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Review Article

Biodegradation of 2- Chlorobenzoic Acid and its other substitutes

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Abstract: Because of the widespread use of these halogenated organic compounds in agriculture and industry, considerable quantities of these compounds' byproducts are discharged into the environment. These chemicals are known as halogenated pesticides. There is one group that, based on the chemical structures of these compounds, is thought to be the most tenacious and poisonous of all the groups. This group is known as the group. A. hydrophila, which was shown to be capable of utilising chlorobenzoate chemicals as a carbon and energy source, was isolated from wastewater treatment plant effluent in Petra, Jordan. Different biodegradation rates (4-chlorobenzoic acid, 5 M/hr; 3,4-dichlorobenzoic acid, 15.5 M/hr; 2-chlorobenzoic acid, 41 M/hr; 3-chlorobenzoic acid, 65 M/hr) were used to achieve these capacities. The release of chloride, the disappearance of the substrate, and finally the development of bacterial cells on these substrates were used to monitor the ability to degrade. Analyses of the ortho or meta ring-cleavage of these aromatic compounds were conducted on A. hydrophila dioxygenases that were physiologically activated by chlorobenzoic acid compounds. The fact that only one 2-dioxygenase activity was found suggests that the ortho route is used for the cleavage. The optimal conditions for 2-CBA chemical breakdown were 3 mM substrate concentration, 25 oC, pH 7, and 200 l inoculum size. Differently from how they affected chloride and cell mass synthesis, the carbon sources had an impact on the breakdown of 2-CBA. Utilized nitrogen sources decreased 2-CBA's ability to degrade and its ability to release chlorine. However, the biodegradation between the 40 and 80 hours was slightly enhanced by the nitrogen source L-proline.

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INTRODUCTION

CBAs, also known as hlorobenzoic acids, are capable of being released into the environment by a wide variety of various sources. The application of CBAs in agricultural settings as herbicides or insecticides is one of the possible sources of these chemicals. 2,3,6-CBA was utilized in the role of a herbicide (Horvath, 1971). CBAs have also been found to be generated as intermediates in the process of the degradation of the same herbicides. To be more specific, 2,6-CBA is produced as an intermediary by microorganisms during the breakdown of dichlobenil (Holtze et al., 2008), 2,5-CBA is produced during the degradation of chlorambene (Kamei et al., 1995; Dietrich et al., 2006), and 4-CBA is the ultimate product of the degradation of the insecticide DDT (Vrchotová et al., 2013).

Drugs such as indomethacin, bupropion, and benzafibrate are examples of a different wide set of chemicals that can produce CBAs as a byproduct of their metabolism in live creatures, including mammals. By inhibiting the formation of prostaglandins, which are molecules that produce fever, discomfort, stiffness, and edema, the anti-inflammatory medicine indomethacin is able to alleviate these symptoms. Indomethacin breaks down into 5-methoxy-2-methylindoleacetic acid and the same amount of 4-CBA. These two compounds are the products of the reaction. (Kamanavalli & Ninnekar, 2004). In the case of the antidepressant bupropion, only a minute quantity (0.3% of the total) of 3-CBA is generated in close proximity to threohydrobupropion, erythrohydrobupropion, and hydroxybupropion (O'Byrne et al., 2010). The breakdown of the anti-obesity medicine bezafibrate results in the creation of an equal proportion of 4-CBA and 4-(2-aminoethyl)-, dimethyl-benzeneacetic acid. Bezafibrate is used to treat obesity. (Quintana et al., 2005).

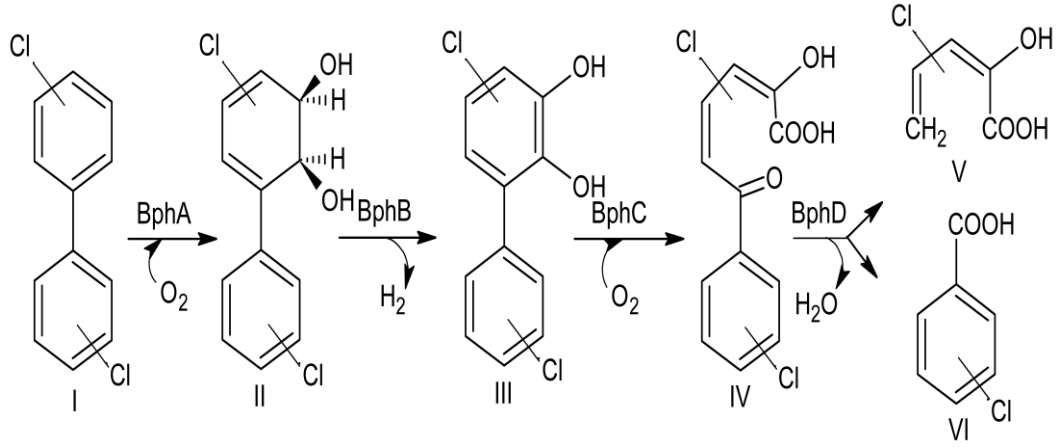

Figure 1. The process by which polychlorinated biphenyls are broken down by aerobic respiring organisms (Cited from Vrchotová et al., 2013).

It is common knowledge that chlorinated biphenyl adducts, or CBAs, are produced during the breakdown of polychlorinated biphenyls (PCBs). Bacteria use a process known as the "upper degradation pathway" to break down PCBs, which results in the formation of CBAs as the final degradation product (Figure 1.) (O'Byrne et al., 2010). CBAs can also be produced when white rot fungi are responsible for the breakdown of PCBs (Kamei et al., 1995; Dietrich et al., 2006). The end product of these metabolic pathways is a variety of CBAs that vary in the position of the chlorine atoms on the benzene ring as well as the quantity of chlorine atoms present, and this variation is dependent on the chlorination of the PCB congeners that have been degraded (Kamei et al., 1995; Dietrich et al., 2006)

The breakdown of chlorobenzoic acids by bacterial action

Environmental chemical pollutants cause significant difficulties for human health as a result of their persistence, toxicity, and transformation into hazardous metabolites. These pollutants include refrigerants, solvents, fire retardants, paints, insecticides, herbicides, plastics, and degreases (Adriaens et al., 1989; Asplund and Grimvall, 1991; Chaudhry and Chapalamadugu, 1991). Many of these environmentally significant xenobiotics that were initially developed for industrial application are halogenated, and halogenation is frequently cited as a factor for the persistence of these xenobiotics (Ampe et al., 1998; Khleifat et al., 2007a; Field and Alvarez, 2008; Khleifat et al., 2010). Both aerobic and anaerobic conditions are suitable for the breakdown of CBAs by bacterial organisms. When anaerobic conditions are present, the initial step in the degradation process is dechlorination, which is then followed by the degradation of the aromatic ring (Khleifat et al., 2007; Khleifat et al., 2009; Tarawneh et al., 2010; Khleifat et al., 2010; Tarawneh et al., 2011; Khleifat et al., 2014; Khleifat et al., 2019; Qaralleh et al., 2021; Khleifat et al., 2006; Khleifat et al., 2021; Jaafreh et al., 2019). The location of the chlorine atom or atoms on the aromatic ring determines the method that will be used for CBAs breakdown when aerobic conditions are present. The dechlorination process is an essential step. The phase of dechlorination can come either before or after the stage of aromatic ring degradation. This is dependent on the structure of the CBA as well as the enzymatic apparatus that the bacteria possess.

Because of their widespread application in agriculture and industry, significant quantities of the halogenated organic compounds that they produce are released into the surrounding environment. According to the chemical structures of such compounds, there is a group that is considered to be the most persistent and poisonous of all the groups. According to Yuroff et al. (2003), herbicides and pesticides are the primary sources of chlorobenzoates (CBAs). CBAs are also released beyond the degradation of chlorinated aromatic compounds, such as chlorinated phenols, chlorobenzoates, and dichlorodiphenyltrichlorethane. Herbicides and pesticides are considered to be the leading sources of chlorobenzoates (CBAs) (DDT). It seems as though the degradation of chlorobenzoate was the stage in the overall PCB degradation process that was the rate limiting step. Due to their pervasive presence (Adriaens and Focht, 1991a and b). because of their low toxicity, chlorobenzoates have been utilized as models to investigate the biodegradation of halogenated aromatic compounds and to shed light on the microbial methods involved in the release of chlorine substituents (Yi et al., 2000; Fritsche and Hofrichter, 2000). Bacteria exhibit a variety of activities, ranging from resistance to naturally toxic substances to sensitivity to even producing some of these substances, such as the biodegradation of organic pollutants. Some of these activities include producing some of these substances (Zeidan et al., 2013; Tarawneh et al., 2011; Khleifat et al., 2006a and b; Khleifat et al., 2009; Khleifat et al., 2008; Khleifat and Abboud, 2003; Althunibat et al., 2016; Aljundi and Khleifat, 2010; Qaralleh et al., 2019; Khleifat et al., 2019). Even while biodegradation has been the subject of a significant amount of research, the majority of which centers on bacterial consortiums, the search for biodegradation is still conducted through the identification of specific bacteria (Abboud et al., 2007).

Biodegradation is the most effective and economical approach to clean up contaminated soil or groundwater in situ, provided that all pollutants (aliphatic or aromatic chemicals) are mineralized in this process. This is because biodegradation breaks down organic matter into simpler compounds (Khleifat et al., 2007a and b). However, in order for biodegradation to be successful, a number of conditions, including favorable physicochemical conditions, the presence of bacteria that are capable of performing the necessary biochemical reactions, and the bioavailability of the harmful substance, essential macro- and micronutrients, co substrates, and electron acceptors, must be satisfied. During the process of biodegradation, the availability of oxygen is a factor that plays a very significant role. Oxygen is capable of directly reacting as a cosubstrate with the organic molecule, in addition to being capable of acting as a terminal electron acceptor (Vogt et al., 2004, Khleifat et al., 2006a-d).

The aerobic bacterial degradation of aromatic substrates typically involves the synthesis of substituted 1,2-dihydroxybenzenes (catechols) or 1,2-dihydroxynaphthalenes as an intermediate step in the degradation process. In the next step of the catabolic route, substituted catechols are oxidized by the cleavage of the aromatic ring. This can take place either between the two hydroxy groups (known as an intradiol or ortho cleavage) or at a bond that is close to the two-hydroxyl groups (extradiol or meta cleavage). Which kind of ring fission is more prevalent, as a general rule, is determined by the other substituents present on the aromatic ring. In most cases, the ortho-cleavage pathway is utilized during the mineralization of chlorocatechols. In contrast, the breakdown of methylcatechols typically occurs by a process known as meta cleavage (Gottschalk and Knackmuss, 1993). Ortho lysis of methyl-substituted catechols during 1,2-dioxygenation frequently results in the creation of dead-end metabolites such as methylmuconolactones (Pieper et al., 1995; Schmidt et al., 1994). The biodegradation of chlorobenzoic acid substitutes, in particular 2-chlorobenzoic acid, was investigated for the first time in this study by Aeromonas hydrophila. This was done by applying various growth conditions, such as carbon starvation, pH, incubation temperature, and aeration/agitation rate. In addition to this, the detection of the dioxygenases enzyme is used to determine the type of degradation pathways that are taking place in bacteria.

Degradation of chlorobenzoic acids by aerobic bacteria

Aerobic bacteria have developed a wide variety of CBAs degrading techniques during the course of evolution. The type of bacteria involved in the aerobic microbial degradation of chlorinated biphenyls (CBAs) as well as the structure and degree of chlorination of the CBAs are both important factors. CBAs can be broken down through the action of chlorocatechole (clc degradation genes) (Chae & Zylstra, 2006; Radice et al., 2007; Zhuang et al., 2003; Francisco et al., 2001; Nakatsu, & Wyndham, 1993; Romanov, V., & Hausinger, 1994) or through hydrolytic dehalogenation with hydroxybenzoic acid as an intermediate like in 4-CBA degradation (fcb degradation genes) [30-32] or through 4,5-dioxygenation like in 3-CBA and 3,4-CBA degradation (cba degradation genes) (Chae & Zylstra, 2006; Radice et al., 2007; Zhuang et al., 2003; Francisco et al., 2001) or through the action of 1,2-dioxygenase reaction. Gram-negative germs, such as Rhodococcus or Bacillus, and gram-positive strains, such as Pseudomonas, Burkholderia, or Achromobacter, are both capable of aerobically degrading CBAs [9]. Gram-positive strains, such as Pseudomonas, are also capable. When it comes to the breakdown of 2-CBA, there are three distinct pathways that have been uncovered so far. Each and every one of them pertains to the dioxygenation reaction that is facilitated by 2-halobenzoate-1,2-dioxygenase (EC 1.14.12.13) (Figure 2). The 1,2-dioxygenase process is the primary activity of the 2-halobenzoate-1,2-dioxygenase enzyme. Catechol is produced as a byproduct of this process, which breaks down 2-CBA. During this process, carbon dioxide and chlorine are released into the atmosphere. In addition to its 1,2-dioxygenase activity, the enzyme 2-halobenzoate-1,2-dioxygenase also possesses 1,6-dioxygenase activity. During the

reaction of 1,6-dioxygenase, 3-chlorocatechol is produced. The 2,3-dioxygenase reaction is the last activity of the 2-halobenzoate-1,2-dioxygenase enzyme. This reaction results in the synthesis of 2,3-dihydroxybenzoic acid. The latter two reactions take place, although only to a very limited extent [9]. Two distinct types of bacteria have been shown to be capable of degrading 2-CBA through the action of 2-halobenzoate-1,2-dioxygenase. The first group was only able to breakdown 2-CBA, but the second group was able to degrade 2-CBA in addition to 2,3-CBA and 2,5-CBA. Both groups utilise the 1,2-dioxygenase reaction for the degradation process. When it comes to 2-CBA, this reaction ultimately results in the production of catechol (Figure 2.). In the degradation process, 2,4-CBA and 2,5-CBA are converted into 4-chlorocatechol (Romanov, V., & Hausinger, 1994)

Biodegradation of 2-CBA by *Aeromonas hydrophila*:

Aeromonas hydrophila bacterium was isolated from the wastewater treatment plant of Petra City which is located in southern Jordan. Morphological and biochemical identification were done as described by (Popovic *et al.,* 2000).The biodegradation of four chlorobenzoic acid 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) were assayed. 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). *Aeromonas hydrophila* was capable of using these substituted compounds as carbon and energy source in different initial rates (4-CBA 5µM/hr, 3,4-DCBA 15.5µM/hr, 2-CBA 41µM/hr and 3-CBA 65µM/hr). *Aeromonas hydrophila* was grown in minimal medium that had these organic compounds as a sole carbon sources. The formation of any bacterial biomass will be a function of exhaustion of these substrates (Khleifat, 2006a, b and c). There was no biodegradation activity in the uninoculated cultures confirming the biodegrading activity made by *Aeromonas hydrophila* cells. The biodegradation was tested using 3 different concentrations (2, 3 and 4mM from each compound) as previously reported (Yun *et al.,* 2007; Marks *et al.,* 1984; Sahm *et al.,* 1986; Dong-In et al., 1997). Our results suggested that such bioremediation bacterium agents might be a suitable polishing agent to reduce residual contaminant levels, which other chemical or physical remediation technologies cannot effectively remediate. The order of initial degradation rate results were in order 3-CBA, 2-CBA, 3,4-CBA and 4-CBA (Figure. 3).

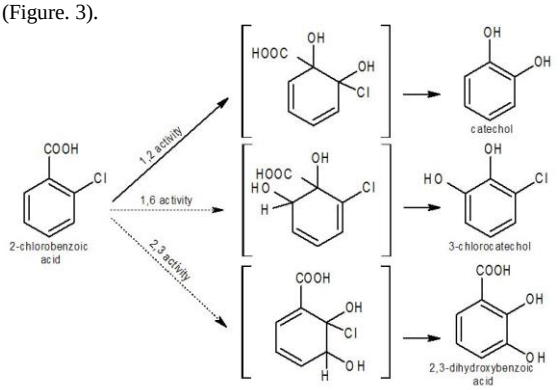

Figure 2: The enzyme 2-chlorobenzoate-1,2 dioxygenase catalyzes the aerobic breakdown of 2-chlorobenzoic acid.

The average degradation rates during the study of CBAs degradation were measured by dividing the net amount of transformed CBAs for (20 h), since after this time 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, 1998; Khleifat, 2006c) to avoid any errors caused by different lengths of lag phases, and the difficulty in ascertaining the time required achieving complete degradation or when the degradation had stopped. The 2-CBA was selected for further experiments because this compound is difficult to degrade by microorganisms because of ortho-position of the chlorine (Urgun-Demirtas *et al.,* 2003). The reduction of 2-CBA levels by *Aeromonas hydrophila* treatments was strongly dependent on time. The experiments were included substrate concentration, detection of dioxygenase activity, temperature, pH, inoculum size, carbon starvation, different glucose concentrations, carbon adaptation, and carbon and nitrogen sources(Alrawashdeh et al., 2019; Al-Sammarraie et al., 2020; Al-Limoun et al., 2020; Al-Tawarah et al., 2020; Alqaraleh et al., 2021)

The higher degradation percentage of 2mM 2-CBA was achieved almost at 40 hours incubation the time point at which the growth reaches 0.33 OD at 600 nm. The concomitant increase in degradation and growth of bacterial cells were also supported by data of chlorine release (Fig.4). However, using (3mM) 2-CBA partial degradation was achieved (Fig.3), but still higher initial degradation rate was observed. Moreover, the consumption of 2-CBA was paralleled with data results of chloride release. Raising the 2-CBA concentration to 4 mM (Fig.4) a less initial degradation rate (Fig.5) and chloride production was also observed. Similarly, the growth was also raised based on OD_{600nm} measurements. Our results were not in agreement with that of (Champagne *et al*., 1998), in which they described that the 2-CBA biodegradation mostly occurs by co-metabolism. They reported that the concentrations of 2-CBA are below the toxic levels and consequently less than the required level of carbon and energy source to maintain microbial biomass within the system or to provide energy for rate limiting reactions.

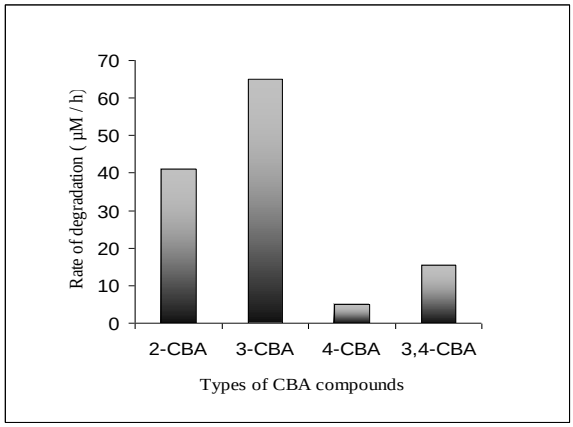

Figure 3. Initial rate of degradation by *Aeromonas hydrophila* (during 20 h) grown in MSM supplemented with 2mM of each types of CBA compounds, under incubation temperature of 37 ºC, agitation rate, 150 rpm and pH 7.

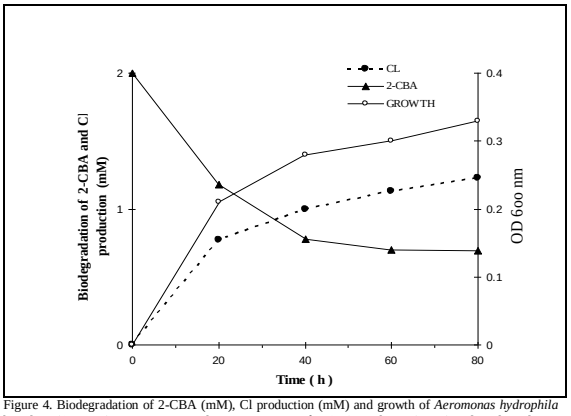

Figure 4. Biodegradation of 2-CBA (mM), Cl production (mM) and growth of *Aeromonas hydrophila* based on ($OD_{600\ nm}$) measurements. The concentration of 2-CBA used was (2mM) and its degradation was monitored using (230nm) and (525nm) for 2-CBA disappearance and chlorine production, respectively, as described in design and methodology. *Aeromonas hydrophila* was grown under incubation temperature of 37 ºC, agitation rate, 150 rpm and pH 7

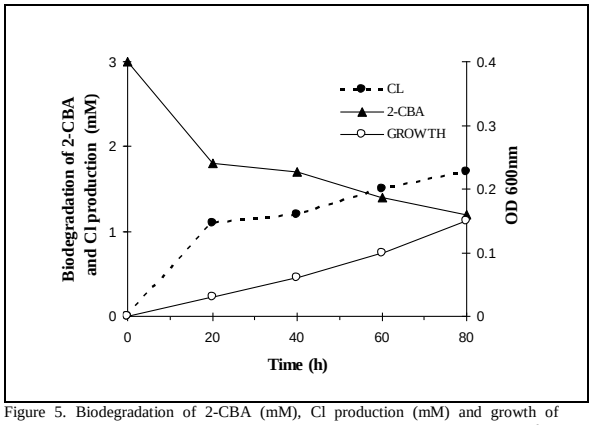

Figure 5. Biodegradation of 2-CBA (mM), Cl production (mM) and growth of *Aeromonas hydrophila* based on OD $_{600\ nm}$ measurements. The concentration of 2-CBA used was (3mM) and its degradation was detected using (230nm) and (525nm) for 2-CBA disappearance and chlorine production, respectively, as described in design and methodology. *Aeromonas hydrophila* was grown under incubation temperature of 37 ºC, agitation rate, 150 rpm and pH 7.

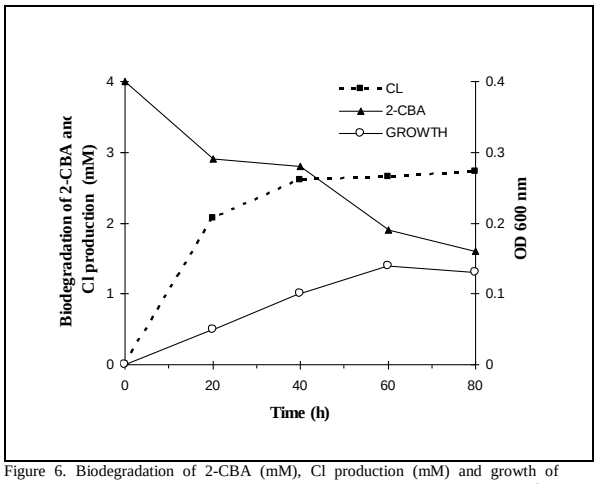

Figure 6. Biodegradation of 2-CBA (mM), Cl production (mM) and growth of *Aeromonas hydrophila* based on OD $_{600\ nm}$ measurements. The concentration of 2-CBA used was (4 mM) and its degradation was detected using (230nm) and (525nm) for 2-CBA disappearance and chlorine production, respectively, as described in design and methodology. *Aeromonas hydrophila* was grown under incubation temperature of 37 ºC, agitation rate, 150 rpm and pH 7.

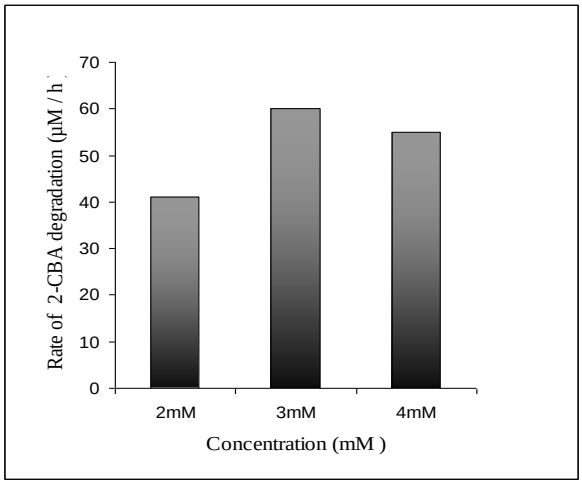

Figure 7. Initial rate of 2-CBA degradation by *Aeromonas hydrophila* (during 20 h) when it's grown in MSM supplemented with (2mM, 3mM and 4mM) of 2-CBA, under incubation temperature of 37 ºC, agitation rate, 150 rpm and pH 7.

Biodegradation of 3-CBA by *Aeromonas hydrophila*:

As with 2-CBA, three different concentrations were also used for 3-CBA compound. Using (2 mM) concentration of 3-CBA, 65% degradation was a achieved (data not shown). Using 3 mM concentration of 3-CBA under the same conditions of previous experiments, a 48.5% almost of 3-CBA degradation was attained. This achievement was almost attained in 20 hours of incubation time. Of course, this biodegradation was accompanied by the formation of growth biomass as determined by optical density measurements. A gradual increase in chloride production was also observed. The results of using (4 mM) concentration of 3-CBA were obtained at 20 h point of incubation. The degradation percentage 40% and higher initial degradation rate (80 µM/h) as shown in (Fig.8).

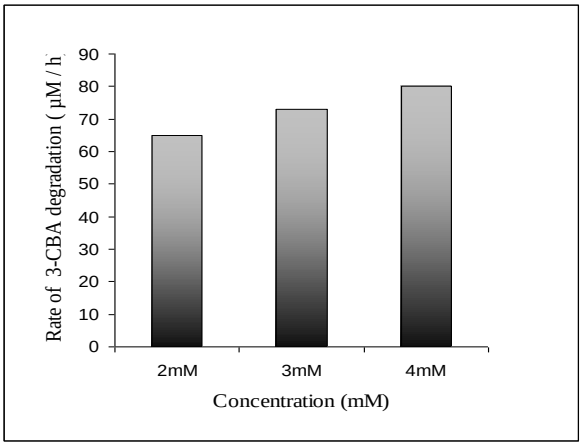

Figure 8. Initial rate of 3-CBA degradation by *Aeromonas hydrophila* (during 20 h) when it's grown in MSM supplemented with (2mM, 3mM and 4mM) of 3-CBA, under incubation temperature of 37 ºC, agitation rate, 150 rpm and pH 7.

Biodegradation of 4-CBA by *Aeromonas hydrophila*

Generally, the biodegradation of this substrate as compared with the other compounds tested (2-CBA, 3-CBA and 3,4-CBA) by this bacterial isolate is very poor. But still the bacterial isolate is capable of using these four substituted CBA compounds with different extents. Using (3 mM) 4-CBA as carbon source, the same degradation ability results were obtained with (2 mM). The Cl production was generally higher than that obtained with (2 mM) 4-CBA. As (4 mM) concentration used, similar results achieved with lower concentration (2 and 3 mM) were also observed here. The manner of degradation ability was concentration-dependent as shown in (Fig.9).

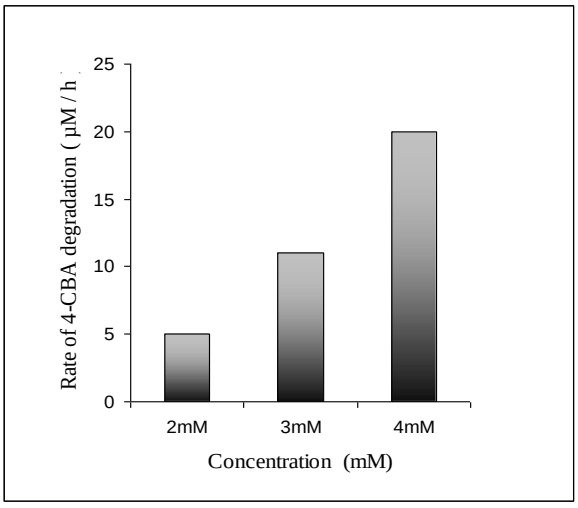

Figure 9. Initial rate of 4-CBA degradation by *A. hydrophlia* (during 20 h) when it's grown in MSM supplemented with (2mM, 3mM and 4mM) of 4-CBA, under incubation temperature of 37 ºC, agitation rate, 150 rpm and pH 7.

Biodegradation of 3,4-CBA by *A. hydrophila*

It was found that Less than 20% of 3,4-CBA concentration disappeared within 20 h and no more degradation was shown. However, the chloride production and growth biomass quantity were observed in parallel**.** About 30 µM/h degradation was achieved when using 3,4-CBA as carbon and energy sources and after 20 hours of incubation (Fig.10) and

resulted in same degradation percentage as compared with that of (2 mM) concentration at the same point. The degradation percentage results of (4 mM) 3,4-CBA compound was increased more than of the other two concentrations. These results indicate that the manner of degradation ability was concentration-dependent.

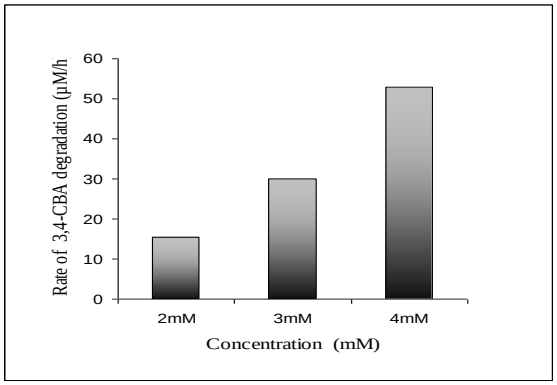

Figure 10. Initial rate of 3,4-CBA degradation by *A. hydrophila* (during 20 h) when it's grown in MSM supplemented with (2mM, 3mM and 4mM) of 3,4-CBA, under incubation temperature of 37 ºC, agitation rate, 150 rpm and pH 7.

Detection of dioxygenase activity

Chloride ions release was due to 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) shown between all substituted CBAs compounds 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). 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 either of our tested four CBA compounds (Harayama and Rekik, 1989; Arensdorf and Focht, 1994 and 1995; Bugg et al., 1997; Mars et al., 1998). However the 2nd one is ortho (intradiol) cleavage catechol 1,2-dioxygenase activities which was detected here for all compounds (2-CBA, 3-CBA, 4-CBA and 3,4-CBA) investigated with different extents (Fig.11). Each CBA compound was separately 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 5-6 minutes.

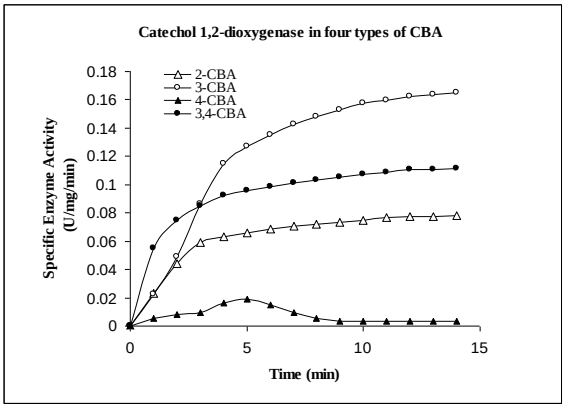

Figure 11. The activity of catechol 1,2-dioxygenase as a function of time. *Aeromonas hydrophila* was harvested after 24 hours of incubation in NB with (0.5 mM) of each type of CBA, under incubation temperature of 37 ºC, agitation rate, 150 rpm and pH 7. Enzyme assay was described in design and methodology.

Effect of incubation temperature on 2-CBA biodegradation

The experimental data on the degradation percentage at different incubation temperature showed different results at 25, 30 and 37 °C. The temperature 25°C was the best for cell biomass, chloride production and the initial degradation rate. It is clear that the temperature becomes vital above 25 °C. The temperature effect was paralleled clearly on the degradation percentage (Fig.12a), chlorine production (Fig.12b), as well as the cell mass (Fig.12c). Thus, it seems that biodegradation of 2-CBA could occur optimally at room temperature for *Aeromonas hydrophila* cells. Temperature obviously had a physiologically powerful impact on the fate of the 2-CBA compound, as the 25 °C generated the best conditions for their degradation, or this could be exclusively the outcome of a temperature effect on enzyme activities (Leven and Schnürer, 2005; Khleifat et al., 2014). It has been reported that the temperature could play an equivalent or larger role than nutrient availability in the degradation of benzoic acid and phenol (Margesin and Schinner, 1997).

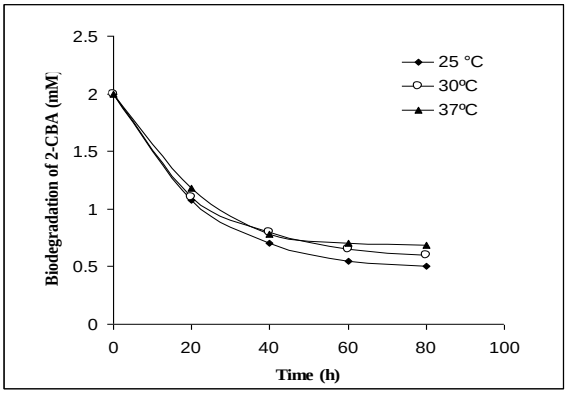

Figure 12a. The effect of different temperature on the biodegradation of 2-CBA (mM) by *Aeromonas hydrophila* when it's grown on MSM supplemented with (2mM) 2-CBA, under 25, 30 and 37 ºC incubation temperatures, agitation rate of 150 rpm and pH, 7.

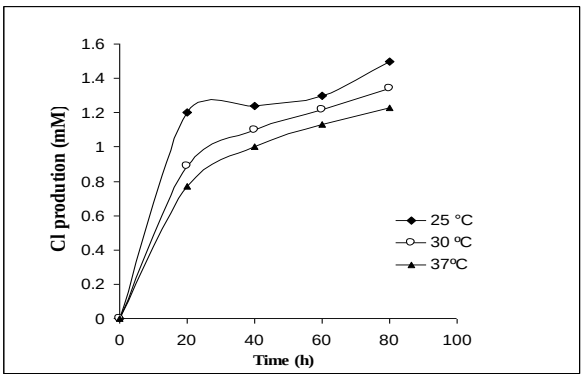

Figure 12b. The effect of different temperature on the Cl production (mM) by *Aeromonas hydrophila* when it's grown on MSM supplemented with (2mM) 2-CBA, under 25, 30 and 37 ºC incubation temperatures, agitation rate of 150 rpm and pH, 7.

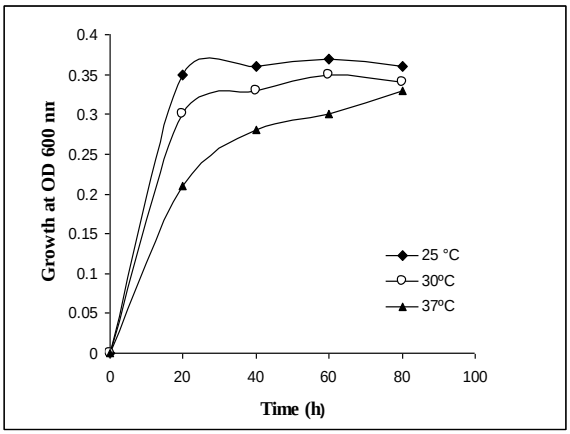

Figure 12c. The effect of different temperature on the growth of *Aeromonas hydrophila* in when it's grown on MSM supplemented with (2mM) 2-CBA, under 25, 30 and 37 ºC incubation temperatures, agitation rate of 150 rpm and pH, 7.

Effect of pH of culture media on biodegradation of 2-CBA:

The percentages of 2-CBA degradation brought about by *A. hydrophila* under different pH levels are shown in figures 13a-c. It is shown from the figure that *Aeromonas hydrophila* optimally degrades the 2-CBA at a pH of 7.0. The pH 7 was considered as a control experiment. However, the pH effect on the other two cases; growth biomass (Fig.13a) and chlorine production (Fig.11b) was different in being the best pH was 8. It is possible that the enzymes for 2-CBA degradation have their optimum enzymatic activities at pH 7.0 (Fig.13c). It was reported that optimum pH for the biodegradation of different aromatic compounds was different from one bacterium to another, for example, pH ranges between 8 and 11 for biodegradation of phenol and catechol by the bacterium *Halomonas campisalis* (Alva and Peyton, 2003) and for the biodegradation of phenol by *Klebsiella oxytoca*, the pH was 6.8 (Shawabkeh *et al*., 2007; (Khleifat et al., 2000; Khleifat et al., 2001; Khleifat et al., 2006; Khleifat et al., 2021; Al Qaisi et al., 2014; Aljundi et al., 2010a; Khleifat et al., 2022a; Khlaifat et al., 2019; Khleifat et al., 2019).

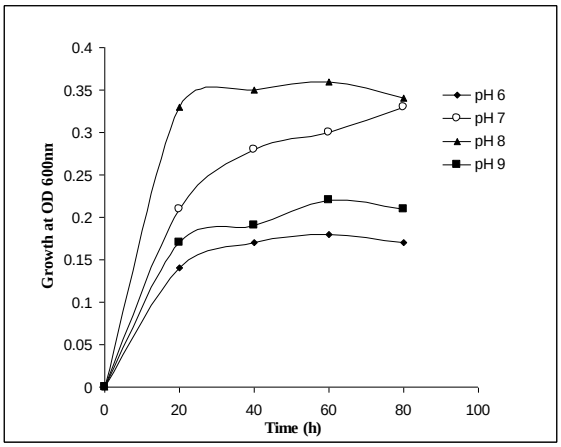

Figure 13a. The effect of different pH on the growth of *Aeromonas hydrophila* when it's grown on MSM supplemented with (2mM) 2-CBA, under incubation temperature of 37 ºC, agitation rate, 150 rpm and different pHs (6, 7, 8 and 9).

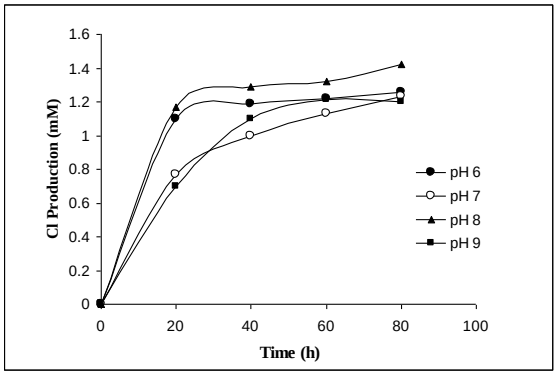

Figure 13b. The effect of different pH on the Cl production (mM) by *Aeromonas hydrophila* when it's grown on MSM supplemented with (2mM) 2-CBA, under incubation temperature of 37 ºC, agitation rate, 150 rpm and different pHs (6, 7, 8 and 9).

Effect of cell density on biodegradation of 2-CBA

The effect of the inoculum's volume on the rate of 2-CBA degradation was tested to decide whether the decrease in induction time during the initial starvation period is a result of increased cell densities or not. When the initial 2-CBA concentration of (2 mM) was used (Figs. 14a-c) with three cell densities measured by OD600 nm and normalized for number of cells/ml. These cells's densities were 0.5X, X, 1.5X and 2X). Generally the three parameters: (1) cell mass (Fig. 14a) (2), chloride (Fig. 14b) production and (3) degradation activity (Fig. 12c) was positively paralleled. The degradation rates was (10, 40, 67 and 71 µM/h) for (0.5X, 1X, 1.5X and 2X) respectively. Different induction times were shown based on the cell-concentration-dependent manner. Also percentage of 2-CBA degradation was differently achieved over different time periods with being the 2X completely degraded the 2-CBA in (80 h) of incubation. Thus starting inocula mass was found to be a major aspect in the time required for 2-CBA-degradation percentage. The same results are consistent with those previously described by others (Khleifat, 2006c; (Khleifat et al., 2007; Allimoun et al., 2015; Al Qaisi et al., 2014; Aljundi et al., 2010a; Aljundi et al., 2010b; Khlaifat et al., 2019; Khleifat et al., 2019). Furthermore, we demonstrated that inoculum's size enhance degradation activity of chlorobenzoic acids most likely by enhancing or increasing the activity of a degradative enzymes in the *Aeromonas hydrophila* .

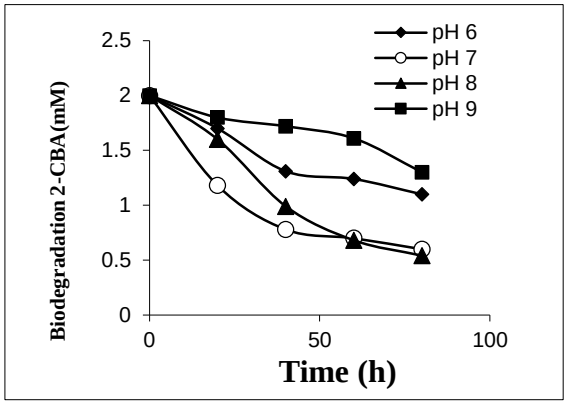

Figure (13c). The effect of different pH on the biodegradation of 2-CBA (mM) by *Aeromonas hydrophila* when it's grown on MSM supplemented with (2mM) 2-CBA, under incubation temperature of 37 ºC, agitation rate, 150 rpm and different pHs (6, 7, 8 and 9).

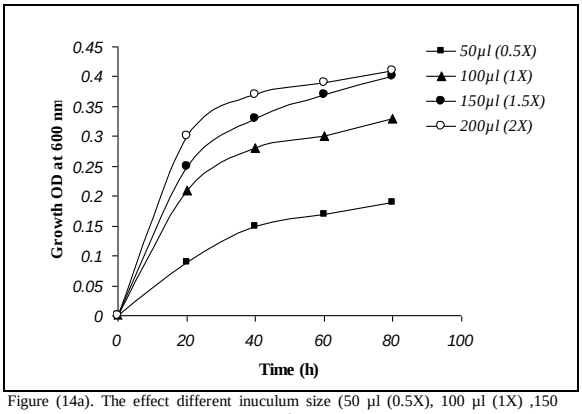

Figure (14a). The effect different inuculum size (50 µl (0.5X), 100 µl (1X) ,150 µl(1.5X) and 200 µl (2X)) on the growth of *A. hydrophila* when it's grown on MSM supplemented with (2mM) 2-CBA, under incubation temperature of 37 ºC, agitation rate, 150 rpm and pH 7.

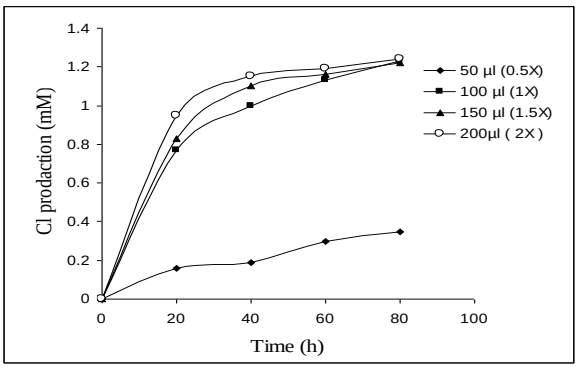

Figure (14b). The effect different inuculum size (50 µl (0.5X), 100 µl (1X), 150 µl (1.5X) and 200 µl (2X)) on the Cl production (mM) by *A. hydrophila* when it's grown on MSM supplemented with (2mM) 2-CBA, under incubation temperature of 37 ºC, agitation rate, 150 rpm and pH 7.

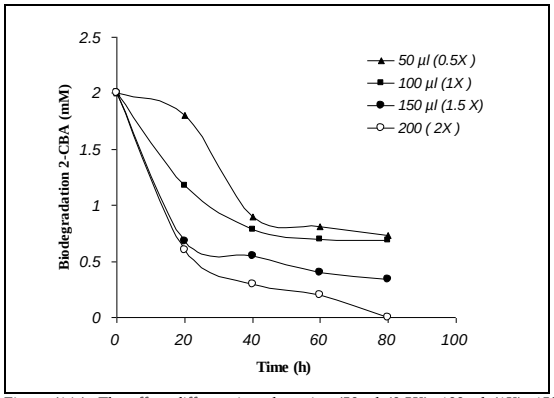

Figure (14c). The effect different inuculum size (50 µl (0.5X), 100 µl (1X) ,150 µl(1.5X) and 200 µl (2X)) on the 2-CBA degradation (mM) of *Aeromonas hydrophila* when it's grown on MSM supplemented with (2mM) 2-CBA, under incubation temperature of 37 ºC, agitation rate, 150 rpm and pH 7.

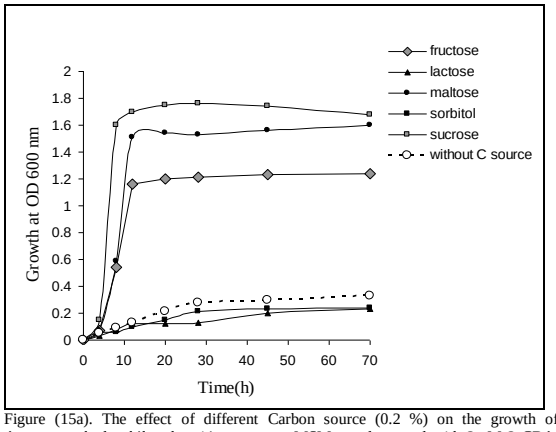

Figure (15a). The effect of different Carbon source (0.2 %) on the growth of *Aeromonas hydrophila* when it's grown on MSM supplemented with 2mM 2-CBA, under incubation temperature of 37 ºC, agitation rate, 150 rpm and pH 7.

Effect of Carbon Sources on Biodegradation of 2-CBA

To further investigate the CBAs-degrading capacity of *Aeromonas hydrophila*, the effect of different carbon sources on the 2-CBA degradation was examined as mentioned in the design and Methodogy. In all cases the growth of *Aeromonas hydrophila* cells was in accordance with the standard microbial batch growth culture (Figs.15a-c). Sucrose, maltose and fructose at 0.2% concentration, enhanced independently the production of cell mass (Fig. 15a) whereas production of chloride was inhibited by all carbon sources investigated (Fig. 15b). These carbon sources affected the 2-CBA degradation (Fig. 13c) differently from that on chloride and cell mass production. Degradation of 2-CBA was only enhanced by maltose and fructose as additional carbon source. The degradation repression by lactose and sucrose and the neutral effect of sorbitol was occurred although the cell biomass increased; this might be a result of catabolite repression of these carbon sources (Khleifat et al., 2007). The same result was shown in the study of the growth of *Ralstonia eutropha*, in which the fructose-grown cells in the presence of phenol minimized the respiration rate, compared with that of only phenol-grown cells (Leonard and Lindley, 1998; Alqudah et al., 2014; Al-Limoun et al., 2019; Al-Qaisi et al., 2021; Alqaraleh et al., 2021; Hajleh et al., 2022).

Effect of Nitrogen Sources on Biodegradation of 2-CBA:

The effect of different nitrogen sources (yeast extract, l-proline, casein and trypton) at 0.2% concentration on the biodegradation of 2-CBA was investigated (Figs.16a, b and c). All nitrogen sources tested resulted in the cell mass increase by 4-8 fold compared to that of control. L-proline caused an enhancement in bacterial growth (Fig.16) after a longer lag phase then the l-proline-grown cells outgrew the other nitrogen sources grown cells at 60 h time point of incubation. Also, this nitrogen source was inhibitory at the first 40 h of incubation and then a slight enhancement effect between the 40-80 h was shown. The biodegradation results were agreed with that of the growth kinetics outcomes shown in (fig.13a). At the same time, the nitrogen sources that inhibit the degradation activity (Fig. 13b) of the 2-CBA cause the same inhibition in the chlorine release as well (Fig. 16c). Previous studies have reported that there an optimal amount of yeast extract should be supplemented for the optimal rate of phenol biodegradation (Lob and Tar, 2000; Khleifat, 2006c, Khleifat et al., 2015). (Topp *et al.*, 1988) 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 2-CBA by *Aeromonas hydrophila* could be attributed to the attenuation of 2-CBA toxicity by available nutrients and consequently the build-up of more cell mass (Loh and Wang, 1998). In our case, an optimal amount of L-prolin probably should be adjusted to have better degradation ability of the 2-CBA.

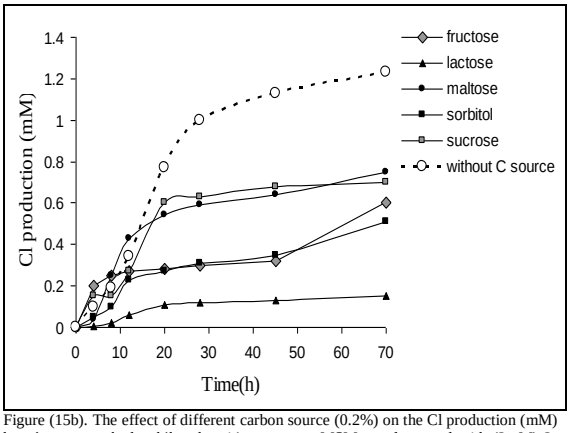

Figure (15b). The effect of different carbon source (0.2%) on the Cl production (mM) by *Aeromonas hydrophila* when it's grown on MSM supplemented with (2mM) 2-CBA, under incubation temperature of 37 ºC, agitation rate, 150 rpm and pH 7.

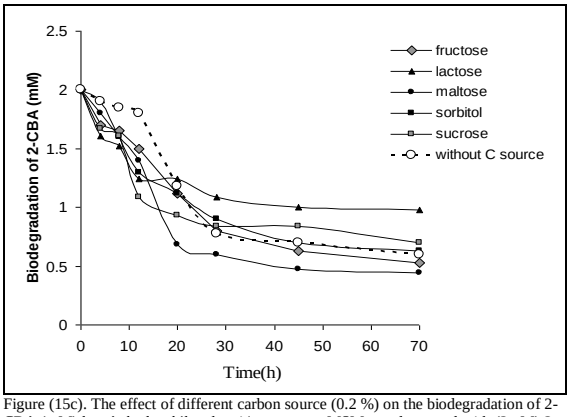

Figure (15c). The effect of different carbon source (0.2 %) on the biodegradation of 2-CBA (mM) by *A. hydrophila* when it's grown on MSM supplemented with (2mM) 2-CBA, under incubation temperature of 37 ºC, agitation rate, 150 rpm and pH 7.

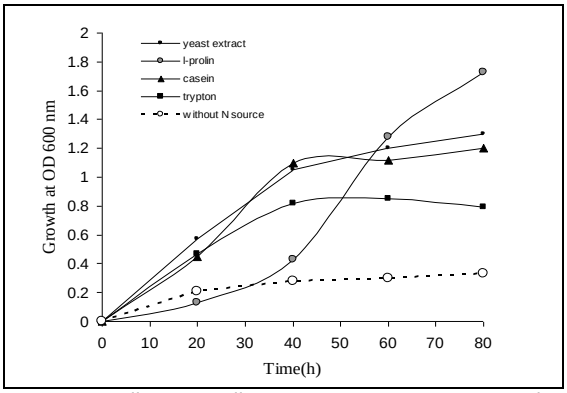

Figure (16a). The effect addition different nitrogen source (0.2%) on the growth of *A. hydrophila* when it's grown on MSM supplemented with (2mM) 2-CBA, under incubation temperature of 37 ºC, agitation rate, 150 rpm and pH 7.

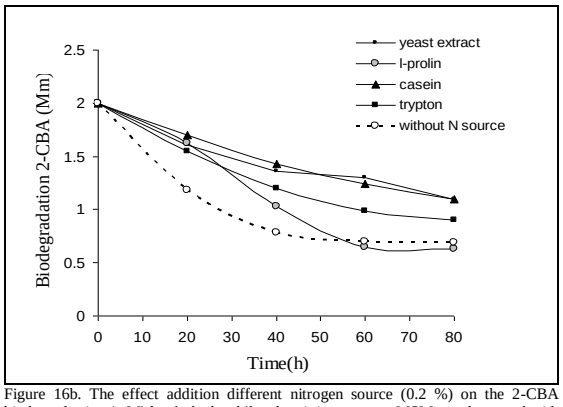

Figure 16b. The effect addition different nitrogen source (0.2 %) on the 2-CBA biodegradartion (mM) by A. *hydrophila* when it is grown on MSM supplemented with 2mM 2-CBA, under incubation temperature of 37 ºC, agitation rate, 150 rpm and pH 7.

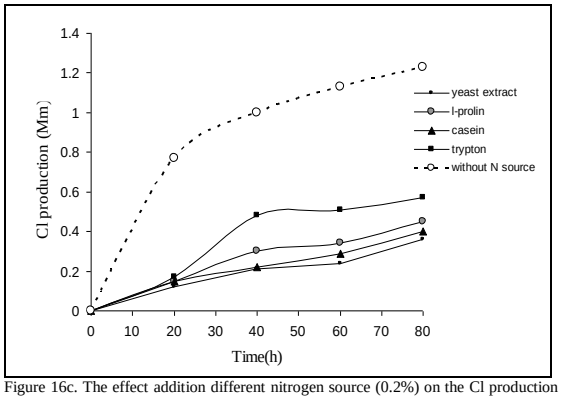

Figure 16c. The effect addition different nitrogen source (0.2%) on the Cl production (mM) by A. *hydrophila* when it's grown on MSM supplemented with (2mM) 2-CBA, under incubation temperature of 37 ºC, agitation rate, 150 rpm and pH 7.

CONCLUSION

Halogenated organic chemicals are widely used in agriculture and industry, therefore their byproducts are released into the environment. Halogenated insecticides. The chemical structures of these chemicals suggest one group is the most tenacious and dangerous. The group is this. A. hydrophila, which uses chlorobenzoate compounds for carbon and energy, was isolated from wastewater treatment plant effluent in Petra, Jordan. These capacities were achieved using different biodegradation rates (4-chlorobenzoic acid, 5 M/hr; 3,4-dichlorobenzoic acid, 15.5 M/hr; 2-chloro, 41 M/hr; 3-chlor, 65 M/hr). Degradation was monitored by chloride release, substrate loss, and bacterial cell growth. A. hydrophila dioxygenases activated by chlorobenzoic acid compounds were used to analyze the ortho or meta ring-cleavage of aromatic compounds. Only one 2-dioxygenase activity suggests ortho cleavage. 3-mM substrate concentration, 25 oC, pH 7, and 200 l inoculum size were appropriate for 2-CBA chemical breakdown. Carbon sources affected 2-CBA breakdown, unlike chloride and cell mass synthesis. Nitrogen sources reduced 2-CBA's degradation and chlorination. L-proline slightly accelerated biodegradation between 40 and 80 hours.

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