Partial Purification and Characterization of 2,4-Dichlorophenoxyacetic Acid Degrading Bacteria Harboring Alpha Ketoglutarate Dioxgenase Enzyme

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Abstract

The catabolic adaptability demonstrated by microorganisms is important in the bioremediation of polluted environments. 2,4-dichlorophenoxyacetic acid/α-ketoglutarate dioxygenase is the first enzyme in the catabolic pathway for the 2,4-dichlorophenoxyacetic acid (2,4-D). In the present study, dioxygenase enzymes were inducible and the serial adaptation of four studied bacterial isolates steadily evolved strains exhibited higher and significantly different rates of 2,4-dichlorophenoxyacetic acid metabolism. For biomass production relative to the original starting strain, 2,4-dichlorophenoxyacetic acid/α-ketoglutarate dioxygenase was partially purified to apparent homogeneity from four bacterial isolates that were studied, using ammonium sulphate precipitation and gel filtration on sephadex G-100. 2,4-dichlorophenoxyacetic acid concentration resulted in a lower yield of the cell biomass of the *in vitro* culture of each of the bacterial isolates and consequently a lower enzyme yield. There was a gradual increase in the enzyme activity from the different bacterial isolates at pH 5.5, maximum enzymes activity at pH 7.5 and no activity at pH 10.0. Optimum temperature for enzyme activity was at 35°C but activity was reduced to zero at 60°C. In conclusion the four bacterial isolates and their cell-free extracts have the potential to bio-mineralized 2,4-dichlorophenoxyacetic acid..

Keywords: 2,4-Dichlorophenoxyacetic Acid; Dioxygenase; α-Ketoglutarate; Catalytic Properties; Enzyme Activity.

1. Introduction

Chlorinated aromatic compounds are environmental pollutants due to their widespread use in the ecosystem. 2,4-Dichlorophenoxyacetic acid (2,4-D) is a chlorinated phenoxy compound which functions as a systemic herbicide and is used to control many types of broadleaf weeds. The wide use of this compound has prompted interest in its biodegradation (Igbinosa, 2006; Igbinosa *et al.*, 2007a,b). *Alcaligenes eutrophus* and other bacteria can degrade 2,4-D through 2,4-dichlorophenol, 3,5-dichlorocatechol, and *ortho* cleavage of this catechol (Fukumori and Hausinger, 1993). Alternatively, *Axotobacter chroococcum* first removes the chloride in the 2 position to produce 4-chlorophenoxyacetate, 4-chlorophenol, and 4-chlorocatechol, again with *ortho* cleavage of this catechol (Balajee and Mahadevan, 1990). 2,4-D biodegradation, by this pathway branch, may produce a byproduct antibiotic protoanemonin, which can be degraded to cis-acetylacrylate by a dienelactone hydrolase of *Pseudomonas* sp. strain B13 (Brückmann *et al.*, 1998). *Comamonas testosteroni* JH5 can cleave 4-chlorocatechol by a *meta* pathway, forming 5-chloro-2-hydroxymuonate semi aldehyde which can be further transformed by a 2-hydroxymuconic semi-aldehyde dehydrogenase or a 2-hydroxymuconic semi-aldehyde hydrolase. Dehydrogenation is the major route, however; the hydrolase step is also of physiological significance. Both branches lead to 5-chloro-2-oxopent-4-eneoate (Selifonov, 1992). *Pseudomonas cepacia* P166 can move the chloride in 5-chloro-2-oxopent-4-eneoate through 5-chloro-4-hydroxy-2-oxopentanate to chloroacetate. Chloroacetate accumulates transiently, and stoichiometric dehalogenation is observed (Arensdorf and Focht, 1995).

Alcaligenes eutrophus JMP 134 is the most extensively studied 2,4-D degrader (Don and Pemberton, 1981). *A. eutrophus* JMP 134 catalyzes the conversion of 2,4-D to 2,4-dichlorophenol (2,4-DCP) by the action of the product of 2,4-dichlorophenoxyacetic acid/α-ketoglutarate dioxygenase (*tfdA*). TfdA catalyzes a hydroxylation reaction and it has been repeatedly reported to be 2,4-D monoxygenase, which in fact is not the case (Hausinger and Fukumori, 1995). Rather, *tfdA* is a Fe(II) and α-ketoglutarate-dependent dioxygenase. 2,4-D/α-ketoglutarate dioxygenase is an Fe(II) and α-ketoglutarate-dependent enzyme that catalyzes the first step in degradation of the herbicide 2,4-D. This enzyme

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couples the oxidative decarboxylation of α-ketoglutarate to the hydroxylation of a side chain carbon atom. The resultant hemiacetal spontaneously decomposes to form 2,4-dichlorophenol (2,4-DCP), succinate, glyoxalate and carbon dioxide (Hogan *et al.,* 2000). The enzyme *tfdA* possesses multiple essential histidine residues, whereas catalytically essential cysteine and lysine groups do not appear to be present (Hogan *et al.,* 2000). *TfdA* resembles numerous other *a*-ketoglutarate-dependent dioxygenases from plants, animals, fungi, and bacteria that catalyze similar hydroxylation reactions at inactivated carbon center. Petroleum degrading bacteria were isolated and biochemical characterizes by Sanni and Ajisebutu (2003a, b). Igbinosa *et al.* (2007a, b) assessed their potentials in the mineralization of 2,4-dichlorophenoxyacetic acid in an attempt to develop active bacterial strains for bioremediation of 2,4-D contaminated system. The present paper describes the partial purification and biochemical characterization of 2,4-D / α-ketoglutarate dioxgenase from four petroleum degrading bacteria.

2. Materials and Methods

2.1. Bacteria Strains and Culture Condition

Characterization of the bacterial strains (SOGU 11, SOGU 16, SERU 2 and SERU 11), identified as *Achromobacter* sp, *Corynebacterium* sp, *Pseudomonas* sp and *Arthobacter* sp, respectively, using a standard culture-based and biochemical reaction, was previously described by Sanni and Ajisebutu (2003a, b). All bacterial isolates were grown in Luria-Bertani (LB)-medium, which contained, per liter: 5g yeast extract, 10g casein peptone, 10g NaCl, pH was adjusted to 7.0 before autoclaving. Subsequently, 10% inocula from LB cultures were transferred to 50 mL freshly prepared Minimal Salts Medium (MSM) containing, per liter, 2.75g K_2HPO_4; 2.25g KH_2PO_4; 1.0g $(NH_4)_2SO_4$; 0.2g $MgCl_2.6H_2O$; 0.1g NaCl; 0.02g $FeCl_3.6H_2O$ and 0.01 $CaCl_2$ (pH 6.8 to7.0) with 5 mM 2,4-D as sole carbon and energy sources. Erlenmeyer flask containing 500 mL of the medium was incubated at 30°C on a rotary shaker at 200 rpm.

2.2. Serial Adaptation of Isolates Between Glucose and 2,4-Dichlorophenoxyaceic Acid (2,4-D)

The standardized bacterial isolates were inoculated into minimal salt medium enriched with 5 mM 2,4-D. The flasks containing the medium were incubated on a horizontal shaker at 200 rpm for 24 h at room temperature (28 ± 2°C). The cultures were later harvested as previously described by Igbinosa (2006) and the bacterial isolates were designated as A_o generation. These A_o generation bacteria were re-cultured in 5mM glucose-enriched medium, harvested and then used to inoculate another fresh medium enriched with 2,4-D. The cultures were then incubated and harvested to generate the A_1 generation. This procedure was repeated to produce the A_2, A_3, and A_4 generations and, in each case, the cultures were harvested by centrifuging at $3,500 \times g$ for 15 min, washed in sterile normal saline solution (0.85% w/v NaCl) and finally stored in sterile normal saline solution. The different generations were standardized to an optical density of 0.1 values using a spectrophotometer at 540 nm wavelength. The standardized A_o, A_1, A_2, A_3 and A_4 generations were then cultivated in another batch of minimal salts medium enriched with 5 mM 2,4-D. The flasks were incubated on horizontal shaker (200 rpm) at room temperature and sampled for growth of the bacterial isolates at different time intervals by taking the optical density reading. This was done using a spectrophotometer at 540 nm wavelength and the growth pattern monitored.

2.3. Assay of 2,4-D/α–ketoglutarate Dioxygenase Enzyme

The cells of each bacterial isolate were grown at 30°C in Peptone Tryptone Yeast extract Glucose Medium (PTYG Medium) This medium consists of 0.25g peptone, 0.25g tryptone, 0.5g yeast extract, 0.5g glucose, 0.03g $MgSO_4$, 0.0035g $CaCl_2$ per liter (pH 7.2) with 2.5 mM 2,4-D. Each of the bacterial cells was harvested by centrifugation at $6,000 \times g$ for 20 min and suspended in a solution containing 25 ml of 20 mM Tris, 1 mM EDTA, and 0.4 mg/l leupeptin (pH 7.2). The assay was carried out according to Fukumori and Hausinger (1993). A 0.2 mL of each of the suspended bacterial isolate solution above was added to 1.0 mM α–ketoglutarate, 50.0 μm ascorbate, 50.0 μm $(NH_4)_2$ Fe $(SO_4)_2$, and 1.0 mM 2,4-D in 10.0 mM imidazole buffer (pH 6.75), incubation was at 30°C for 1 min. The reaction was terminated with the addition of Ethylenediamine tetra acetic acid (EDTA). The absorbance was measured at 510 nm after the addition of 10.0 μL of 8% potassium ferricyanide. One unit of enzyme activity was defined as the amount of enzyme that formed 1.0 μmol of phenol derivative per minute at 30°C.

Enzyme activity unit [μmol phenol min^{-1} ml^{-1}]

$$= \frac{OD_{510} \times Df}{10\ \mu mol \times V \times t}$$

where OD_{510} = absorbance reading

V = Volume of enzyme used

t = time of incubation = 1 min

Df = Dilution factor of the enzyme solution

2.4. Protein Determination

Protein concentrations of the crude extract were determined by the method of Lowry *et al*. (1951). Protein concentration was extrapolated from the standard curve using Bovine Serum Albumin (BSA) as standard.

2.5. Extraction of the Crude Enzyme

The clear crude extract was brought to 15% ammonium sulphate saturation by the addition of solid ammonium sulphate (84 g/L). The precipitate formed was collected by centrifugation at $12,000 \times g$ for 20 min, and was discarded. The supernatant was taken up to 65% saturation by adding ammonium sulphate (350 g/L) over a period of 1 h with continuous stirring and left overnight. The resulting precipitate was collected by centrifugation at $6,000 \times g$ for 30 min and immediately dialyzed against several changes of 50 mM citrate buffer (pH 5.0) containing 10mM $Na_2S_2O_3$. The dialysate was centrifuged at $6,000 \times g$ for 30 min to remove insoluble material (Igbinosa, 2006).

2.6. Determination of Molecular Weight of the Crude Enzyme

The molecular weight of the native enzyme was estimated by gel filtration on a sephadex G-100 column (40 × 2.5cm) in 20 mM Tris, 1.0 mM EDTA, 100 mM NaCl (pH 7.2). The standard proteins used in calibrating the column were bovine serum albumin Mr, 68,000; 7.0 mg/mL, egg albumin Mr, 45,000; 10.0 mg/mL, trypsinogen Mr, 24,000; 10.0 mg/mL, catalase Mr, 58,000; 10.0 mg/mL. A volume of 2.0 mL of each of these protein standards was passed through the column in successions, at a flow rate of 40.0 mL/h. Fractions of 3.0 mL were collected and elution was monitored by measuring absorbance at 280 nm for each protein standard. The void volume (V_o) of the column was determined with Blue dextran 2000 (2.0 mg/mL) and its elution was monitored by measuring absorbance 600 nm. According to Andrew (1964), the K_{av} value of each eluted protein was calculated from the equation.

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

where V_e = elution volume of protein, which is measured from the start of the sample application to the inflection point (or the half height) of the rising part of the elution peak.

V_o = void volume, which is the elution volume of blue dextran.

V_t = total gel bed volume, calculated as the product of the cross sectional area and the bed height.

The K_{av} values of the protein standard were plotted against their corresponding molecular weight on a semi log graph sheet. The molecular weight of the enzyme was extrapolated from its K_{av} value.

2.7. Determination of Kinetic Parameters

The kinetic parameters (V_{max} and K_m) were determined for the crude enzyme 2,4-D/α-ketogluarate dioxygenase activity at various concentrations of 2,4-D from 0.01 mM - 2.0 mM. The apparent K_m and V_{max} of the enzyme for 2,4-D were determined by plotting the reciprocals of the initial velocities against the reciprocal of 2,4-D concentration (Lineweaver-Burk 1934).

2.8. Effect of pH and Temperature on Enzyme Activity

The effect of pH on the enzyme was determined by carrying out the assay of the enzyme using the following 3 buffers at different pH values, 0.2 M citrate buffer (pH 4.0 - 6.0), 0.2 M phosphate buffer (pH 6.5 - 8.5), and 0.2 M Borate buffer (pH 8.0 - 10.0) but at constant ion strength of 0.05 M, in assay mixture.
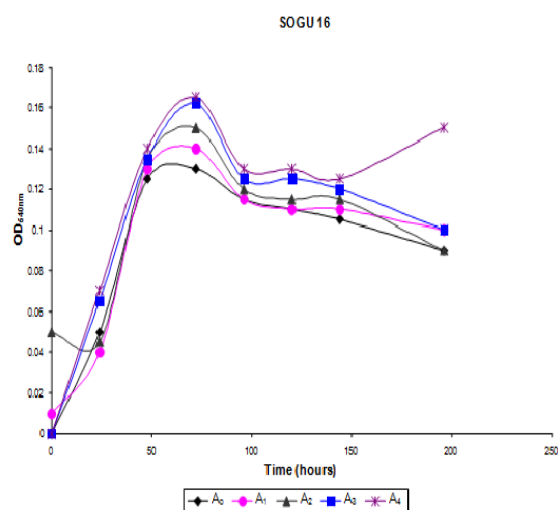
2.9. Stability of Enzyme Activity

For the pH stability test, the enzyme was incubated at different pH at 30°C for up to 40 h and the residual activity was measured after readjustment of the pH to 7.0. To determine the effect of temperature on the stability of the enzyme, the enzyme solution was incubated in 100mM (pH 7.0) buffer for up to 40 h at various temperatures between (4, 20, 40 and 60°C), then the residual enzyme activity was assayed. To examine the optimum temperature for enzyme activity, this was measured at various temperatures (20, 30, 35, 40, 45, 50 and 60°C).

3. Results and Discussion

3.1. Serial Adaptation of the Isolates to 2,4-D

The ability of each bacterial isolate to grow in the 2,4-D enriched media may be due to a selection process leading to the emergence of strain, which is subsequently able to resist the toxic effect of the compound. The successive growth yield in SOGU16 (*Corynebacterium* sp) showed highest yield of 0.165 cells/h that was observed in 5th generation at 72 h with the least yield being 0.00 cells/h that was observed in the 1st generation at zero hour (Fig. 1). In SOGU11 (*Achromobacter* sp), maximum yield of 0.165 cells/h was observed in the 5th generation at 72 h and a minimum yield of 0.01 cells/h in the 1st generation at zero hour (Figure 1). In SERU11 (*Arthrobacter* sp), isolate maximum growth yield of 0.150 cells/h was observed in the 5th generation at 120 h and a minimum yield of 0.05 cells/h in the 1st generation at zero hour (Figure 2). In SERU2 (*Pseudomonas* sp), isolate maximum growth yield of 0.185 cells/h was observed in the 3rd and 4th generation at 48 h while a minimum growth yield of 0.00 cells/h was observed in the 1st generation at zero hour (Figure 2). In all the isolates, there was no significant variation between the different generation times ($p < 0.05$). The ability of bacterial isolate to grow in the 2,4-dichlorophenoxyacetic acid enriched media may be due to a selection process leading to the emergence of strain (mutants) which are subsequently able to resist the toxic effect of the compound. It was reported that Dichloroacetic acid (DCA) inhibit *Pseudomonas putida* pp3 which are sensitive (DCA[S]), but DCA resistant mutants (DCA[R]) arise readily if the growth environments contains between 5 and 100 mM DCA. The results of the growth yield of different generations of the bacterial isolates obtained by serial adaptation to the 2,4-dichlorophenoxyacetic acid revealed that the subsequent generations obtained showed steady increase in rate of metabolism for biomass production through the series of generations. In general, the more distant the generation was from the original stains, the higher the yield became, which were significantly different ($p < 0.05$) from each other.
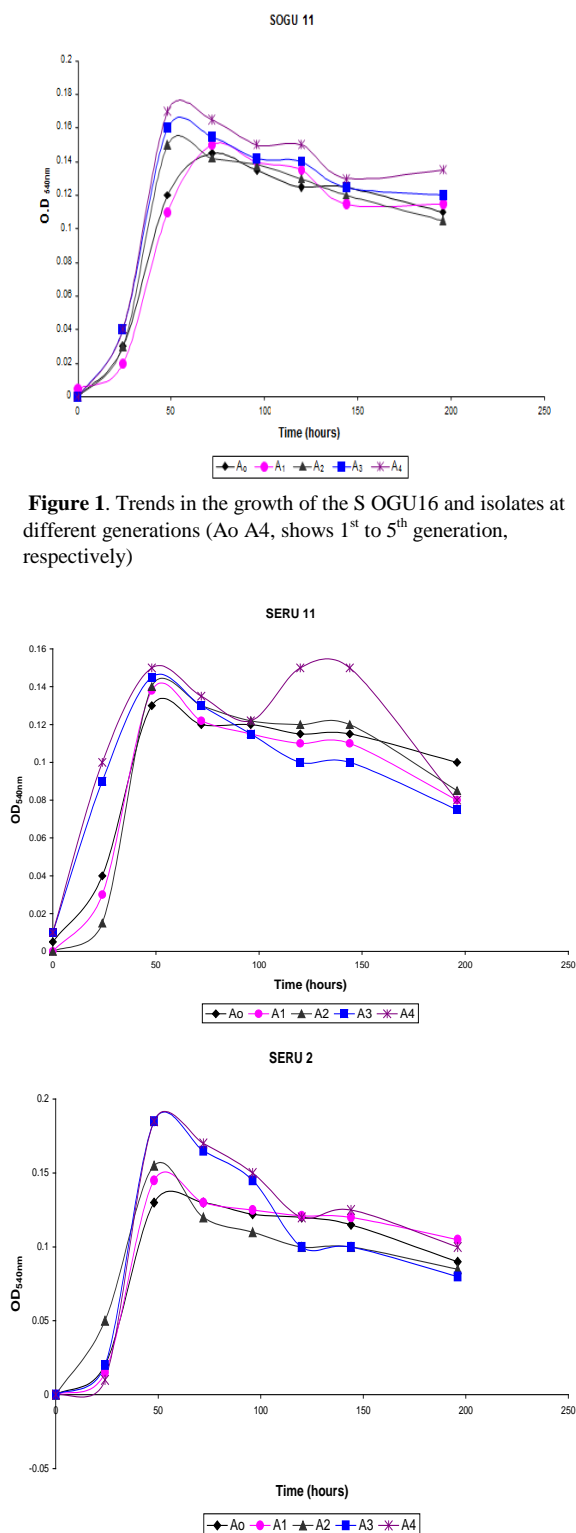
Figure 1. Trends in the growth of the S OGU16 and isolates at different generations (Ao A4, shows 1st to 5th generation, respectively)

Figure 2. Trends in the growth of the SERU 11 and SERU 2 isolates at different generations (Ao – A4, shows 1st to 5th generation, respectively).

3.2. Partial Purification of the Crude Enzyme

A summary of the result of the partial purification of the 2,4-D\α-ketoglutarate dioxygenase from the four different bacteria isolates (SOGU 16, SOGU 11, SERU 11 and SERU 2 *Corynebacterium* sp, *Achromobacter* sp,

and *Arthrobacter* sp, *Pseudomonas* sp respectively) are presented in Table 1. The presence of protease inhibitor during the early stages of 2,4-D/α–ketogulatarate dioxygenease partial purification enhanced the stability of the enzyme. The partial purification gave a low specific activity of the enzyme. The loss of activity during the affinity chromatography step seemed to differ from those of previous reports for *Alcaligenes eutrophus* JMP134 (Fukumori and Hausinger, 1993) and *Psendomonas cepacia* CSV90 (Bhat *et al.*, 1993). This might be attributed to the fact that some of the proteins removed might be involved in the stability of the enzyme as reported by Fersht (1989), although the step gave a good increase in the partial purification fold. The specific activity of the partial purified bacterial isolate, as shown on (Table 1), was lower than that obtained for *A. eutophics* JMP134 (16.9 units/mg protein) (Fukumori and Hausinger 1993). However, the partial purified 2,4-D/α-ketoglutarate dioxygenase was stable at 4°C. This was similar to *A. eutrophus* JMP134 and *P cepacia* CSV90 as reported by Fukumori and Hausinger (1993) and Bhat *et al.* (1989), respectively.

Table 1. Partial purification of 2,4-D/α-ketoglutarate dioxygenase

Procedure	Total volume (ml)	Total protein (mg)	Total activity (µmol/min)	Specific activity µmol/ min/mg rotein)	Purification fold	% yield
Crude Extract						
SOGU 16	600	950.0	1750	1.84	1	100
SOGU 11	600	878.1	1725	1.96	1	100
SERU 11	600	980.2	1758.0	1.79	1	100
SERU 2	600	967.4	1980.1	2.05	1	100
Ammonium Sulphate precipitation (70 %)						
SOGU 16	100	318.4	1210	3.80	2.07	69.0
SOGU 11	110	305.2	1208.6	3.96	2.02	70.0
SERU 11	110	310.0	1150.1	3.71	2.07	65.0
SERU 2	110	295.0	1301.0	4.41	2.15	66.0
Sephadex G-100						
SOGU 16	75	245.1	1011.5	4.132	2.24	58.0
SOGU 11	75	110.1	1094.6	5.21	2.83	63.0
SERU 11	80	195.6	980.0	5.01	2.80	56.0
SERU 2	65	189.2	966.8	5.11	2.50	49.0

3.3. Effect of pH on Enzyme Activity

The residual enzymatic activity of the different bacterial isolates after incubation at various pH values was determined by using standard assay condition. The enzymes from the different bacterial isolates showed a gradual increase from pH 5.5 to a maximum at 7.5; there was no activity at pH 10.0. Hausinger and Fukumori (1995) reported that optimum pH for 2,4-D is 6.5 to 7.5. Also, an optimum pH of 8.0 was reported for 2-monochloropropionic acid and 2,2-dichloropropionic acid (Slater, 1992). It would appear that chlorination process

is favored in an alkaline medium. It is suggestive that a change in pH alters the state of ionization of charged amino acids that may play crucial roles in substrate binding or the catalytic event itself, as well as decreasing the saturation of the enzyme with substrate as a result of decrease in affinity.

3.4. Effect of Temperature on Enzyme Activity

The optimum temperature for the enzyme activity was 35°C. The activity reduced to zero at 60°C. The optimum temperature of 2,4-D dioxygenase activity was between 30°C and 35°C, which fell within the range reported by Hausinger and Fukumori (1995). Microorganisms, found effective in bioremediation, have been shown to perform well in the temperature range of 10°C- 40°C (Cookson, 1995). The effect of temperature on the enzyme catalyzed reaction revealed that the enzyme has its optimum catalysis at temperature of 35°C. Temperatures of 30°C and 35°C have been reported for an optimum activity of dioxygenase enzyme (Bhat, 1993).

3.5. Stability of Enzyme Activity

Maintaining low temperatures during bacterial growth and enzyme isolation is important for obtaining high levels of activity. It was observed that the colonies grown at higher temperatures were turbid, whereas those grown at a moderate temperature remained clear. The partially purified 2,4-D/α-ketoglutarate dioxygenase was found to be very sensitive to thermal inactivation. At a protein concentration of 0.5 μm, 2,4-D/α-KG dioxygenase enzyme remained stable only up to 30°C and is completely inactivated within 10 min at 40°C. Loss of the enzyme activity, at relatively high temperatures, may also be related to the denaturation of the enzyme at such temperatures since enzymes are proteins and may be denatured at certain temperatures (Martin *et al.*, 1983; Hausinger and Fukumori, 1995).

3.6. Catalytic Properties of 2,4-D/α-Ketoglutarate Dioxygenase

Activity of 2,4-D/α-KG dioxygenase requires ferrous ion and is enhanced by ascorbic acid. The ferrous ion requirement could not be met by other divalent metal ion including Co(II), Cu(II), Li(II), Mg(II), Mn(II), Ni(II), and Zn(II) when added as sulfate salt at a concentration of 100 μm. There was a time-dependent decrease in the enzyme activity and ferrous ion alone was unable to sustain enzyme catalysis over long-time periods. The rate of activity loss was greatly reduced by the inclusion of ascorbic acid in the assay. The specific requirement for α-KG and Fe(II), and the stimulation of activity by ascorbate, are typical characteristics of α-KG-dependent-dioxygenases (Abbott and Udenfriend, 1974).

3.7. Molecular weight Estimation by Sephadex G–100 Chromatography

The molecular weight of 2,4-D/α-ketoglularate dioxygenase in the different bacterial isolates was estimated by running the enzyme through a standardized sephadex G–100 column and estimated to be M_r 36,000 ± 1,400; M_r 35,000 ± 765; M_r 40,000 ± 1,850 and M_r 36,000 ± 950 for SOGU 16, SOGU 11, SERU 11 and SERU 2, respectively, for the native enzyme. These findings differ from the findings in the work of Fukumori and Hausinger (1993) who reported the native enzyme to be M_r 50,000 ± 2,500 and from those in the work of Bhat *et al.* (1993) where the molecular weight of the native enzyme was given as M_r 56,000 by light scattering method. It was suggested that the conversion was found to occur after cell disruption rather than during the cell cultivation. This same procedure was used here to demonstrate that the molecular weight of the protein present in *A. eutrophus* JMP134 cell extract was M_r 32,000.

3.8. Kinetic Parameters

The kinetic parameters of each of the bacterial isolates were estimated. The double-reciprocal plots were linear and gave patterns, which intersected at various points to the left of the ordinate above the abscissa. The apparent K_m and V_{max} values for 2,4-D and α-ketoglutarate as substrate and co-substrate, respectively, are summarized in Table 2. In addition to 2,4-D, the enzyme catalyzes the α-ketoglutarate dependent release of the expected bacterial enzyme (Table 2). The enzyme exhibits a great affinity for 2,4-D. This was similar to *A. eutrophus* JMP 134, as reported by Fukumori and Hausinger (1993). Similarly, α-ketoglutarate is the preferred co-substrate because the enzyme exhibits a high affinity for it, as observed in the present study.

Table 2. K_m and V_{max} values of 2,4-D and α-ketoglutarate as substrate of 2,4-D/ α-ketoglutarate dioxgenase for the different bacteria isolates

Isolates	2,4-D		A-KG	
	K_m (μm)	V_{max}	K_m (μm)	V_{max}
SOGU 16	18.0 ± 1.5	5.01 ± 0.45	3.50 ± 0.64	1.51 ± 0.05
SOGU 11	16.8 ± 1.0	4.5 ± 0.35	3.20 ± 0.55	1.0 ± 0.03
SERU 11	17.5 ± 1.3	4.0 ± 0.32	4.00 ± 0.80	1.45 ± 0.04
SERU 2	19.8 ± 1.8	5.50 ± 0.65	3.80 ± 0.75	1.50 ± 0.06

Results were the average of five determination ± the standard error of mean (SEM)

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