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Biodegradation of 2-Chlorobenzoic Acid by *Acinetobacter calcoaceticus*: Optimization of Growth Conditions and Growth Kinetics

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The bacterium *Acinetobacter calcoaceticus* was isolated from Petra wastewater plant, Jordan and investigated for its biodegradation ability of different chlorobenzoic acid compounds (2-CBA, 3-CBA, 4-CBA, and 3, 4-CBA). As a result of testing these four substituted chlorobenzoic acid compounds as being sole carbon and energy sources by this bacterium and 2-CBA was further used for optimization conditions because it is the most toxic one among the tested compounds. The optimization conditions included substrate concentration, incubation temperature, pH, inoculum size, carbon starvation, carbon adaptation and carbon and nitrogen sources. The best incubation temperature and pH were 37^{o}C and 7.0, respectively. The carbon sources lactose, maltose and sucrose led to the decrease of 2-CBA degradation and simultaneous addition of sorbitol and fructose caused complete degradation of 2-CBA within 70 h incubation. Yeast extract, L-prolinee, casein and trypton as nitrogen sources caused a repression in 2-CBA degradation. Haldane model was best fit for determination of specific growth rate on various substrate concentrations. The maximum growth rates on 2 mM 2-CBA reached only 0.25. The results of optimization experiments confirm that the cell volume is the key factor in obtaining the best results. These results suggest that *A. calcoaceticus* carry potential genes for different chlorobenzoic acid -degrading enzyme(s) system, which could have a common origin.

Keywords: Biodegradation, *Acinetobacter calcoaceticus*, 2-chlorobenzoic acid

INTRODUCTION

Each year, millions of tons of toxic compounds were used globally as pesticides in agricultural production. The release of chlorinated organic compounds into the environment receives a widespread concern due to their toxicity to humans and wildlife, their relative persistence in aquatic sediments and soils and their bioaccumulation. (Al-Limoun et al., 2018; Allimoun et al., 2015; DeWeerd and Bedard, 1999; Khleifat, 2007; Khleifat et al., 2018; Khleifat et al., 2010). A huge number of bacterial and fungal genera possess the capability to degrade organic pollutants. Microbial degradation is one of the possible ways for removing aromatic compounds from the environment. Over the last 20 years there have been numerous reports on bacterial degradation of chlorinated biphenyls. *Alcaligenes* sp, *Pseudomonas* sp , *Rhodococcus*sp., *Acinetobacter* sp , and *Burkholderia* sp. are among the most commonly reported and extensively studied bacteria known to degrade CBA (Arensdorf and Focht, 1994; Krooneman et al., 1996; Tros et al., 1996, Alqudah et al., 2014). The predominant degraders of organopollutants in the toxic zone of contaminated areas are chemo-organotrophic species able to use a huge number of natural and xenobiotic compounds as carbon

sources and electron donors for the generation of energy. Although many bacteria are able to metabolize organic pollutants, a single bacterium does not possess the enzymatic capability to degrade all or even most of the organic compounds in a polluted soil. The degradation is strongly dependent on the availability of oxygen in the medium containing CBA (Krooneman et al., 1996). This is because oxygen is required not only as a terminal electron acceptor in the respiratory electron transfer chain but also as a substrate in the degradation-pathways (Romanov and Hausinger, 1994). There are factors that inhibited or enhanced CBA compounds degradation. Such as, substrate concentration, temperature, pH, inoculum size, carbon starvation, carbon adaptation, and nitrogen sources, the microbial population in a given site may not have the capability to degrade all the CBA compounds (Khleifat, 2006a, 2006b, 2006c).

In order to overcome these adverse effects, some technologies capable of breaking down these compounds have been developed by engineers. One technology, which uses aerobic conditions in which oxygen is the electron acceptor, is simply a metabolism of aromatic compounds by microorganisms. During this aerobic metabolism of aromatic compounds, the microorganisms use molecular oxygen to hydroxylate aromatic compounds and to perform oxidative cleavage of the aromatic ring (Assinder and Williams, 1990; Duan et al., 2019; Stiborova et al., 2015). During the hydroxylation and cleavage of aromatic compounds, microorganisms produce enzymes called monooxygenases or dioxygenases that transform aromatic compounds into central intermediates such as catechol (1,2-dihydroxybenzene), protocatechuate (3,4-dihydroxybenzoate), and gentisate (2,5-dihydroxybenzoate). These intermediates are then cleaved by dioxygenases (Domaradzka et al., 2015; Gupta et al., 2015).

On the other hand, a technology based on anaerobic conditions, in which light or inorganic electron acceptors such as nitrate, sulfate, and carbon dioxide are used, is also used to degrade aromatic compounds. During anaerobic metabolism, microorganisms oxidize aromatic compounds to carbon dioxide mainly via benzoyl-CoA, the most common central intermediate, which is dearomatized by the enzyme called benzoyl-CoA reductase and via other central intermediates such as resorcinol (1,3-dihydroxybenzene) and phloroglucinol (1,3,5-trihydroxybenzene) (Adelaja et al., 2015; Ghattas et al., 2017). Of the electron acceptors used in the anaerobic metabolism of aromatic compounds, nitrate has been paid the most attention because nitrate is naturally produced in wastewater treatment plants using the nitrification process. Another reason that nitrate is commonly used as the electron acceptor in full-scale wastewater treatment plants is that sulfate-reducing microorganisms need very strict reducing environments, which are relatively expensive to maintain(Schink et al., 2000).

Bacteria, usually display different activities like resistance to antimicrobial agents and essential oils of medicinal plants (Qaralleh et al., 2009; Tarawneh et al., 2009), heavy metals bioremediation (Abboud et al., 2009; Aljundi and Khleifat, 2010; Khleifat et al., 2009), whey disposal (Abboud et al., 2010) and biodegradability of highly toxic compounds such as phenol, 2-CBA and detergents (Khleifat et al., 2008). These activities could be chromosomal or plasmid dependent (Khleifat et al., 2009). *A. calcoaceticus* has formerly been isolated from the wastewater treatment plant in Petra, Jordan (Khleifat et al., 2009). In this study, the degradation of 2-chlorobenzoic acid (2-CBA) by *A. calcoaceticus* was investigated for the first time under altered growth conditions. The conditions comprised cell densities, pH, incubation temperature and additional different carbon and nitrogen sources. Growth kinetics was determined by using a mathematical model.

MATERIALS AND METHODS

Bacterial Strains

The bacterium *A. calcoaceticus* used in this study was already isolated from the wastewater treatment plant in Jordan (Abboud et al., 2007). The bacterium was identified using the REMEL kit (RapIDTM ONE and RapIDTM NF Plus systems) procedure for biochemical characteristics.

Media and culture conditions

Nutrient broth (NB) was used in this experiments. NB composed 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 K2HPO4 (10 mM), NaH2PO4 (3 mM), (NH4)2SO4 (10 mM), and MgSO4 (1 mM). Minimal salt medium (MSM) contained trace elements with final concentrations (in milligrams per liter) as follow: CaSO4, 2; FeSO4 $7H_2O$, 2; MnSO4.H2O, 0.2; $CuSO_4$, 0.2; ZnSO4.7H2O, 0.2; COSO4. 7H2O, 0.1; NaMoO 4.2 H_2O, 0.1; H_3BO_3,

0.05. The impact of various nitrogen sources on the 2-CBA degradation was measured after modifying the MSM broth medium by eliminating the ammonium sulphate from MSM. Nitrogen sources involved yeast extract, l-prolinee, casein and trypton that were added separately at concentration of 0.2% (w/v) whereas carbon sources include glucose, fructose, sorbitol, maltose, lactose and sucrose (Khleifat, 2010; Khleifat et al., 2010).

Analytical procedures

Chloride determination.

Inorganic chloride was determined turbidimetrically by measuring AgCl precipitation. Samples (1ml) were acidified with (10 µl) of 10 N H_2SO_4 and centrifuged at 14.000 rpm for 5 min to remove material that precipitated due to acidification alone. Precipitation of AgCl was then measured by adding (10µl) of 0.1 M $AgNO_3$ (in 5 M H_3PO_4) and immediately measuring absorbance at A_{525} by using (UV/VIS spectrometer perkinelmer lambda 25). Chloride concentration was quantified by reference to a standard curve that was linear from 0.5 to 4 mM (Hickey and Focht, 1990; Khleifat et al., 2015).

Effect of different growth conditions on the 2-CBA biodegradation ability

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

Cells were grown on MSM medium supplemented with different concentrations of 2-CBA (2mM, 3mM and 4mM). The culture was incubated at 37ºC, pH 7 and shaked at 150 rpm overnight. Different pHs (6, 7, 8 and 9) of the growth media were used to test the effect of variations in pH on the degradation ability of 2-chlorobenzoic acid by the same bacterium. The effect of different incubation temperatures (25, 30, and 37°C) upon the degradation of 2-chlorobenzoic acid by bacteria was also investigated.

Effect of cell density on 2-CBA degradation

A. calcoaceticus cells were obtained logarithmically as previously reported. Cell concentrations based on OD600 nm values were set to be 0.1, 0.2, 0.3 and 0.4 expressed in 0.5X, 1X, 1.5X and 2X to follow the effect of cell density on 2-CBA degradation. Bacterial cells of *A. calcoaceticus* were cultured in the MSM medium containing 2 mM of 2-CBA at 37 ° C, with a shaking rate of 150 rpm. The range of 2-CBA biodegradation was scrutinized through the estimation of 2-CBA disappearance as a role per time, as reported before (Abboud et al., 2010; Khleifat et al., 2008).

Enzyme assay

Cells were grown in 1.5 liters of nutrient broth for (24 hours), then they centrifugation at (6.000 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 6.000 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).

RESULTS

Biodegradation of 2-CBA by *Acinetobacter calcoaceticus*:

In this study, the bacterium *Acinetobacter calcoaceticus* was isolated from the City of Petra which is located in southern Jordan. In the course of this study a four substituted chlorobenzoic acid compounds were tested for their biodegradability by this bacterium. These compounds include 2-chlorobenzoic acid (2-CBA), 3- chlorobenzoic acid (3-CBA), 4-chlorobenzoic acid (4-CBA) and 3,4-dichlorobenzoic acid compounds (3,4-CBA). Our results showed that *A. calcoaceticus* could use these four different as carbon and energy sources with different extents (Table 1). The uninoculated culture was used as a negative control to assess if CBAs could be degraded, however, there was no degradation activity revealed, which in turn supported the results of biodegradation activity that was achieved by *A. calcoaceticus* cells. *A. calcoaceticus* showed ability of degrading all four chlorobenzoate substrates with different rates. The degradation rates were 49.5, 55, 10µM/h and 5 for 2-CBA, 3-CBA, 4-CBA and 3,4-CBA, respectively (Table 1). 2-CBA was used for the rest of the optimization experiments because the 2-CBA is environmentally the most toxic one as compared with the rest compounds tested. The experiments included substrate concentration, temperature, pH, inoculum size, carbon starvation, carbon adaptation, and carbon and nitrogen sources.

Table 1. Degradation of 2-CBA, 3-CBA , 4-CBA and 3,4-CBA by *A. calcoaceticus*

Substrate	Growth (OD_{600})	CL released (mM)	Degradation rates (µM/h)
2-CBA	0.7	1.75	49.5
3-CBA	0.32	0.70	55
4-CBA	0.38	0.80	10
3,4-CBA	0.28	0.30	10

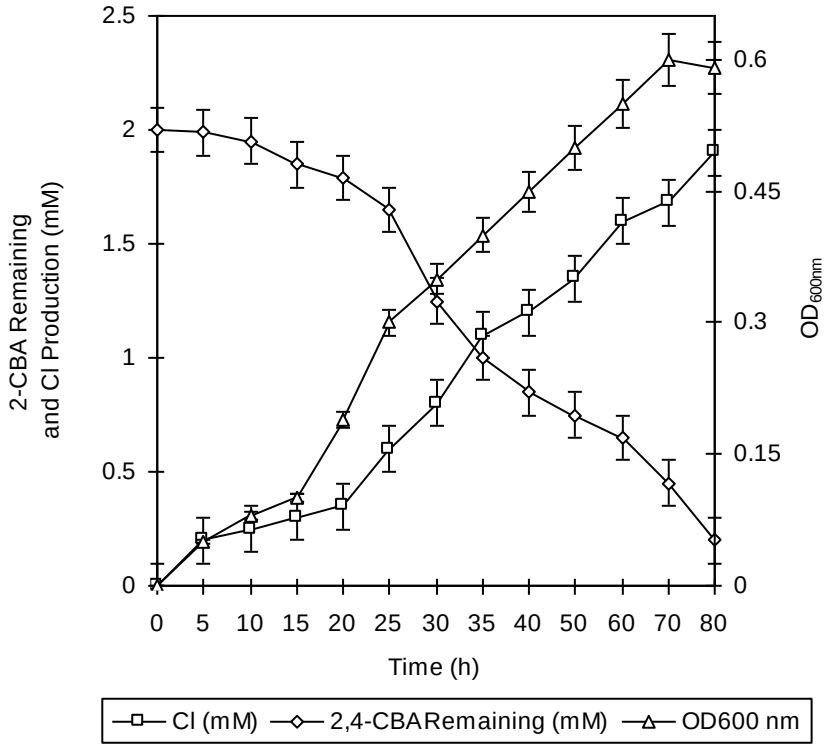


Figure 1. Degradation of 2-CBA by *A. calcoaceticus* as a function of time.

Effect of substrate concentration

The complete degradation of 2mM 2-CBA was completed after 80 hours of incubation time (Fig. 1). The growth reaches 0.6 OD at 600 nm with a degradation rate of 49.5 µM/h was achieved. The associated increase in degradation and growth of bacterial cells were also supported by data of chloride release. When using 3mM 2-CBA, a 45µM/h degradation was achieved with growth rate of 23 h^{-1} (Fig. 2), Moreover, the consumption of 2-CBA was paralleled with data results of chloride release. Raising the 2-CBA concentration to 4 mM, a less degradation rate (10µM/h) and chloride production was also noticed. In the same way, the OD600nm measurements, which represent the growth, was also elevated to almost 1.0 OD600nm (data not shown).

Growth Kinetics

Ten diverse primary 2-CBA concentrations (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 mM) were used. The increase of initial concentration of 2-CBA caused increase in the growth rate of *A. calcoaceticus* up to a value of 0.25 h-1 (Fig. 2) then beyond this concentration the bacterial cells started to decrease with further increasing the concentration of 2-CBA. This decrease is due to the inhibitory feature of cells that was occurred as a function of increase in the 2-CBA concentration. Numerous kinetic models were tried to conclude such tendency however; Haldane model is the best one that could be applied to characterize the inhibitory effect, which carries the form of following equation.

$$\mu = \frac{\mu_{\max} C_s}{K_I + C_s + C_s^2 / K_p}$$

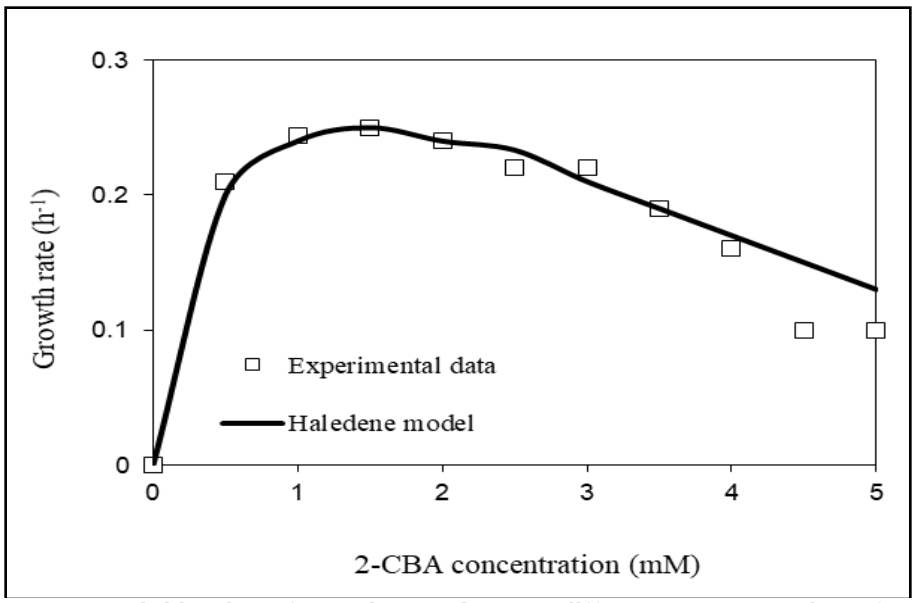


Figure 2; Growth kinetics of *A. calcoaceticus* on different concentration of 2-CBA

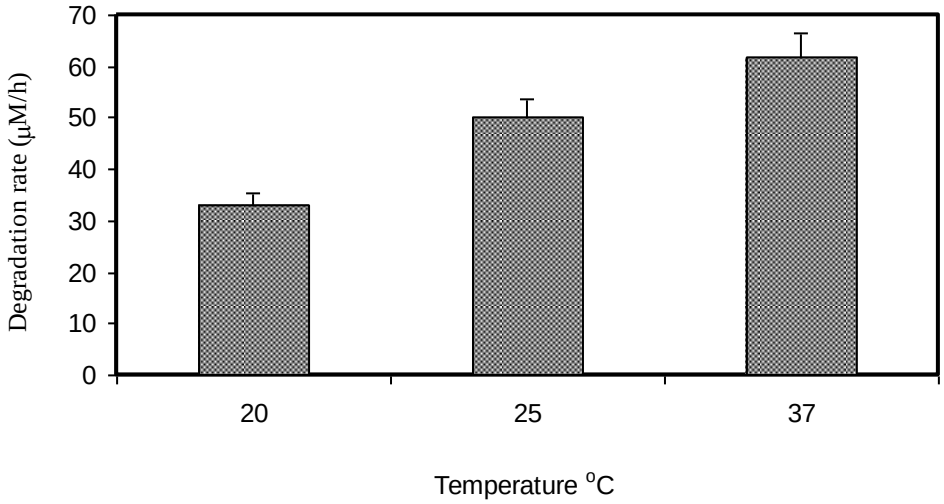


Figure 3; **Effect of incubation temperature on the relative biodegradation percentage by *A. calcoaceticus***

Effect of Incubation Temperature

Three different incubation temperature (20, 25, and 37°C) were tested to assess their effect on the biodegradation of 2-CBA. The experimental data on the degradation percentage at different incubation temperature (Fig 3) showed significant difference in percentage degradation of the 2-CBA. There was significant difference in percentage degradation of the 2-CBA. The incubation temperature clearly became vital below 37 °C. The effect of incubation temperature on the degradation percentage, chlorine production as well as the cell mass was clearly similar. As a result, it appears that biodegradation of 2-CBA could come about at all examined incubating temperatures; with 37 °C is being the best temperature for growing of *A. calcoaceticus* cells and 2-CBA biodegradation.

Effect of pH of Culture Media on Biodegradation of 2-CBA

A. calcoaceticus could degrade 2-CBA at a wide range of pH (Fig 4), from 6 to 9, with an optimum value of 7. It is possible that the enzymes for 2-CBA degradation have their optimum enzymatic activity at pH 7. It was reported that optimum pH for the biodegradation

of different aromatic compounds was different from one bacterium to another.

Effect of Carbon and Nitrogen Sources

Effect of different carbon source such as lactose, maltose, sucrose, fructose and sorbitol at fixed concentration (0.2%) and glucose with different concentrations. In addition, nitrogen source such as sorbitol, fructose and their combinations at 0.2% concentration, were utilized to find out their efficiency to degrade the 2- CBA. In table 2 showed the 2-CBA degradation rates proceed faster at ranges between 1.2- and 1.5-fold higher than that of the control. The simultaneous addition of sorbitol and fructose caused complete degradation of 2-CBA within 70 h incubation. In all cases the growth of *A. calcoaceticus* cells was in accordance with the standard microbial batch culture. At the same time, glucose, maltose and sucrose led to the decrease of the 2-CBA biodegradation. Nitrogen sources (Table 3 and figs. 5a-c) supplied caused a repression in 2-CBA degradation by 2.8, 1.4, 3.3 and 3.3 for yeast extract, L-proline, casein and trypton, respectively. The *ortho* CBA 1,2-dioxygenase in *A. calcoaceticus* has been detected indicating that the chloroaromatic ring fission occurs through the ortho pathways, not through the meta pathways (Fig. 6). lactose, maltose, sucrose, fructose and sorbitol and glucose at fixed concentration (0.2%). Sorbitol, fructose and their combinations at 0.2% concentration, as additional carbon sources.

Effect of carbon starvation on 2-CBA degradation

A two mM of 2-CBA degradation by pre-starved *Acinetobacter calcoaceticus* cells was shown to be faster than that of non-starved cells in both the logarithmic and stationary phases (Figs. 7a-c). The starved cells required only 20 h to achieve the complete degradation percentage compared to the control cells that were achieved within 40-60h (Fig. 1). Meantime, the cell growth exhibit similar profile in reaching the stationary phase earlier than the control (within less than 20 h). The 2mM 2-CBA, while the non-starved cells needed longer time ranges, of between 48 and 60 h. When our isolate was pre-starved for 24 and 48 h, the degradation ability of 2-CBA had clearly commenced after a shorter acclimation period, become slightly faster, and was completely accomplished in a shorter time, compared with that of the non-starved cells.

Effect of cell density on 2-CBA degradation

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 (Fig 8 a and b) with three cell densities (0.5, 1.0, 1.5 and 2 OD_{600}) expressed as 0.5. 1, 1.5 and 2X, the same induction time was shown for the four cases. However, those four cell densities caused different degradation rates measured as ppm/h. Also last 2-CBA degradation results were achieved over different periods. Thus starting inocula mass was found to be a major aspect in the time required for total 2-CBA degradation. The time required for complete degradation was reduced to 40 h of incubation. Although best size of starting inocula for biodegradation ability of *A. calcoaceticus* was 2X whereas best growth obtained for same cells was with inoculum size of 1X. We observe from the results that the application of all conditions optimally led to a complete biodegradation in 40 hours instead of 70-80 in most cases. This confirms that the cell volume is the key factor in obtaining the best results (Fig. 9)

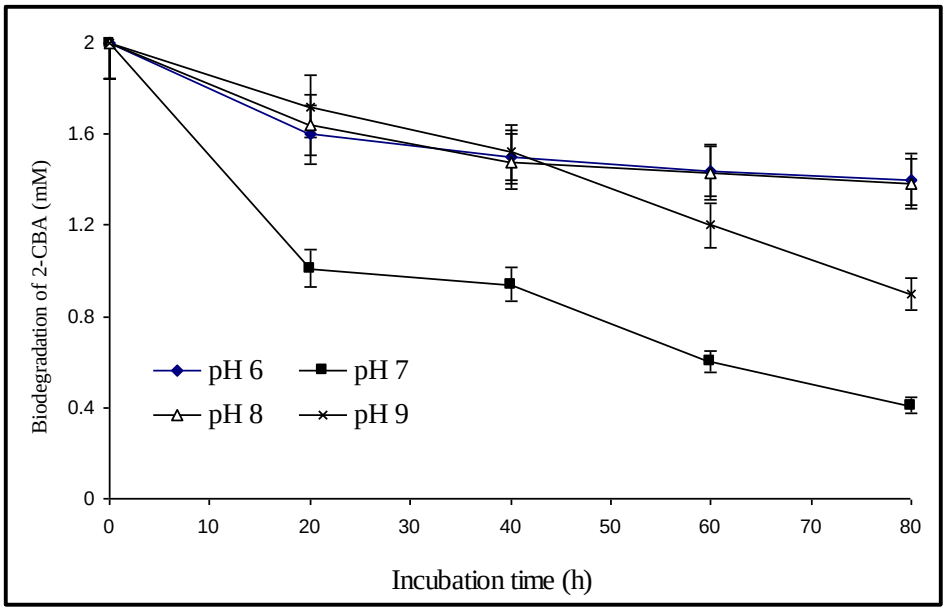


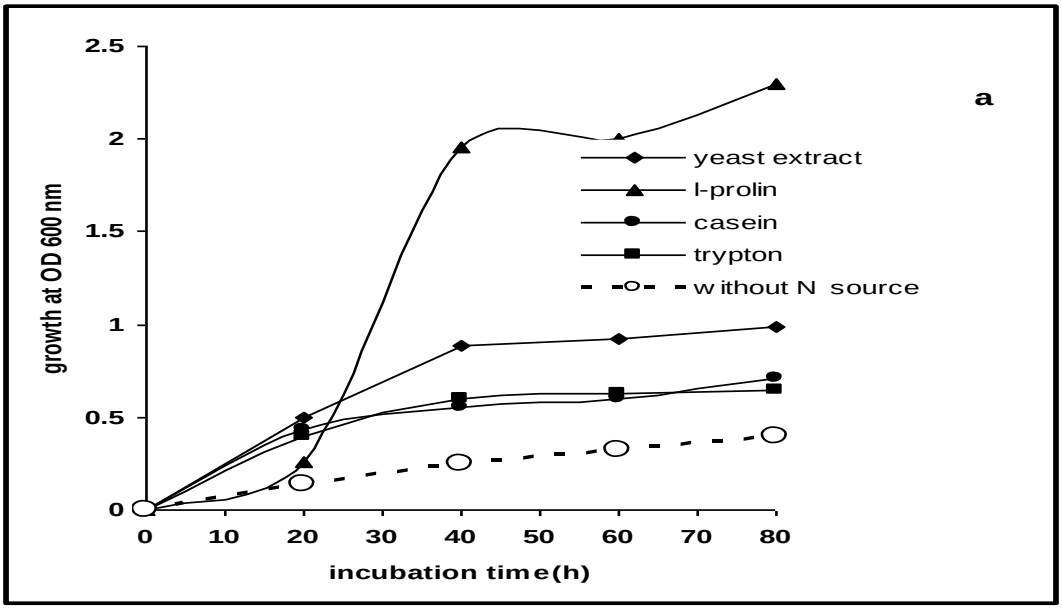
Figure 4: Effect of reaction pH on the relative biodegradation percentage as a function of time by *A. calcoaceticus*

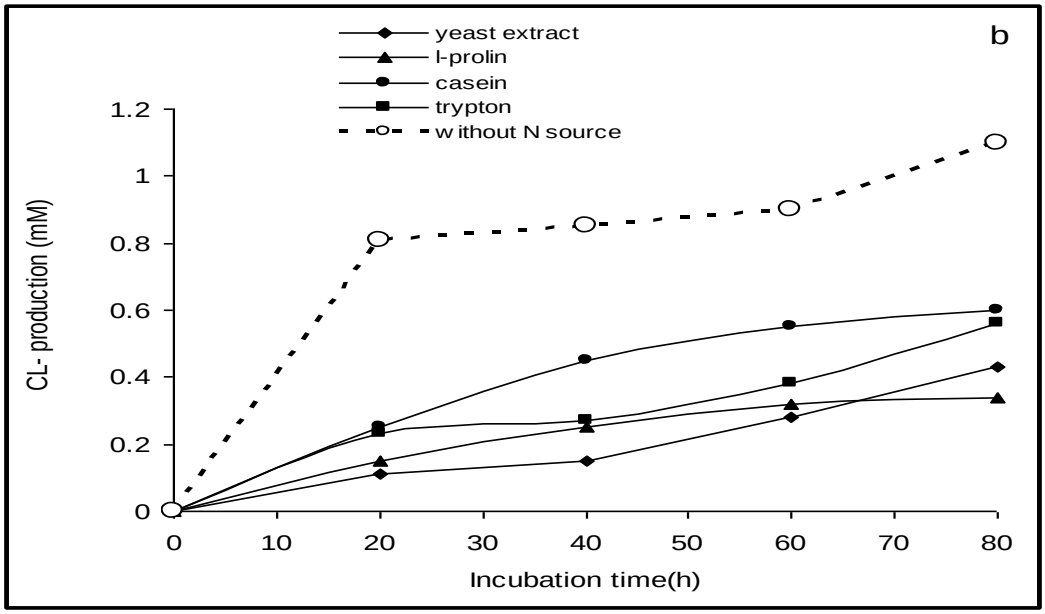
Table 2: Effect of different carbon source on the degradation rate of 2-CBA by *A. calcoaceticus* cells

Carbon source	Degradation rate (µM/h)	Cell mass (OD_{600})
Control	49.5	0.6
Glucose	35	0.63
Fructose	60.5	0.52
Lactose	48	0.68
Sucrose	40	0.80
Maltose	36.5	0.82
Fructose+ Sorbitol	73.2	0.70
Mannitol	48	0.84
Sorbitol	58	1.65

Table 3: Effect of different nitrogen source on the degradation rate of 2-CBA by *A. calcoaceticus* cells

Nitrogen source	Rate of degradation µm/h
Control	49.5
Yeast extract	18
l-prolinee	37
Casein	16.5
Trypton	15


a
growth at OD 600 nm
incubation time(h)
yeast extract
l-prolin
casein
trypton
without N source
0
0.5
1
1.5
2
2.5
0
10
20
30
40
50
60
70
80


b
CL- production (mM)
Incubation time(h)
yeast extract
l-prolin
casein
trypton
without N source
0
0.2
0.4
0.6
0.8
1
1.2
0
10
20
30
40
50
60
70
80

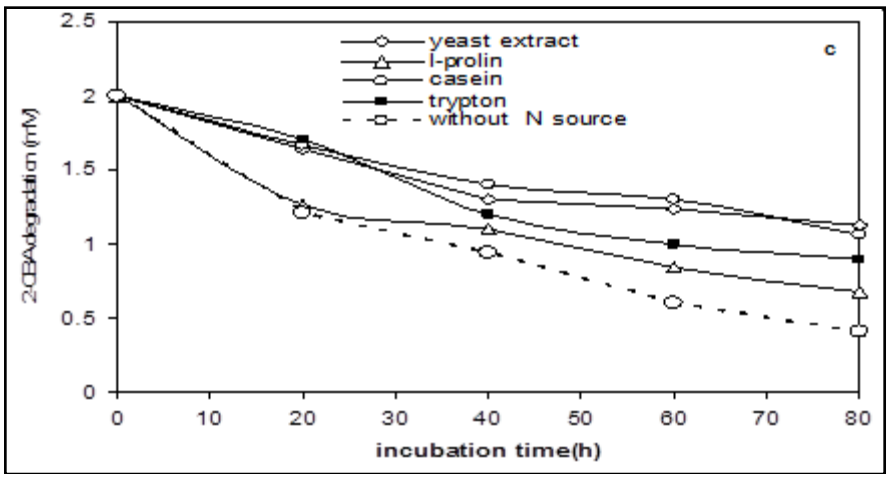


Figure 5; Effect of different nitrogen source on the degradation rate of 2-CBA by *A. calcoaceticus* cells (a) cell mass based on OD_{600} nm, (b) Cl production and (c) 2-CBA biodegradation

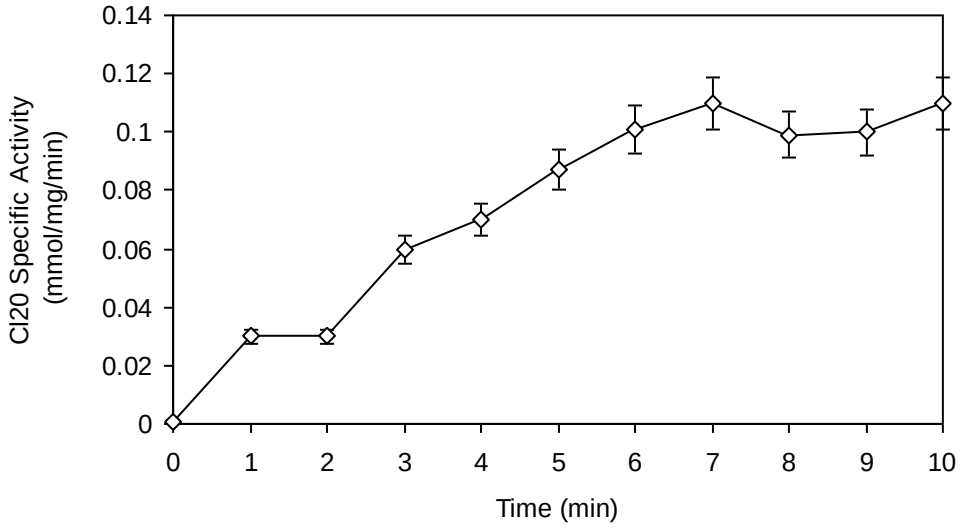
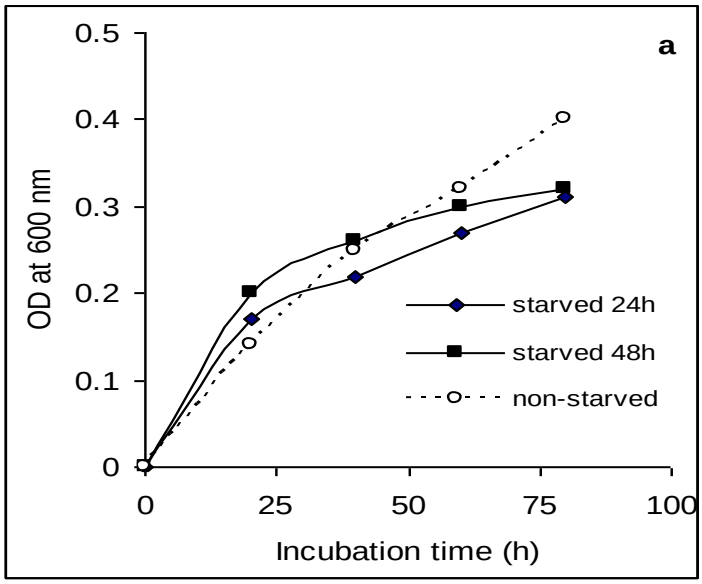
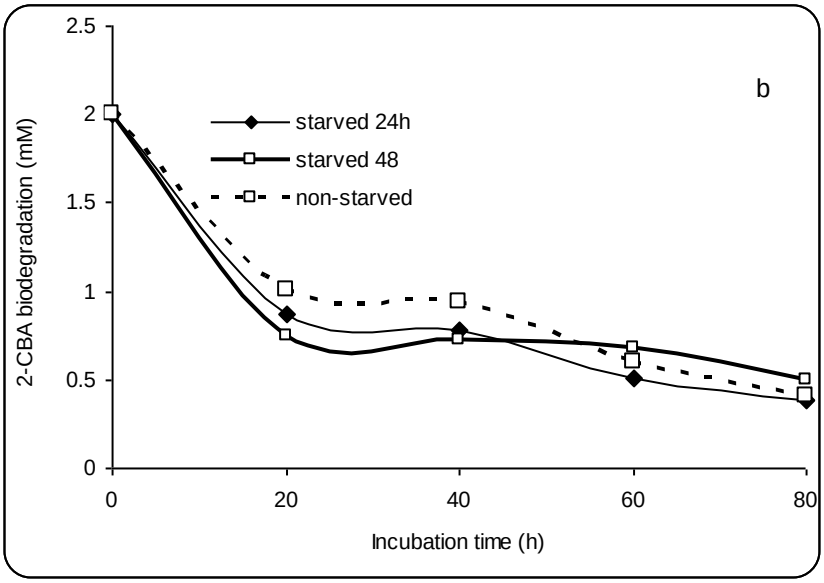


Figure 6; Measurement of Cl20 activity for the cell extracts of *A. calcoaceticus* grown in nutrient broth plus 0.5 mM 2-CBA. All data are average of three trials with error bars indicate standard deviations; where not visible they are smaller than the diameters of the points.


a
OD at 600 nm
0.5
0.4
0.3
0.2
0.1
0
0
25
50
75
100
Incubation time (h)
starved 24h
starved 48h
non-starved


b
2-CBA biodegradation (mM)
2.5
2
1.5
1
0.5
0
0
20
40
60
80
Incubation time (h)
starved 24h
starved 48
non-starved

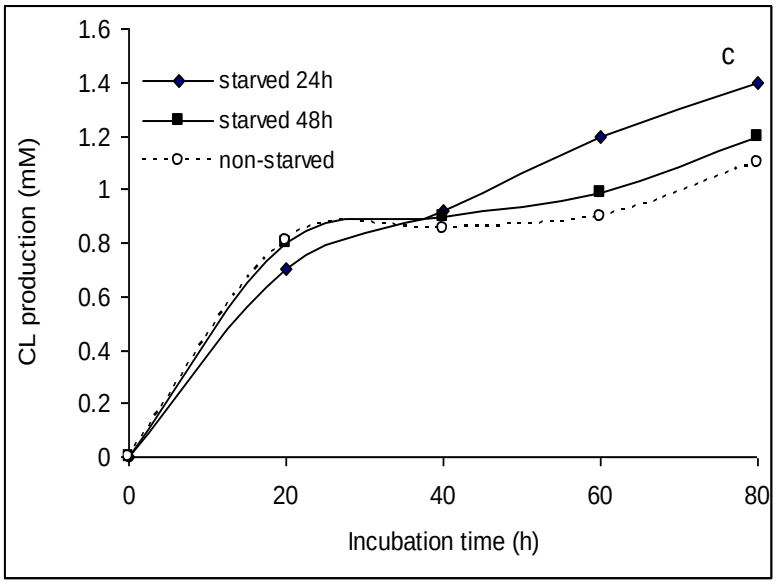
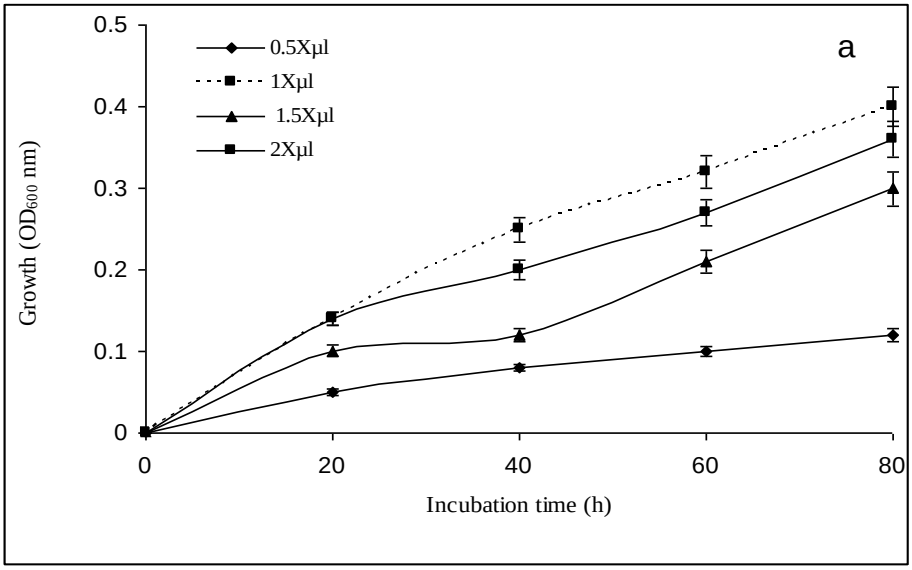


Figure 7; Effect of carbon starvation on the degradation rate of 2-CBA by *A. calcoaceticus* cells (a) cell mass based on OD_{600} nm, (b) Cl production and (c) 2-CBA biodegradation



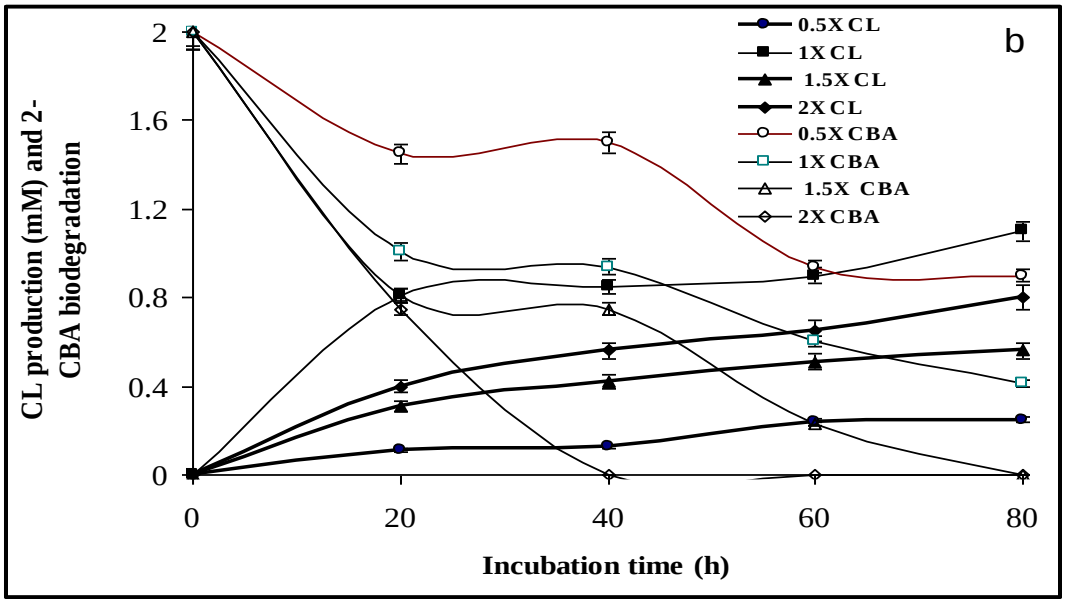


Figure 8. Effect of cell volume on the degradation rate of 2-CBA by *A. calcoaceticus* cells (a) cell mass based on OD_{600} nm, (b) Cl production and 2-CBA biodegradation

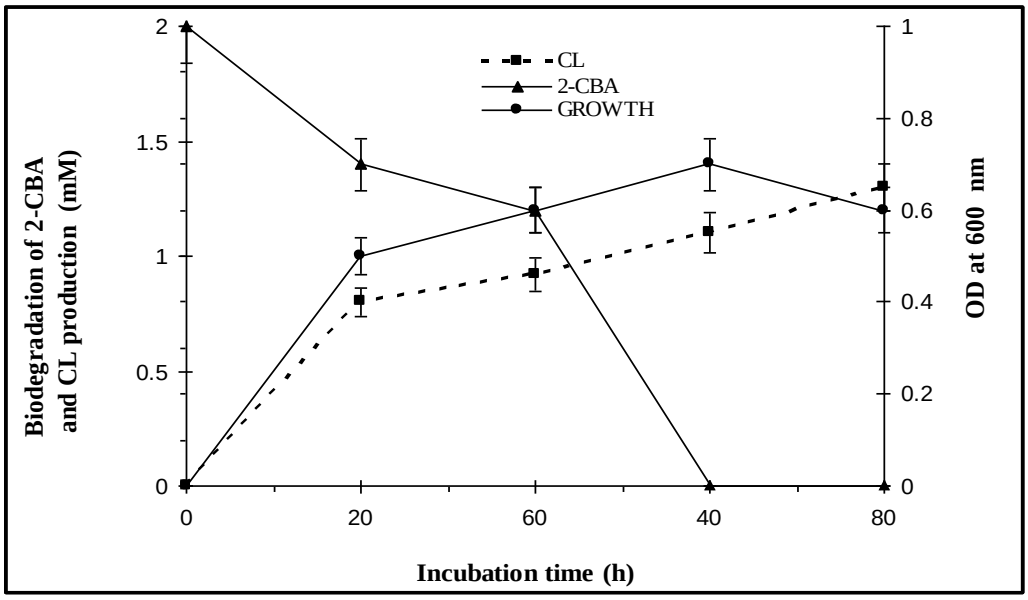


Figure 9; Effect of collectively optimal conditions on the biodegradation of 2-CBA by *A. calcoaceticus* cells

DISCUSSIONS

Several researches about the biodegradation of chlorobenzoates had formerly been reported. However, no simultaneous studies regarding substituted chlorobenzoic acid biodegradation by a pure culture of *A. calcoaceticus* was referred. It was reported that *A. calcoaceticus* cells are well known in being implicated in the biodegradation of different pollutants such as biphenyl or chlorobiphenyl, aniline, phenol, benzoate, crude oil and acetonitrile[40] or as a member of two-facultative anaerobic bacterial consortium in the degradation of some surfactants such as linear alkylbenzene sulfonate (LAS) (Khleifat, 2006a, 2006b). In this survey, *A. calcoaceticus* bacterium

was isolated from the City of Petra, which is located in southern Jordan. Frequently, investigators secluded chlorobenzoate degrading bacteria from polluted sites of agricultural or industrially stations. As it is noted, the results obtained from this work contradict with the said of investigators who hypothesized that free-chloro-compounds environments do not harbor chloroaromatic degraders (Banks, 1994; Brunsbach and Reineke, 1993; Fulthorpe et al., 1996; McCullar et al., 1994). On the contrary, Fulthorpe et al., (1996) reported that microbial populations in environments have not been exposed to chloro-compounds was prevalent in the ability of using these compounds as carbon and energy sources. The substituted chloroaromatic degraders was also isolated from two pristine soils indicating that 2-, 3-, and 4-chlorobenzoate are degraded by different bacterial populations[45]. Our results showed that *A. calcoaceticus* was capable of using these four different compounds (2-CBA, 3-CBA, 4-CBA and 3,4-CBA) as carbon and energy sources with different extents. As mentioned in Materials and Methods section it should be point out that the degradation ability was tested through measuring the discharge of chloride, removal of the substrate and eventually the bacterial cells growth on that substrate (Adebusoye et al., 2008). To warrant the utilizing of these compounds as carbon and energy sources by this bacterium, a minimal medium that had these organic compounds as only carbon sources was implied and thus the forming of any cell mass will be a result of consumption for these substances(Khleifat, 2006a, 2006c). In the exploratory screening, there were 3 different concentrations used from each constituent; 2, 3 and 4mM as reported with several studies (Marks et al., 1984; Yun et al., 2007). The four substituted CBA substrates were revealed in being degradable by tested bacterium *A. calcoaceticus* for different extents of rates. The biodegradation rates obtained were 49.5, 55, 10 and 5 µM/h for 2-CBA, 3-CBA, 4-CBA and 3,4-CBA, respectively. 2-CBA was selected for further optimization experiments in view of the fact that 2-CBA is the utmost toxic one through these tested compounds (Champagne et al., 1998). The optimization experiments were consisted of incubation temperature, substrate concentration, pH, inoculum size, carbon starvation, carbon adaptation, and carbon and nitrogen sources. It was observed that increasing the initial concentration of 2-CBA caused the growth rate to increase upto 0.25 h^{-1} beyond which growth rate started to decrease with further increasing the concentration of 2-CBA. This is due to the feature of cells inhibition that was occurred with additional increase in the 2-CBA concentration (Tros et al., 1996). The biodegradation of 2-CBA appear to be occurred at room temperature; whereas 37 °C is the best incubation temperature for *A. calcoaceticus* cells. So incubation temperature obviously had a physiologically vigorous influence on the metabolic track of the 2-CBA compound, thus mesophilic temperature (37 °C) produced best conditions for the degradation, or this could be entirely the effect of a temperature on activities of related enzyme (Leven and Schnürer, 2005). It has been mentioned that the incubation temperature likely function an equal or more substantial role than the plenty of nutrient during the degradation of benzoic acid and phenol (Margesin and Schinner, 1997; Onysko et al., 2000). Alteration of pH of the 2-CBA containing uninoculated culture caused no impact on the amount of existed 2-CBA. The *A. calcoaceticus* optimally degrades 2-CBA at a pH of 7.0. It is likely that the 2-CBA degrading enzymes have their most favorable degradation activities at pH 7.0. The best convenient pH for the biodegradation activity of different aromatic compounds was different from one bacterium to another, for instance, the best pH for biodegradation of 4-CBA by *Arthrobacter* is 6.8 (Marks et al., 1984); the optimum pH for biodegradation of phenol and catechol by the bacterium *Halomonas campisalis* ranges between 8 and 11 (Alva and Peyton, 2003) and best pH for phenol biodegradation by *Klebsiella oxytoca* was 6.8)Shawabkeh et al., 2007.(

The carbon sources Sorbitol, fructose and their combinations at fixed concentration (0.2%) allowed 2-CBA degradation rates to proceed faster at ranges between 1.2- and 1.5-fold higher than that of the control. The simultaneous addition of sorbitol and fructose caused reduction in the time required for complete degradation of 2-CBA from 80 h to 70 h of incubation time. In all cases the growth of *A. calcoaceticus* cells was in conformity with the standard batch microbial culture (Ampe et al., 1998; K-C Loh and Tan, 2000). Fructose and sorbitol, however, caused almost growth inhibition which reflects that the procedure of growth inhibition by fructose and sorbitol in *A. calcoaceticus* is intermediated through a regulation controlled by fructose (Tapiainen et al., 2001). Degradation of 2-CBA was repressed by glucose, lactose, maltose, sucrose and mannitol although the increase of

cell biomass; this could be an effect of catabolite repression caused by these carbon sources (Khleifat et al., 2006). The same result was shown in the study of the growth of *Ralstonia eutropha*, in which the cells grown on fructose plus phenol reduced the rate of respiration, in contrast with the culture media that contain phenol alone (Léonard and Lindley, 1999). Similar situation occurs during phenol biodegradation with other bacteria has been shown with different carbon sources, such as acetate (Ampe et al., 1998) and glucose (K-C Loh and Tan, 2000). Different glucose concentrations (data not shown) were also used as additional carbon sources (0.1, 0.15, 0.2 and 0.25%). All glucose concentration tested resulted in the inhibition of biodegradation rate, chloride production and bacterial growth (data not shown). There are several other reports of pH regulation in glucose- and organic pollutant-containing media(Fakhruddin and Quilty, 2005; Wang and Loh, 1999). Glucose supplementation of the medium above certain limit caused a big decline in pH and inhibition of substrate degradation stipulating the function of pH in the biodegradation of 2-CBA by this bacterium (Banta and Kahlon, 2007). Earlier studies have reported that an ideal amount of yeast extract should be supplemented to obtain the optimal biodegradation rate of phenol (K-C Loh and Tan, 2000). Topp et al., 1988, talked about an ideal amount of carbon supplemented for the degradation of pentachlorophenol. The reason for enhancing the degradation rate of 2-CBA by *A. calcoaceticus* can be attributed to alleviate the toxicity of 2-CBA by accessible nutrients and thus the origination of more cell mass (Kai-Chee Loh and Wang, 1997; Topp et al., 1988). At least four different aerobic routes for CBA mineralization, and a fifth track in Alcaligenes sp. strain L6 (via gentisate and not involving (chloro) catechol) have been identified8. The first step in the ortho-dehalogenation pathway path was the dihydroxylation of hydroxyl by ortho-CBA 1,2-dioxygenase, followed by the removal of halogenation step. This leads to 2-CBA conversion to catechol, which is easily metabolized to muconate(Romanov and Hausinger, 1994). In the modified orthocleavage path of 2-CBA it happens through oxidation by chlorocatechol 1,2-dioxygenase. The calcined chalkatecule is produced by chloro-CIS, CIS-Mooconate; followed by cycloisomerization accompanied by chloride release (Vollmer and Schlömann, 1995) .

The Ortho-cleaving of chlorocatechol leads to production of chloro-cis,cis-muconates; which is pursued by cycloisomerization concomitant with chloride release[18]. On conditions that meta-cleavage happen, 2-CBA is transformed to 3-chlorocatechol. After making the acylchloride from 3- chlorocatechol by catechol 2,3-dioxygenase causes inactivation of the catechol dioxygenases(Arensdorf and Focht, 1994). Thus distinguishing between the *meta* and *ortho* pathways, these known characteristic enzymes, C23O, were measured for the path of the meta and C12O for the ortho path. The activities of both enzymes were measured in *A. calcoaceticus* cells that were grown at M9 media that contain 0.5 mM 2-CBA. While the cleavage pathways of *ortho* (C120) and meta (C230), are known to be present together for the mineralization of chloro-aromatic compounds in many bacteria (Müller et al., 1996), in *A. calcoaceticus* strain only the *ortho* CBA 1,2-dioxygenase has been detected signifying that the fission of chloroaromatic ring is happening via the *ortho* pathways, not thru the *meta* cleavage tracks. Thus, lactose, maltose, sucrose, yeast extract, casein, trypton, casein can be converted into carbon and nitrogen sources and energy is packaged effortlessly which can provide selective improvement of the bacterial strain, retaining these substrates at an accurate level so as not to repress the 2-CBA *ortho* 1,2--dioxygenase simultaneously maintaining cellular growth capacity (Neumann et al., 2004). *A. calcoaceticus*, which suffers from a lack of response to other carbon sources in the medium /environments, can provide a great quality if this microorganism is used in the degradation of 2-CBA in a media containing microbial consortium such as wastewater or soil. Typically, the induction of 2-CBA degradation may occur when these sources of carbon and nitrogen are added as a result of increased cell biomass using more easily metabolized carbon and nitrogen sources such as fructose and sorbitol, which cause faster CBA-2 degradation rates after a short adaptation period of (Leung et al., 2005).

It has been shown that a 2 mM of 2-CBA degradation by the pre-starved cells of the *A. calcoaceticus* was faster than non-starved cells in both the logarithmic and stationary phases. Meanwhile, cell growth shows a similar image in access to the stationary phase at a time earlier than the control (in less than 20 hours). When *A. calcoaceticus* was pre-starved for 24 and 48 h, the ability of 2-CBA degradation began to develop clearly after a shorter adaptation period and became slightly faster, and was completed in a shorter time, compared to the ability of non-

starved cells. This may be the result of an early expression of the 2-CBA degrading genes (Reardon et al., 2002). In some situations, carbon starvation in *E. coli* causes the expression of peptide transporter protein (A, CstA), even if there are no stimuli of their own or because of the induced selective enzymes required for biodegradation (Leung et al., 2005). In general, the common carbon starvation response to undifferentiated gram-negative bacteria is an increase in their ability to assimilate environmentally produced nutrients (Leung et al., 2005). Carbon-starved cells are more sensitive to 2-CBA toxicity than non-starved cells. Perhaps the explanation for this is that the amount of ingredients (enzymes) in the cell are the same, and the difference is at the time of expression, ie early or late expression of the 2-CBA catabolic genes (Khleifat, 2006a). More attention should be paid to the problem.

Although best starting cell volume for biodegradation ability of *A. calcoaceticus* was 2X whereas best growth obtained for same cells was with inoculum size of 1X. Implementation of whole conditions optimally derived the achievement of complete biodegradation in shorter time (40 h) than of the most other cases. Our results are consistent with those previously described by others (Abboud et al., 2007; Khleifat, 2006b). This confirms that the cell volume is the key factor in obtaining the best results.

CONCLUSION

Based on the chloride released rate and enzymes activities, a pathway for degradation of 2-chlorobenzoic acids by that *A. calcoaceticus* was proposed. The results of optimization experiments confirm that the cell volume is one of the key factor in obtaining the best results.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

AAA and IKA contributed to the design of the experiments and performed the experimental work. IKA authors carried out laboratory tests. AAA wrote the manuscript, all authors revised and approval the final version.

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