chloride ions similar to (Yun et al., 2007). The 1st one is catechol 2,3-dioxygenase activity, which is responsible for extradiol cleavage of the meta-pathway in microorganisms that was not detected in 3,4 - DCBA compound. However, the 2nd one is ortho (intradiol) cleavage catechol 1,2-dioxygenase activities which was detected here for 3,4-DCBA compound investigated with different extents (Fig. 12). 3,4-DCBA compound was separately added to the nutrient broth grown cells in 0.5mM concentration to induce the production of enzymes. The time required to detect the maximal catechol 1,2-dioxygenase activities was shown to be 5- 6 minutes.

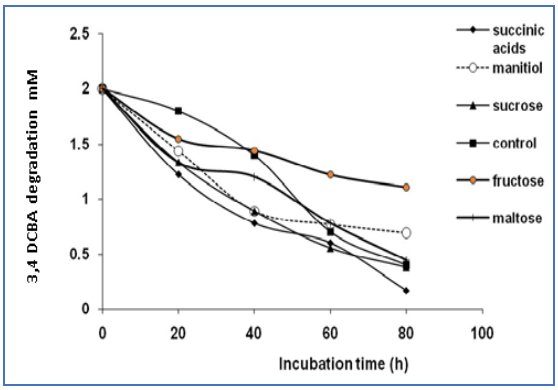

Figure 11 Effect of addition of different carbon source, on the biodegradation level of 3,4- DCBA(2mM) by *Edwardsiella tarda*. The bacterial cells were grown on (2mM) 3,4-DCBA in minimal media (M9 media) plus each correspondingly nitrogen source. The temperature, aeration rates & pH were, 42 ºC, 250 rpm,7.5, respectively. Data are average of three independent experiments.

Table 3 Degradation percentage of 3,4 DCBA at Different C-source

C-source	20 h	40 h	60 h	80 h
Mannitol	28%	55%	56%	71%
Fructose	20%	28%	39%	45%
Sucrose	34%	56%	72%	81%
Maltose	34%	40%	55%	75%
Succinic acid	39%	61%	70%	90%
Control	10%	55%	65%	80%

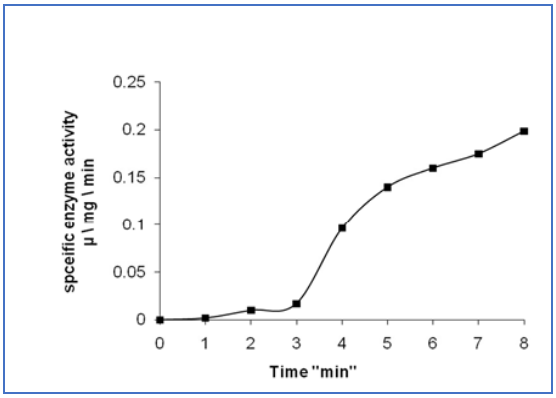

Figure 12 The activity of catechol 1,2-dioxygenase as a function of time. *E. tarda* was harvested after 24 hours of incubation in NB with (0.5 mM) of 3,4-DCBA, at incubation temperature 37 ºC, agitation rate of 150 rpm and pH 7.5. Enzyme assay was described in design and methodology.

4. CONCLUSION

Results from this work for the biodegradation of 3,4-DCBA by *E. tarda* would suggest of using this bacterium for the removal of a higher concentration of 3,4-DCBA. We investigated the influence of supplementary carbon and nitrogen sources on improving the biodegradation rates of 3,4-DCBA by *E. tarda*. Several conditions were optimized to increase the degradation efficiency in the medium such as the availability of oxygen, pH, and temperature, adaptation as well as starvation. Based on the chloride released rate and enzymes activities, a pathway for degradation of 3,4-DCBA by *E. tarda* was proposed: degradation of 3,4-DCBA occurs via the formation of catechol as central intermediates which are further mineralized through a modified ortho-pathway.

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