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Original Article

Biodegradation of Chlorobenzoic Acid Substitutes, Particularly, 2- Chlorobenzoic Acid by *Aeromonas hydrophila*

Khaled Tarawneh[a], Farah M. AL-Quraishi[a], Haitham Qaralleh[b], Amjad Al Tarawneh[c,] Muhamad O. Al-limoun[a], Khaled M Khleifat[a]

[a]Department of Biology, Mutah University, Karak, Jordan, [b]Department of Medical Laboratory sciences, Mutah University, Karak, Jordan; [c]Prince Faisal Center for Dead Sea, Environmental and Energy Research (PFC-DSEER), Mutah University, Karak, Jordan,

*Corresponding Author: alkh_kha@hotmail.com

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Abstract: Bacterium *Aeromonas hydrophila* (*A. hydrophila*) was isolated from the Petra Wastewater Treatment Plant effluent in southern Jordan. It was identified by using morphological and biochemical characteristics. *A. hydrophila* was found to be able of using chlorobenzoate compounds as carbon and energy source. These capabilities were with different biodegradation rates (4- chlorobenzoic acid 5µM/hr, 3,4-dichlorobenzoic acid 15.5µM/hr, 2- chlorobenzoic acid 41µM/hr and 3- chlorobenzoic acid 65µM/hr). The degradation ability was monitored through the release of chloride, disappearance of the substrate and finally the growth of bacterial cells on these substrates. *A. hydrophila* dioxygenases physiologically induced by chlorobenzoic acid compounds, were analyzed for both ortho or meta ring-cleavage of these aromatic compounds. Only 1, 2-dioxygenase activity was detected which suggest that the cleavage is through the ortho pathway. The best results of degradation of 2-CBA compound were obtained with 3mM substrate concentration, 25°C, pH 7and 200µl inoculum size. The carbon sources affected the 2-CBA degradation differently from that on chloride and cell mass production. Nitrogen sources used reduced the degradation activity of the 2-CBA as well as in the chlorine release from 2-CBA. However, the nitrogen source L-proline had a slight enhancement effect on the biodegradation between the 40-80h.

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INTRODUCTION

Considerable pollution and human health problems due to environmental chemical pollutants as a aresult of their persistence, toxicity, and transformation into hazardous metabolites of such as refrigerants, solvents, fire retardants, paints, pesticides, herbicides, plastics and degreases (Adriaens et al., 1989; Asplund and Grimvall, 1991; Chaudhry and Chapalamadugu, 1991). Many of these environmentally important xenobiotics, introduced for industrial use, are halogenated, and halogenation often is implicated as a reason for their persistence (Ampe et al., 1998; Khleifat et al., 2007a; Field and Alvarez, 2008; Khleifat et al., 2010).

These large volumes of their halogenated organic chemicals are liberated into the surroundings due to their wide agricultural and industrial usage. Such these compounds are classified as the most enduring and toxic group according to their chemical structures. Herbicides and pesticides are considered the leading sources of chlorobenzoates (CBAs) (Yuroff et al., 2003), which are also released beyond the degradation of chlorinated aromatic compounds, such as chlorinated phenols, chlorobenzoates and dichlorodiphenyltrichlorethane (DDT). The chlorobenzoate degradation appeared to be the rate limiting step in the overall PCB degradation process. Due to their ubiquitous presence (Adriaens and Focht, 1991a and b). low toxicity, the chlorobenzoates have been used as models to study the biodegradation of halogenated aromatic compounds, and to clarify the microbial strategies involved in the release of chlorine substituents (Yi et al., 2000; Fritsche and Hofrichter, 2000). Bacteria exhibit different activities ranging from resistance to natural toxic substances to sensitive to even producing some of these substances including the biodegradation of organic pollutants (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). Although biodegradation has been extensively studied and mostly are centered on bacterial consortia, the search for biodegradation is still sought through distinct individual bacteria (Abboud et al., 2007).

The most effective and economic way to clean up contaminated soil or groundwater *in situ* is biodegradation, provided that all contaminants

(aliphatic or aromatic compounds) are mineralized in this process (Khleifat et al., 2007a and b). However, many requirements must be met to ensure successful biodegradation, including favorable physicochemical conditions; the presence of bacteria able to perform the necessary biochemical reactions; and the bioavailability of the harmful substance, essential macro- and micronutrients, co substrates, and electron acceptors. The availability of oxygen plays a particularly important role in the biodegradation process. Oxygen can react as cosubstrate directly with the organic molecule, and it can be used as terminal electron acceptor (Vogt *et al.,* 2004, Khleifat et al., 2006a-d).

Aerobic bacterial degradation of aromatic substrates very often proceeds via intermediate formation of vicinal dihydroxy arenes such as substituted 1,2-dihydroxybenzenes (catechols) or 1,2-dihydroxynaphthalenes. Further, down the catabolic pathway, substituted catechols are oxidized by cleavage of the aromatic ring either between the two hydroxy groups (intradiol or *ortho* cleavage) or at a bond adjacent to the two-hydroxyl groups (extradiol or *meta* cleavage). As a rule, which mode of ring fission predominates depends on the other substituents on the aromatic ring. Chlorocatechols generally are mineralized via the *ortho*-cleavage pathway. Degradation of methylcatechols, in contrast, generally proceeds via *meta* cleavage (Gottschalk and Knackmuss, 1993). 1,2-Dioxygenation (*ortho* cleavage) of methyl-substituted catechols often results in the formation of dead-end metabolites such as methylmuconolactones (Pieper *et al*., 1995; Schmidt *et al*., 1994). In this study, biodegradation of chlorobenzoic acid substitutes, particularly, 2-chlorobenzoic acid by *Aeromonas hydrophila* was investigated for the first time through applying different growth conditions, including carbon starvation, pH, incubation temperature and aeration/agitation rate. Besides, the detection of dioxygenases enzyme to determine which the type of degradation pathways being occurred in bacteria.

MATERIALS AND METHODS

Bacterial Strains

The bacterium used in these experiments was *Aeromonas hydrophlia* that was already isolated from Petra waste water plant, Jordan. Its biochemical identity was re-verified using the REMEL kit (RapID^{TM} ONE and RapID^{TM} NF Plus systems) procedure as well as the morphological characteristics which were always microscopically checked.

Media and culture conditions

Cells were grown at 37 8C in nutrient broth (NB) for enzyme assay or in a minimal salt medium (MSM) for all 2-CBA degradation experiments (Khleifat, 2007a and b; 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; $CoS04.\ 7H_2O$, 0.1; $NaMoO_4.2H20$, 0.1; H_3BO_3, 0.05. The N sources in the
MSM broth medium were modified for the specific purposes of the experiments. For example, ammonium sulphate was excluded from MSM when testing the effect of different nitrogen sources on the 2-CBA degradation. Different carbon (glucose, fructose, sorbitol, maltose, lactose and sucrose) and nitrogen (yeast extract, l-prolin, casein and trypton) sources were added independently up to a concentration of 0.2% (w/v), to assess their effect on the biodegradation rate of 2-CBA by *A. hydrophlia* cells.

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, 2-CBA concentration in culture medium was determined spectrophotometrically by monitoring absorbance at 235 nm. Cells were grown in MSM containing 2, 3 and 4 mM 2-CBA. 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 2-CBA 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 2-CBA were measured by dividing the net amount of transformed 2-CBA for 24 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, 1998) 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 different growth conditions on the 2-CBA biodegradation ability

Effect of 2-CBA concentration, pH, incubation temperature and agitation rate

The effect of the different substrate concentrations (1, 2, and 3 mM) on the 2-CBA degradation by *A. hydrophlia* was examined. The growth medium was an MSM medium incubated at 37 ºC, under a 150 rpm shaking rate and a pH of 7.0. Different pHs (6, 7, 8 and 9) of the growth media was used to assess the effect of variations in pH on the degradation ability of 2-CBA by the same bacterium (Aljundi et al., 2010). The effect of different incubation temperatures (25, 30 and 37 ºC) upon the percentage degradation of 2-CBA by *A. hydrophlia* was investigated. The medium used was an MSM medium.

Effect of cell density on 2-CBA degradation

A. hydrophlia cells were obtained from the mid-log phase culture as described previously. Cell densities were adjusted to OD_{600} nm values of 0.1, 0.2, 0.3 and 0.4 as 0.5X, 1X, 1.5X and 2X to check the effect of cell density on 2-CBA degradation. The *K. oxytoca* cells were grown in the 2 mM-2-CBA-containing MSM medium at 37 ºC, with an agitation rate of 150 rpm. The 2-CBA degradation was assayed as a function of time, as previously described.

Enzyme assay

A. hydrophlia 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).

RESULTS AND DISCUSSIONS

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. 1).

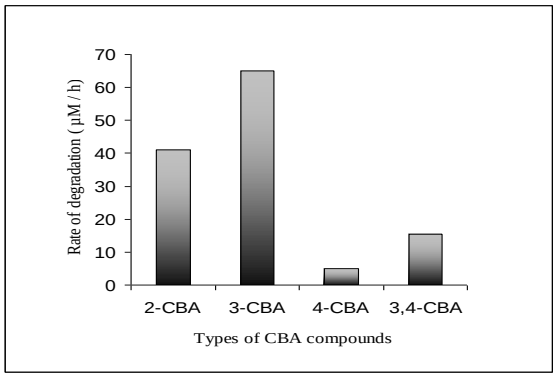

Figure 1. Initial rate of degradation by *Aeromonas hydrophila* (during 20 h) grown in MSM supplemented with 2mM of each types of CBA compounds, under incubation temperature of 37 ºC, agitation rate, 150 rpm and pH 7.

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.

Biodegradation of 2-CBA by *Aeromonas hydrophila*:

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.2). 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.

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.6).

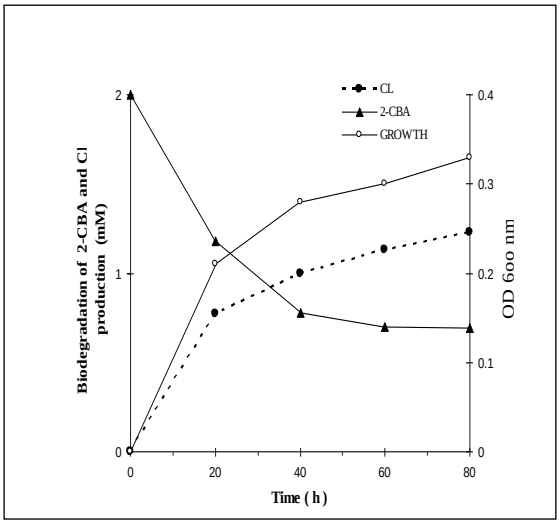

Figure 2. Biodegradation of 2-CBA (mM), Cl production (mM) and growth of *Aeromonas hydrophila* based on ($OD_{600\ nm}$) measurements. The concentration of 2-CBA used was (2mM) and its degradation was monitored using (230nm) and (525nm) for 2-CBA disappearance and chlorine production, respectively,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

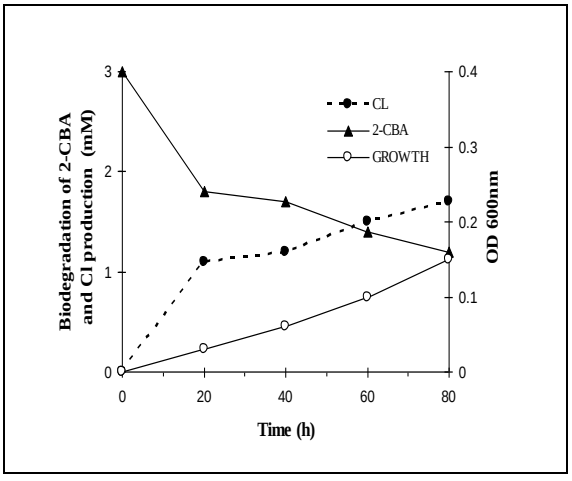

Figure 3. Biodegradation of 2-CBA (mM), Cl production (mM) and growth of *Aeromonas hydrophila* based on $OD_{600\ nm}$ measurements. The concentration of 2-CBA used was (3mM) and its degradation was detected using (230nm) and (525nm) for 2-CBA disappearance and chlorine production, respectively, as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

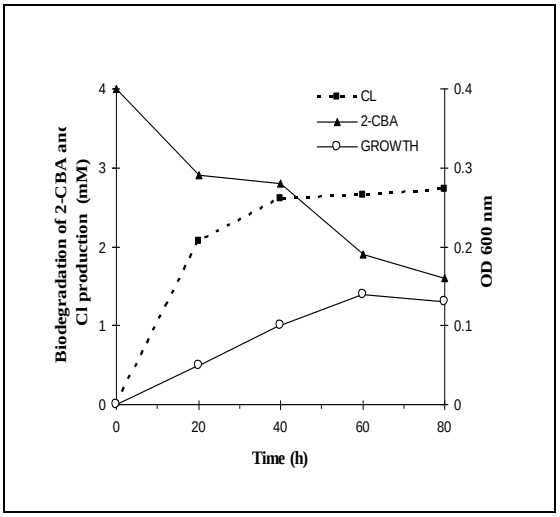

Figure (4). Biodegradation of 2-CBA (mM), Cl production (mM) and growth of *Aeromonas hydrophila* based on $OD_{600\ nm}$ measurements. The concentration of 2-CBA used was (4 mM) and its degradation was detected using (230nm) and (525nm) for 2-CBA disappearance and chlorine production, respectively,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

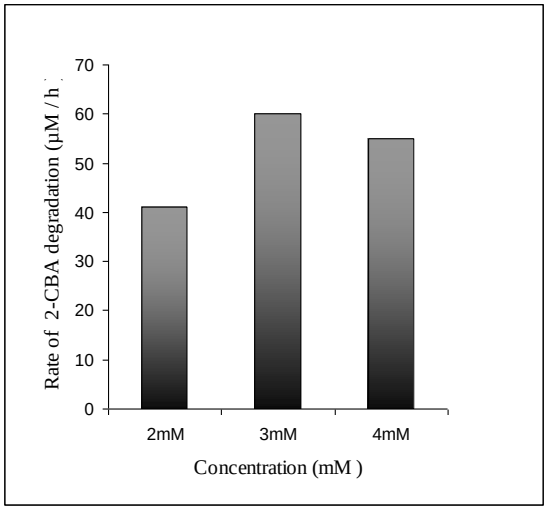

Figure 5. Initial rate of 2-CBA degradation by *Aeromonas hydrophila* (during 20 h) when it's grown in MSM supplemented with (2mM, 3mM and 4mM) of 2-CBA, under incubation temperature of 37 ºC, agitation rate, 150 rpm and pH 7.

Biodegradation of 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.7).

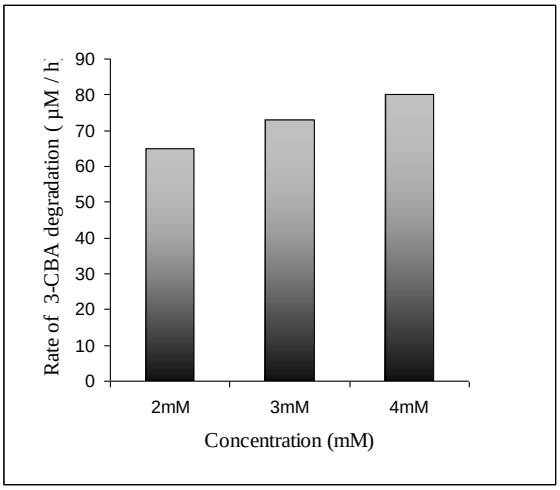

Figure 6. Initial rate of 3-CBA degradation by *Aeromonas hydrophila* (during 20 h) when it's grown in MSM supplemented with (2mM, 3mM and 4mM) of 3-CBA, under incubation temperature of 37 ºC, agitation rate, 150 rpm and pH 7.

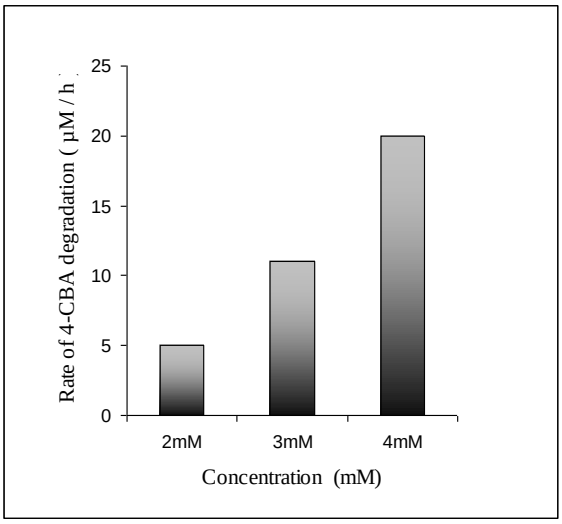

Figure 7. Initial rate of 4-CBA degradation by *A. hydrophlia* (during 20 h) when it's grown in MSM supplemented with (2mM, 3mM and 4mM) of 4-CBA, under incubation temperature of 37 ºC, agitation rate, 150 rpm and pH 7.

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.8) 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.

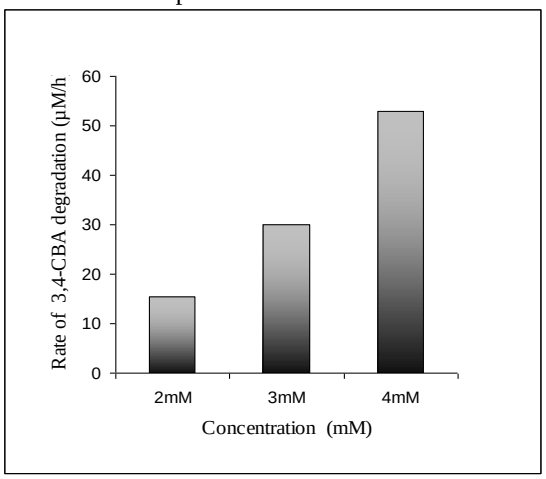

Figure 8. Initial rate of 3,4-CBA degradation by *A. hydrophila* (during 20 h) when it's grown in MSM supplemented with (2mM, 3mM and 4mM) of 3,4-CBA, under incubation temperature of 37 ºC, agitation rate, 150 rpm and pH 7.

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.9). 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.

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.10a), chlorine production (Fig.10b), as well as the cell mass (Fig.10c). 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).

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 11a-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.11a) 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.11c). 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).

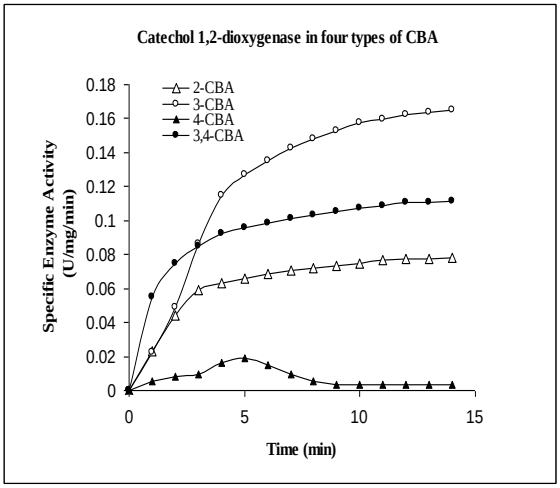

Figure 9. The activity of catechol 1,2-dioxygenase as a function of time. *Aeromonas hydrophila* was harvested after 24 hours of incubation in NB with (0.5 mM) of each type of CBA, under

incubation temperature of 37 ºC, agitation rate, 150 rpm and pH 7. Enzyme assay was described in design and methodology.

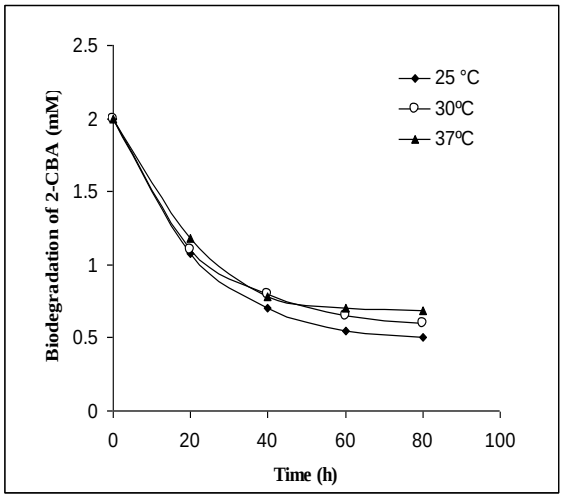

Figure 10a. The effect of different temperature on the biodegradation of 2-CBA (mM) by *Aeromonas hydrophila* when it's grown on MSM supplemented with (2mM) 2-CBA, under 25, 30 and 37 ºC incubation temperatures, agitation rate of 150 rpm and pH, 7.

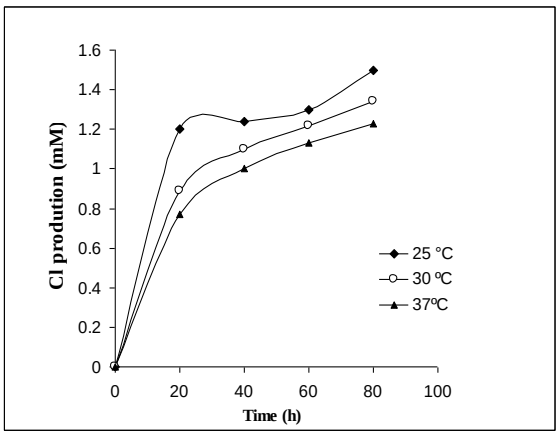

Figure 10b. The effect of different temperature on the Cl production (mM) by *Aeromonas hydrophila* when it's grown on MSM supplemented with (2mM) 2-CBA, under 25, 30 and 37 ºC incubation temperatures, agitation rate of 150 rpm and pH, 7.

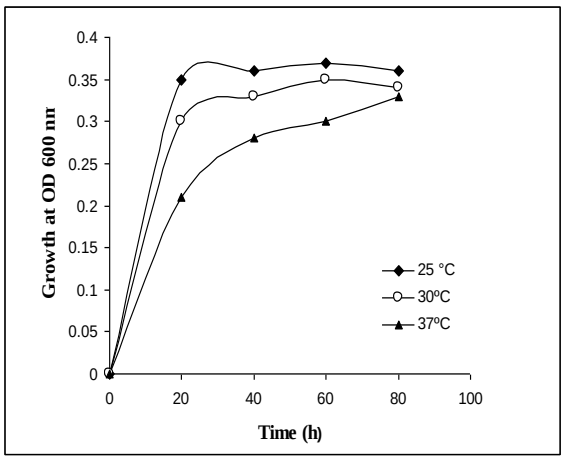

Figure 10c. The effect of different temperature on the growth of *Aeromonas hydrophila* in when it's grown on MSM supplemented with (2mM) 2-CBA, under 25, 30 and 37 ºC incubation temperatures, agitation rate of 150 rpm and pH, 7.

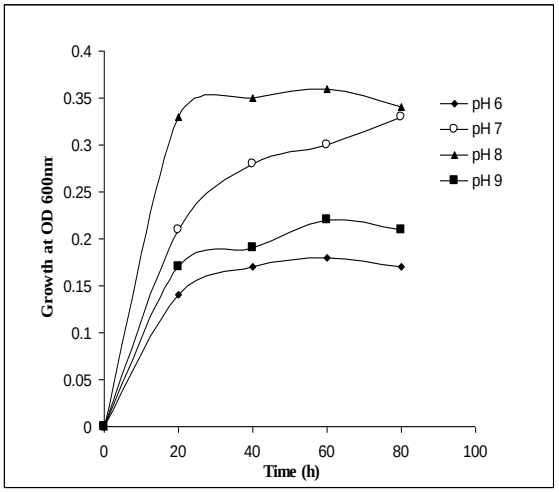

Figure 11a. The effect of different pH on the growth of *Aeromonas hydrophila* when it's grown on MSM supplemented with (2mM) 2-CBA, under incubation temperature of 37 ºC, agitation rate, 150 rpm and different pHs (6, 7, 8 and 9).

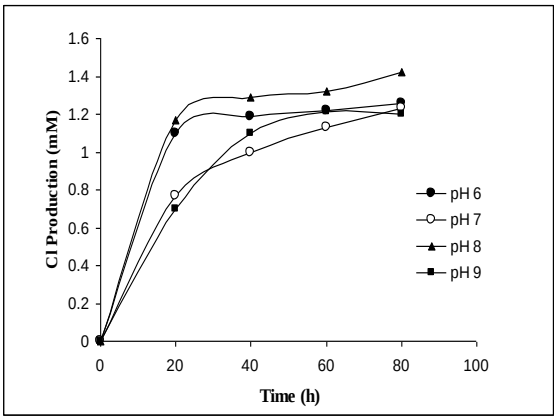

Figure 11b. The effect of different pH on the Cl production (mM) by Aeromonas hydrophila when it's grown on MSM supplemented with (2mM) 2-CBA, under incubation temperature of 37 ºC, agitation rate, 150 rpm and different pHs (6, 7, 8 and 9).

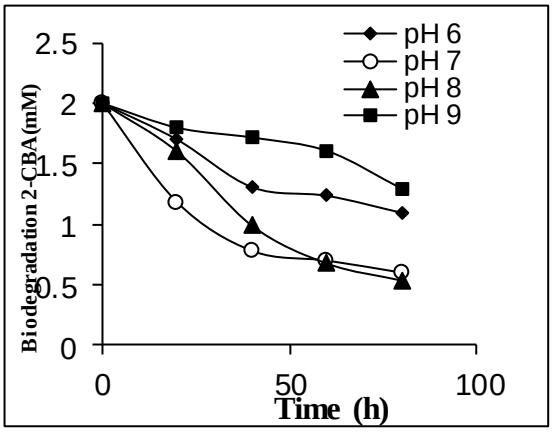

Figure (11c). The effect of different pH on the biodegradation of 2-CBA (mM) by Aeromonas hydrophila when it's grown on MSM supplemented with (2mM) 2-CBA, under incubation temperature of 37 ºC, agitation rate, 150 rpm and different pHs (6, 7, 8 and 9).

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. 12a-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. 12a) (2), chloride (Fig. 12b) 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). 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*.

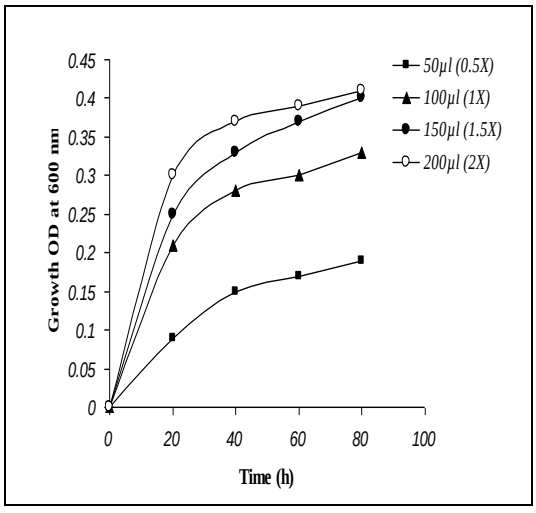

Figure (12a). The effect different inuculum size (50 µl (0.5X), 100 µl (1X) ,150 µl(1.5X) and 200 µl (2X)) on the growth of *A. hydrophila* when it's grown on MSM supplemented with (2mM) 2-CBA, under incubation temperature of 37 ºC, agitation rate, 150 rpm and pH 7.

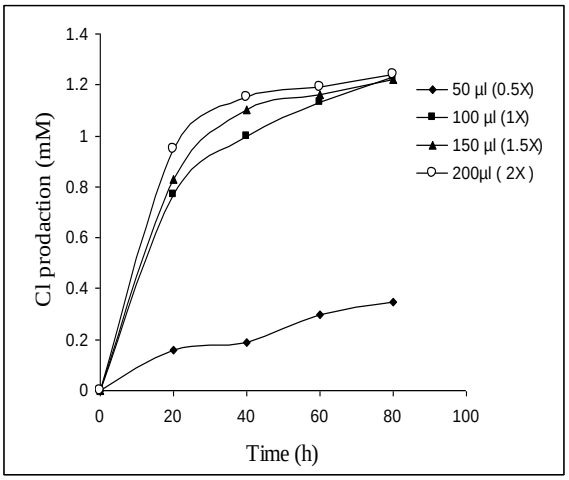

Figure (12b). The effect different inuculum size (50 µl (0.5X), 100 µl (1X), 150 µl (1.5X) and 200 µl (2X)) on the Cl production (mM) by *A. hydrophila* when it's grown on MSM supplemented with (2mM) 2-CBA, under incubation temperature of 37 ºC, agitation rate, 150 rpm and pH 7.

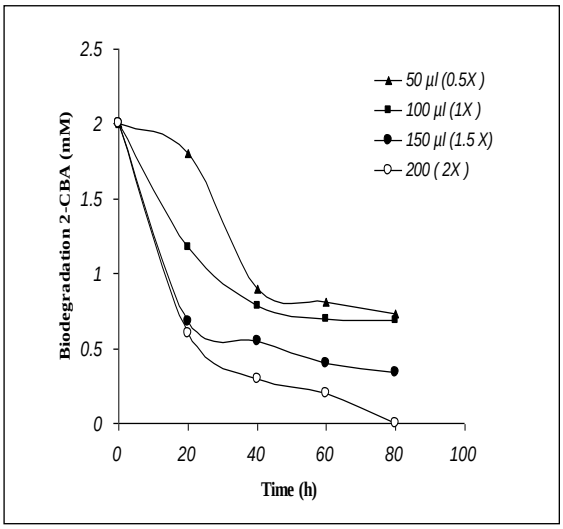

Figure (12c). The effect different inuculum size (50 µl (0.5X), 100 µl (1X) ,150 µl(1.5X) and 200 µl (2X)) on the 2-CBA degradation (mM) of *Aeromonas hydrophila* when it's grown on MSM supplemented with (2mM) 2-CBA, under incubation temperature of 37 ºC, agitation rate, 150 rpm and pH 7.

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.13a-c). Sucrose, maltose and fructose at 0.2% concentration, enhanced independently the production of cell mass (Fig. 13a) whereas production of chloride was inhibited by all carbon sources investigated (Fig. 13b).

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).

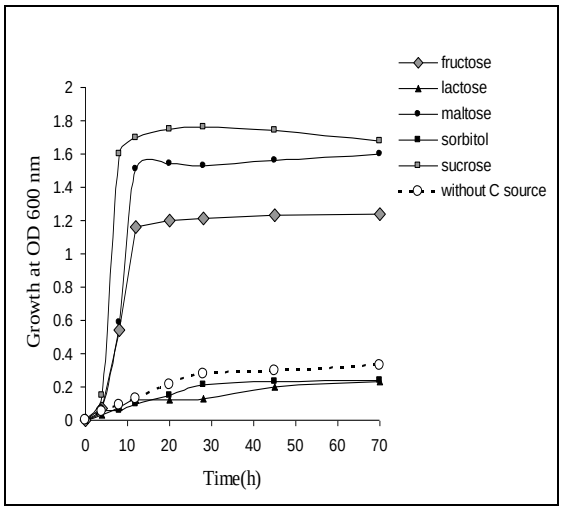

Figure (13a). The effect of different Carbon source (0.2 %) on the growth of *Aeromonas hydrophila* when it's grown on MSM supplemented with 2mM 2-CBA, under incubation temperature of 37 ºC, agitation rate, 150 rpm and pH 7.

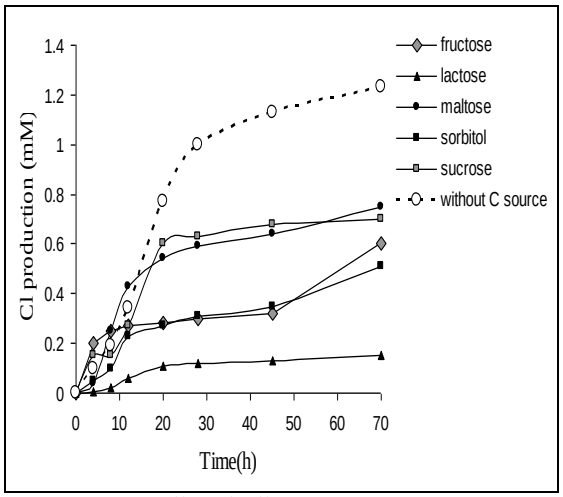

Figure (13b). The effect of different carbon source (0.2%) on the Cl production (mM) by *Aeromonas hydrophila* when it's grown on MSM supplemented with (2mM) 2-CBA, under incubation temperature of 37 ºC, agitation rate, 150 rpm and pH 7.

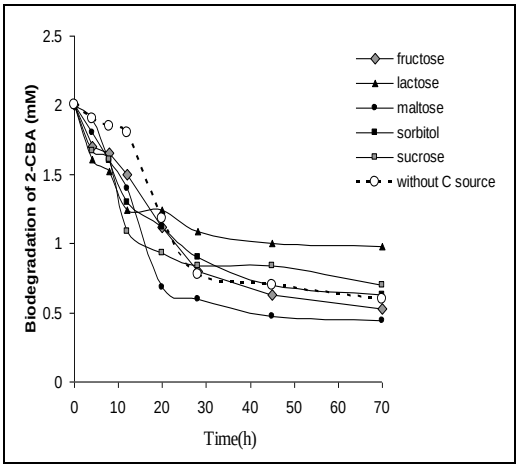

Figure (13c). The effect of different carbon source (0.2 %) on the biodegradation of 2-CBA (mM) by *A. hydrophila* when it's grown on MSM supplemented with (2mM) 2-CBA, under incubation temperature of 37 ºC, agitation rate, 150 rpm and pH 7.

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.14a-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.14a) 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.14b).

At the same time, the nitrogen sources that inhibit the degradation activity (Fig. 14b) of the 2-CBA cause the same inhibition in the chlorine release as well (Fig. 14c). 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.

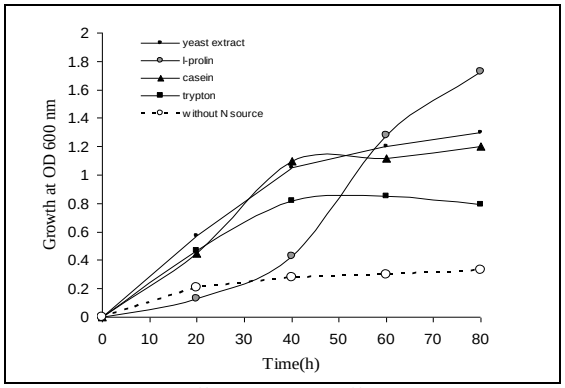

Figure (14a). The effect addition different nitrogen source (0.2%) on the growth of *A. hydrophila* when it's grown on MSM supplemented with (2mM) 2-CBA, under incubation temperature of 37 ºC, agitation rate, 150 rpm and pH 7.

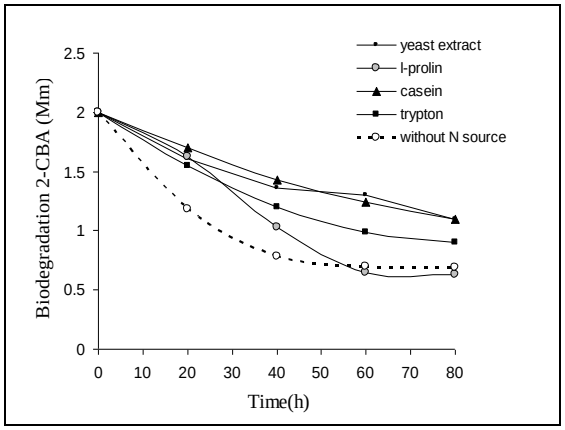

Figure 14b. The effect addition different nitrogen source (0.2 %) on the 2-CBA biodegradartion (mM) by A. *hydrophila* when it is grown on MSM supplemented with 2mM 2-CBA, under incubation temperature of 37 ºC, agitation rate, 150 rpm and pH 7.

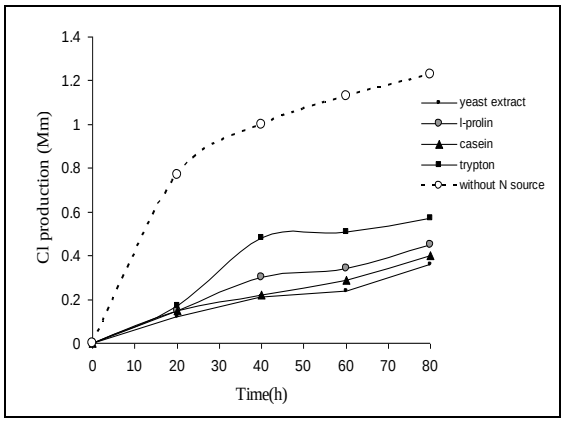

Figure 14c. The effect addition different nitrogen source (0.2%) on the Cl production (mM) by A. *hydrophila* when it's grown on MSM supplemented with (2mM) 2-CBA, under incubation temperature of 37 ºC, agitation rate, 150 rpm and pH 7.

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