# Production, purification and partial characterization for an oxalate decarboxylase (OxDcase) from the probiote *Lactobacillus plantarum* KSK-II

**Essam Kotb***

Department of Microbiology and Botany, Faculty of Science, Zagazig University, Zagazig, Egypt 44519

***Lactobacillus plantarum*** **KSK-II was the most potent oxalate decomposer among various isolated lactic acid bacteria. There was no detectable extracellular or even intracellular oxalate decarboxylase (OxDcase) productivity in the absence of oxalate. The highest enzyme productivity was obtained at 72 h in a medium containing 0.1 M oxalate, 0.1% (w/v) D-glucose, 0.05% (w/v) soybean flour and 0.1% (w/v) of the prebiotics fructo-oligosaccharides and arabinogalactan. Enzyme purification increased its specific activity to 19.6-fold with 14.8% recovery and molecular weight of 63 kDa. The optimal reaction temperature, pH and p*I* values for OxDcase were 35°C, 5.0 and 3.5 respectively, and it was stable till 70°C and at pH 4.0–7.0 for 1 h. The apparent $K_m$ value of the enzyme was 12.70 mM, the turnover number ($K_{cat}$) was 64.10 $s^{-1}$ and the catalytic efficiency ($K_{cat}/K_m$) was 5.05 $mM^{-1}$ $s^{-1}$. Treatment of oxaluric rats with *L. plantarum* KSK-II and a prebiotic mixture significantly decreased oxalate levels inside their bodies suggesting a successful synbiobtic system in the prevention of oxalate stones. KSK-II OxDcase may also be clinically significant from the perspective of its thermo-tolerance and activation by triton X-100 and the reducing agents (sodium-L-ascorbate, potassium ferrocyanide and *o*-PDA). The non-inhibitory activity of chloride and the oxalate specificity are also significant for clinical applications of the enzyme in measuring of oxalate levels in body fluids.**

**Keywords:** Calcium oxalate, *Lactobacillus plantarum*, oxalate decarboxylase, purification, urolithiasis.

A variety of minerals can be crystallized inside the body. Among them, calcium oxalate (CaOx) may be precipitated anywhere in the urinary system, viz. kidney (nephrolithiasis), ureter (ureterolithiasis) and bladder (cystolithiasis), and cause major problems in animal and human bodies. Urolithiasis is not completely understood, although dietary, gender, age and familial factors have been identified[1,2].

CaOx is also formed by many litter fungi and bacteria in response to environmental pH. Oxalate consumption by decarboxylases and oxidases of other microorganisms leads to the alkalinization of the medium[1]. Oxalate decarboxylase (OxDcase) (E.C. 4.1.1.2) degrades CaOx to formate and $CO_2$ and oxalate oxidase (OxOxase, oxalate : oxygen oxidoreductase, E.C. 1.2.3.4) catalyses the oxidation of CaOx to $H_2O_2$ and $CO_2$ (ref. 3).

OxDcase was first discovered in *C. velutipes* and *C. hirsutus*[4], and then identified in *A. niger*[5], *M. verrucaria*[6], *S. sclerotiorum*[7], *C. versicolor*[8], *C. subvermispora*[9], *A. bisporus*[10], *T. ochracea*[11], *C. minitans*[12] and other fungi. OxDcase has also been reported in *B. subtilis*[1] and several gastrointestinal tract bacterial flora of human body like *O. formigenes*[13], *S. faecalis*[14], *P. rettgeri*[15], *E. lentum*[16] and *Escherichia coli*[17].

Members of lactic acid bacteria (LAB) are classified as safe for human consumption and used extensively as probiotics in promoting human health. An earlier study in humans reported that consumption of a mixture of LAB resulted in a reduction of oxalate[18]. Non-digestible food ingredients have been used as prebiotics for reduction of oxalate as they manipulate the already present intestinal microflora[19]. Fructo-oligosaccharides (FOS) are likely the most widely used prebiotics in domestic animals. Thus, they are incorporated into certain commercial foods.

In this study, we isolated *L. plantarum* KSK-II, a potent oxalate degrader in both *in vitro* and *in vivo* experiments suggesting strain KSK-II as a promising probiotic in prevention of oxaluria and its OxDcase as a clinically significant enzyme from the perspective of its thermo-tolerance, in addition to activation by triton X-100 and several reducing agents. The non-inhibitory activity of chloride and the weak competitive inhibition exerted by carboxylic acids other than oxalate are also significant for clinical applications measuring oxalate levels in body fluids.

## Materials and methods

### *Materials*

The bacterial identification system API 50CH was purchased from BioMérieux (Marcy l'Etoile, France).

*e-mail: ekotb@hotmail.com

Abcam's formate assay kits were obtained from Abcam plc (Cambridge, UK). Arabinogalactan, guar gum, inulin, lactitol, maltodextrin were from Sigma-Aldrich Chemical Co. (USA). Sephadex G-100 FF and DEAE-Sepharose CL-6B were from Amersham Pharmacia Biotec. (Sweden). MRS agar was from Oxoid Ltd (UK). Protein standards for SDS-PAGE were from Invitrogen, Shanghai, China. Other chemicals employed in this study were of analytical grade.

### *Bacterial strain and enzyme production*

Selective isolation of LAB from several dairy and fermented foods was performed on MRS agar and incubated aerobically at 37°C for 48 h. The ability of isolates to degrade oxalate was checked by streaking onto the surface of oxalate clearing medium (OCM)[20] of the following composition (g/100 ml distilled water): 1.5 agar, 1.0 potassium oxalate (KOx) monohydrate, 0.03 $NH_4NO_3$, 0.05 $K_2HPO_4$, 0.01 $MgSO_4{\cdot}7H_2O$, 0.002 $FeC1_3{\cdot}6H_2O$, 0.0015 $Ca(NO_3)_2{\cdot}4H_2O$. Addition of 0.1% yeast extract (w/v) was necessary to enhance bacterial growth. Oxalate degradation was detected at 3 pHs: 5, 7 and 8. After autoclaving and solidification of medium in petri dishes, ten-millilitres of sterile 0.1 M $CaCl_2$ was added to the surface of the medium for 1 min to convert KOx (clear) to CaOx (white cloudy). Clearance around bacterial growth is indicative of oxalate degradation.

For initial enzyme production by positive isolates, the above medium (OCM) was used under submerged conditions with the omission of agar and $CaCl_2$ while, adding few drops of phenol red that brings yellow colour at the initial pH (5.0). Appearance of turbidity and change of phenol red colour to bright pink is indicative of the ability of bacterium to grow and produce $CO_2$ from oxalate thus raising the pH to 8.4. At the end of fermentation period, the remaining oxalate was assessed by titration against 0.1 N $KMnO_4$. Strain *L. plantarum* KSK-II that was previously isolated from *kishk*[21], a traditional Egyptian fermented food was found to be the most efficient oxalate decomposer. It was identified based on the phenotypic and biochemical characteristics outlined in *Bergy's Manual of Systematic Bacteriology*. The identification system API 50CH was applied for strain confirmation.

### *Enzyme assay and protein measurement*

OxDcase activity, unless otherwise stated, was assessed using Abcam's formate assay kit where the end product of oxalate decarboxylation, formate, was oxidized to generate colour at $\lambda_{450}$ that was directly proportional with formate concentration. One unit (U) of enzyme activity was defined as the amount of enzyme producing 1 μg of formate per minute under the standard assay conditions. Protein concentration in samples was estimated by Folin–Ciocalteu's reagent using bovine serum albumin as a standard protein.

### *Effect of initial pH on degradation process by L. plantarum*

The optimal pH value for oxalate degradation was determined by the tested strain of *L. plantarum*. The initial pH of OCM medium was adjusted to pH range of 3.0–12.0 using four buffer systems: 0.1 M glycine-HCl (pH 3.0–3.5), 0.1 M sodium acetate buffer (pH 4.0–5.5), 0.1 M sodium phosphate buffer (pH 6–8) and 0.1 M glycine-NaOH buffer (8.5–12.0). An overnight LB culture of strain KSK-II was inoculated into the fermentation medium at 1% (v/v) and the cultures were shake-incubated at 30°C for 7 days to determine bacterial growth and OxDcase activity.

### *Effect of oxalate concentration on degradation process by L. plantarum*

Strain KSK-II was inoculated (1%, v/v) into OCM containing different concentrations of KOx monohydrate at 0 (control), 50, 100, 200, 400 and 800 mM. The flasks were then shake-incubated at 30°C for 7 days, and three flasks of cultures were sampled at 12 h intervals to determine the final bacterial growth ($\lambda_{600}$), pH and oxalate content.

### *Effect of carbon/nitrogen sources on degradation process by L. plantarum*

The OCM mineral medium was tested with several carbon/nitrogen sources in the presence of 0.1 M KOx monohydrate as enzyme inducer. For each medium, the carbon augment was adjusted to 0.1% (w/v) while the tested nitrogen source was adjusted to 0.05% (w/v) and the original nitrogen sources replaced in OCM. The tested carbon sources included D-glucose, lactose, maltose, D-fructose and soluble starch, while the tested nitrogen sources included $KNO_3$, $NH_4NO_3$, L-alanine, L-glutamic acid, yeast extract, soybean flour and casein. Strain KSK-II was inoculated (1%, v/v) into the different media and the cultures were shake-incubated at 30°C for 72 h to determine the final bacterial growth and enzymatic activity.

### *Effect of prebiotics on in vitro CaOx degradation by L. plantarum*

The optimized OCM mineral medium was tested with selected prebiotics at 0.1% concentration (w/v) for CaOx

degradation *in vitro*. The prebiotics tested were arabinogalactan, guar gum, inulin, lactitol and maltodextrin in addition to a commercial Arabic gum and FOS. Strain KSK-II was inoculated (1%, v/v) and the cultures were shake-incubated at 30°C for 72 h to determine the final bacterial growth and enzymatic activity in comparison with control (absence of prebiotics).

### *Enzyme purification*

The enzyme was purified at 4°C in three steps including salting-out with $(NH_4)_2SO_4$, ion-exchange chromatography with DEAE-Sepharose CL-6B and gel filtration chromatography with Sephadex G-100. Culture broth was centrifuged (7000 *g*, 10 min) and the supernatant fractionated by the addition of 30–70% $(NH_4)_2SO_4$. Proteins were collected by centrifugation (10,000 *g*, 15 min) and dissolved in 2 ml of 20 mM sodium acetate buffer at pH 5.0. After removing the insoluble material, the crude enzyme solution was applied to a DEAE-Sepharose CL-6B column ($1.5 \times 20$ cm$^2$). The column was washed with 20 mM sodium acetate buffer (pH 4.0), developed by $(NH_4)_2SO_4$ gradient (1.7–0 M) at a flow rate of 0.5 ml/min and the active fractions were pooled and dialysed against the same buffer. After dialysis, active fractions were passed through a Sephadex G-100 FF column ($2.5 \times 45$ cm$^2$) using 20 mM sodium acetate buffer (pH 5.0). Finally, active fractions were pooled and concentrated by lyophilization.

SDS-PAGE was carried out to determine the purity and molecular weight of OxDcase, as described by Laemmli[22] using 5 and 15% (w/v) stacking and separating gels respectively.

### *Effect of pH on OxDcase activity and stability*

The purified OxDcase was adjusted to different pH values using 0.1 M glycine-HCl (pH 2.5–3.5), 0.1 M sodium acetate buffer (pH 4.0–5.5) and 0.1 M sodium phosphate buffer (pH 6–8) and the enzyme activity was determined at 35°C using oxalate as a substrate. The pH stability in the same range was detected by incubating the OxDcase solution for 1 h at 35°C with buffer solution, and then residual activity at pH 7.0 was determined. The isoelectric point (p*I*) for the purified OxDcase from *L. plantarum* KSK-II was easily determined as described by Kantardjieff and Rupp[23] with slight modifications. The enzyme preparation was incubated at 4°C with different pHs (2.5–8.0). After 12 h incubation, the enzyme was precipitated by centrifugation at 10,000 *g* for 15 min. The precipitated proteins were quantified using Folin–Ciocalteu's reagent. The p*I* was expressed as the pH range at which the maximum OxDcase precipitation occurred.

### *Effect of temperature on fibrinolytic enzyme activity and stability*

The effect of temperature on the decarboxylase activity of *L. plantarum* KSK-II enzyme was studied at 20–85°C in 0.1 M sodium acetate (pH 5.0) using oxalate as a substrate. The thermal enzyme stability was determined by keeping the enzyme solution alone in the same buffer at the same range of temperatures for 1 h. At the end of the incubation period, the replicate tubes were cooled and assayed for residual activity under the standard conditions.

### *Activation, inhibition and substrate specificity*

The effect of intervening chemicals (reducing agents, triton X-100 and halides) on OxDcase activity was studied by pre-incubation with the enzyme solution for 1 h in 0.1 M sodium acetate (pH 5.0) at 35°C and the residual activity was measured. For tested carboxylic acids (oxaloacetic, citric, succinic, maleic, 2-ketoglutaric, fumaric, malonic and phthalic acid), simultaneous incubation with enzyme and oxalate was done to measure their competitive effect. The activity of OxDcase towards oxalic acid in the absence of reagents was taken as 100%. Enzyme activity in this experiment was measured as a means of remaining oxalate, because the reaction end product, formate, may be produced from any of the tested carboxylic acids during enzyme reaction. A parallel confirmatory experiment was done on solid medium containing 1.5% agar and 0.1% KOx with pH adjusted at 6.0. Five microlitre of enzyme and competitive inhibitor mixtures were applied to each hole after being punched by a capillary tube and incubated at 35°C for 6 h.

### *OxDcase kinetic parameters*

The kinetic parameters of the purified OxDcase towards KOx as a substrate were evaluated in 50 mM acetate buffer (pH 5.0) at 35°C using different concentrations of substrate (5–100 mM) and enzyme concentration ($E_t$) of 0.4 mM. Michalis–Menten constant ($K_m$) and maximum velocity ($V_{max}$) were calculated from a Lineweaver–Burk plot while the turnover number ($K_{cat}$) of the enzyme for the substrate was expressed by the specific activity per mole of enzyme ($K_{cat} = V_{max}/E_t$).

### *In vivo study*

Twenty male albino rats (150 g mean weight) were divided into five groups and kept in the animal house of the Faculty of Science, Zagazig University, Zagazig. One group was fed only with a standard commercial diet and served as a universal control group; other rats were fed as

follows: sodium glycolate along with the diet (group A); 100 mg sodium glycolate mixed with 1 ml of strain KSK-II culture (containing $10^9$ cells) along with the standard diet (group B); the same glycolate–KSK-II cells mixture along with prebiotic mixture (FOS and arabinogalactan) mixed with the standard diet at 0.1% (w/w) (group C); and glycolate along with the prebiotic mixture mixed with the standard diet at 0.1% (w/w) (group D). Glycolate and/or bacterial cells were orally administered once a day using a stomach tube. After the experimental period of 10 days, urine samples were collected for estimation of calcium, phosphate and oxalate. Oxalate was measured by titration against 0.1 M $KMnO_4$.

### *Statistical analysis*

Experiments were performed in triplicates and the data of results were presented as the average of triplicates ± SDs.

## Results and discussion

### *Enzyme production*

At present, OxDcases from several microorganisms have been purified and the gene sequences of some have been sequenced and cloned. In this study, a lactic acid bacterium was tested for OxDcase activity. The bacterium was earlier isolated from a local traditional fermented food called *kishk*[21] and identified as *L. plantarum* KSK-II. Strain KSK-II was the most potent oxalate decomposer among several LAB isolated from dairy and fermented foods. It was identified based on the phenotypic and biochemical characteristics outlined in *Bergy's Manual of Systematic Bacteriology*. The identification system API 50CH (BioMérieux, Marcy l'Etoile, France) was applied for strain confirmation.

Figure 1 *a* shows that acidic pHs were essential for strain KSK-II to decarboxylate oxalate. Optimal pH for maximal enzyme productivity was 6.0 (181 U/ml) (Figure 1 *a*). Enzyme productivity above pH 7.0 was significantly decreased. Only 59 U/ml was secreted at pH 7.5 with 3.1-fold decrease than at pH 6.0. This pattern of oxalate degradation by *L. plantarum* is similar to that of *B. subtilis* Yvrk[1] where acidic conditions may act as a signal or inducer to trigger production of oxalate degrading enzymes such as OxDcase[1] and the permeases responsible for translocation of oxalate into the bacterial cells[12,24]. Extracellular OxDcase may have a role in controlling the pH of the bacterial environment, which has been acidified by oxalate.

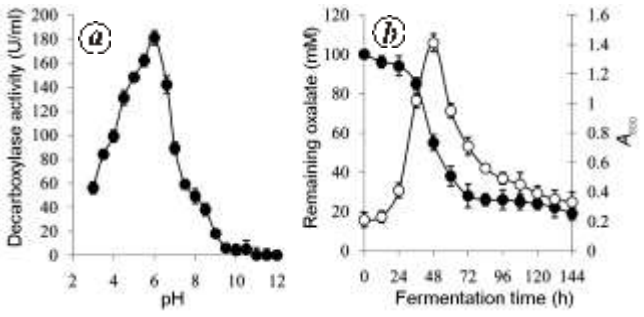


**Figure 1.** ***a***, Effect of initial pH values on OxDcase productivity by *L. plantarum* KSK-II under submerged fermentation for 7 days at 30°C with initial inoculum of 1% (v/v). ***b***, Time course of enzyme production by *L. plantarum* KSK-II under the optimized conditions: (-○-) Bacterial growth (A600) and (-•-) OxDcase productivity as reflected by the content of remaining oxalate (mM).

The influence of oxalate concentration upon enzyme productivity by strain KSK-II was also examined (data not shown). Among all tested oxalate concentrations, maximal enzyme productivity was obtained at 0.1 M oxalate with final pH reached at 8.6 from 6.0. Biomass was highest at 0.4 M of oxalate concentration. It should be emphasized that there was no enzyme productivity at 0% oxalate concentration. Thus, the OxDcase from strain KSK-II is considered as an oxalate inducible enzyme. In view of the findings of other studies, most OxDcases cited in the literature are extracellular and intracellular OxDcases specifically associate with plasma membrane, spore coat and/or inside intracellular vesicles which have been reported in *C. versicolor*[8], *B. subtilis*[25] and *B. subtilis* YvrK[1] respectively. Inducible OxDcases by oxalate, other carboxylic acids or by lowering culture medium pH were also reported. OxDcases of *Pandoraea* sp. OXJ-11[26], *C. velutipes*[27] and *C. versicolor*[8] were produced only in the presence of oxalate. In addition, OxDcase of *B. subtilis* was induced in acidic growth media (particularly at pH 5.0) but not by oxalate[1]. Acid-induced OxDcases might have a role in $H^+$ consumption and regulation of acidity, while oxalate-induced ones might protect an organism from harmful metabolic effects of oxalate[1,27]. On the other hand, it was shown that OxDcase from *A. bisporus*[10] was not induced by either pH or oxalate.

In a complementary experiment, growth curve and change in oxalate content during the growth of strain KSK-II in OCM was performed (Figure 1 *b*). Oxalate degradation was very slow during the first 24 h of fermentation process. Enzyme productivity (as reflected by the remaining oxalate) then increased along with cell growth ($A_{600}$) and enzyme productivity reached a maximum (remaining oxalate was 28 mM, i.e. 72% of initial oxalate was degraded) at 72 h after cell growth of *L. plantarum* KSK-II reached a peak at 48 h of fermentation which indicates secondary metabolite. This result disagreed with that of *A. bisporus* OxDcase activity that was present in both culture medium and mycelium. Enzyme activity in the mycelium peaked at two-weekly intervals after primary growth phase and into secondary metabolism, with activity peaks in the medium occurring 7 days later than in the mycelium[10]. Faecal LAB degraded oxalate within only 48 h (ref. 28) versus 5–7 days in other studies evaluating *in vitro* degradation[14,16].

The influence of different carbon sources was tested at 0.1% (w/v) concentration and it was observed that addition of soluble starch did not have a significant effect on both enzyme production and growth of strain KSK-II (Table 1). In addition to starch, we also studied the effect of rapidly metabolizable carbon sources like glucose, fructose, maltose and lactose. As shown in Table 1, *L. plantarum* KSK-II exhibited maximum productivity of enzyme in culture medium containing 0.1% glucose as carbon source (378 U/ml).

The effect of various nitrogen sources (at 0.05% w/v) on enzyme synthesis in OCM mineral medium containing 0.1 M $KO_x$ and 0.1% D-glucose as carbon source was also studied (Table 1). Organic nitrogen sources greatly enhanced both growth and enzyme productivity by strain KSK-II when compared to inorganic sources. Strain KSK-II exhibited maximum productivity of enzyme (419 U/ml) in culture medium containing soybean flour as nitrogen source (Table 1). In addition to soybean flour, we also studied the effect of L-glutamic acid, L-alanine, casein, yeast extract, $KNO_3$ and $NH_4NO_3$. As shown in Table 1, yeast extract induced high bacterial growth but enzyme production was minimal (196 U/ml). Our results were in contrast with the results of *C. minitans* OxDcase[24], where there was no significant difference between the tested carbon and nitrogen sources towards oxalate degradation; only $NH_4NO_3$ exerted inhibitory effect (degradation was as low as 46.1%).

The effect of prebiotics on CaOx degradation *in vitro* was tested at 0.1% concentration (w/v). The tested prebiotics for CaOx degradation *in vitro* were arabinogalactan, guar gum, inulin, lactitol, maltodextrin, Arabic gum and FOS. All tested prebiotics were found to be enhancers of bacterial degradation of CaOx with maximal degradation being achieved in the presence of FOS and arabinogalactan (Figure 2). However, Arabic gum and inulin showed lower superiority for the bacterium to degrade CaOx. Bacterial variation in response to prebiotics is well documented in the literature[29]. The increase in CaOx degradation reported with different prebiotics was ascribed to the enhancement of prebiotics to bacterial growth (Figure 2). Thus, combination of several prebiotics may be better for *in vivo* ingestion along with probiotics which is in agreement with Weese and coworkers[28].

**Table 1.** Growth and enzyme production by *L. plantarum* KSK-II with several carbon and nitrogen sources

| Carbon/nitrogen source | Enzyme productivity (U/ml) | Bacterial growth ($A_{600}$) |
|---|---|---|
| Carbon | | |
| Blank* | 219 ± 13 | 1.46 ± 0.17 |
| D-glucose | 378 ± 16 | 1.92 ± 0.19 |
| D-fructose | 355 ± 20 | 1.65 ± 0.13 |
| Lactose | 256 ± 17 | 1.54 ± 0.20 |
| Maltose | 274 ± 14 | 1.76 ± 0.14 |
| Soluble starch | 224 ± 13 | 1.35 ± 0.12 |
| Nitrogen | | |
| Blank** | 65 ± 3 | 0.43 ± 0.08 |
| Yeast extract | 196 ± 8 | 1.35 ± 0.15 |
| Soybean flour | 419 ± 25 | 1.68 ± 0.22 |
| Casein | 286 ± 13 | 1.27 ± 0.18 |
| L-alanine | 192 ± 16 | 0.72 ± 0.09 |
| L-glutamic acid | 184 ± 11 | 0.85 ± 0.10 |
| $KNO_3$ | 151 ± 9 | 0.48 ± 0.07 |
| $NH_4NO_3$ | 163 ± 12 | 0.64 ± 0.05 |

Cultivations were performed for 72 h at 30°C in OCM mineral medium with pH 6.0. Values are means of three independent experiments ± standard deviations. *Indicates optimized OCM mineral medium was used without any substitution; **indicates optimized OCM mineral medium was used without $NH_4NO_3$ and addition of yeast extract.

## *Properties of purified enzyme*

OxDcase was purified from the culture supernatant of *L. plantarum* KSK-II by the procedure described in materials and methods. As summarized in Table 2, the enzyme was easily and quickly purified by a combination of various steps. In the first step, fractions with $(NH_4)_2SO_4$ concentrations from 30% to 70% had the highest specific activity. The final specific activity of the enzyme increased and reached 19.6-fold with a recovery of 14.8% based on the initial culture supernatant. The final eluted proteins (Figure 3 *a*) were subjected to SDS-PAGE and only a major band at 63 kDa was observed in the purified sample (Figure 3 *c*), with resemblance to the OxDcases of *C. velutipes* (64 kDa, deglycosylated enzyme 55 kDa)[27] and *A. bisporus* (64 kDa)[10] although higher in molecular mass compared to that reported from the white-rotting *C. versicolor* (59 kDa)[8] and *D. squalens* (52–55 kDa)[30].

The pH activity profile of the purified OxDcase from strain KSK-II had maximum values at pH 5.0 and was pH stable at pH 4.0–7.0 for 1 h at 35°C (Figure 4 *a*), which is different to the OxDcases from *A. bisporus* (pH 3.6 with a pH range of 3–4)[10] and *C. velutipes* (pH 3 with a pH range of 1.5–5.5)[27]. The p*I* of the tested OxDcase was found at pH 3.5 ± 0.2 with resemblance to an OxDcase isozyme from *C. velutipes* (p*I* 3.3)[27], the two isozymes of *A. bisporus* (p*I*s 3.0 and 3.4)[10] and OxDcase of *C. versicolor* (p*I* 3.0)[8]. However, it differed from OxDcase of *C. versicolor* (p*I* 2.3)[8] and the second isozyme of *C. velutipes* (p*I* 2.5)[27].

The optimum temperature for our enzyme activity was at 35°C and it was maximally stable till 70°C for 1 h (Figure 4 *b*). The conformational structure of the enzyme seemed not affected by preincubation below 65°C for 1 h as the remaining activity was over 97% of the initial value. The thermal stability of the enzyme (Figure 4 *b*) was evaluated by preincubating the enzyme in 0.1 M sodium acetate (pH 5.0) without substrate at different temperatures (20–85°C) for 1 h. The conformational structure of OxDcase seemed not affected by preincubation below

**Table 2.** Summary of purification steps

| Purification step | Total activity (U) | Protein content (mg) | Specific activity (U/mg protein) | Purification folds | Recovery (%) |
|---|---|---|---|---|---|
| Culture supernatant | 112,156 | 264 | 425 | 1.0 | 100.0 |
| $(NH4)_2SO_4$ precipitation | 90,216 | 132 | 683 | 1.6 | 80.4 |
| DEAE-Sepharose CL-6B | 41,413 | 6 | 6,902 | 16.2 | 36.9 |
| Sephadex G-100 FF | 16,653 | 2 | 8,326 | 19.6 | 14.8 |

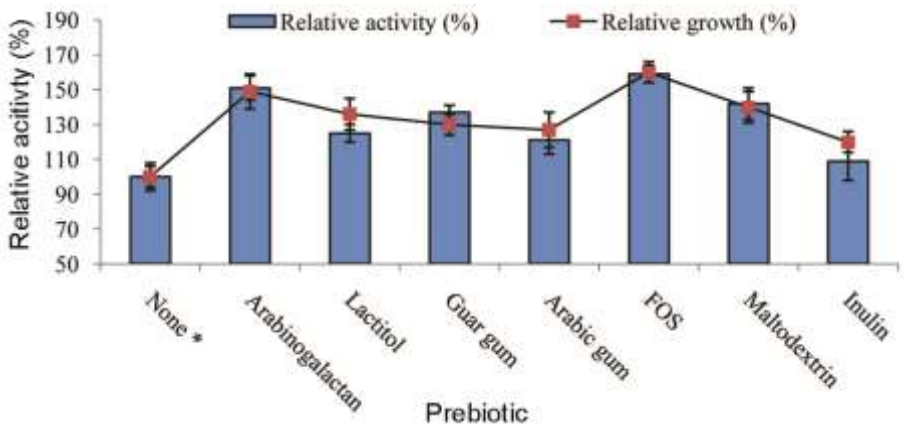


**Figure 2.** Effect of prebiotics on *in vitro* CaOx degradation by *L. plantarum* KSK-II. The optimized OCM mineral medium was mixed with selected prebiotics at 0.1% concentration (w/v). Strain KSK-II was inoculated (1%, v/v), and the cultures were shake-incubated at 30°C for 72 h to determine the final bacterial growth and enzymatic activity in comparison with control (absence of prebiotics).

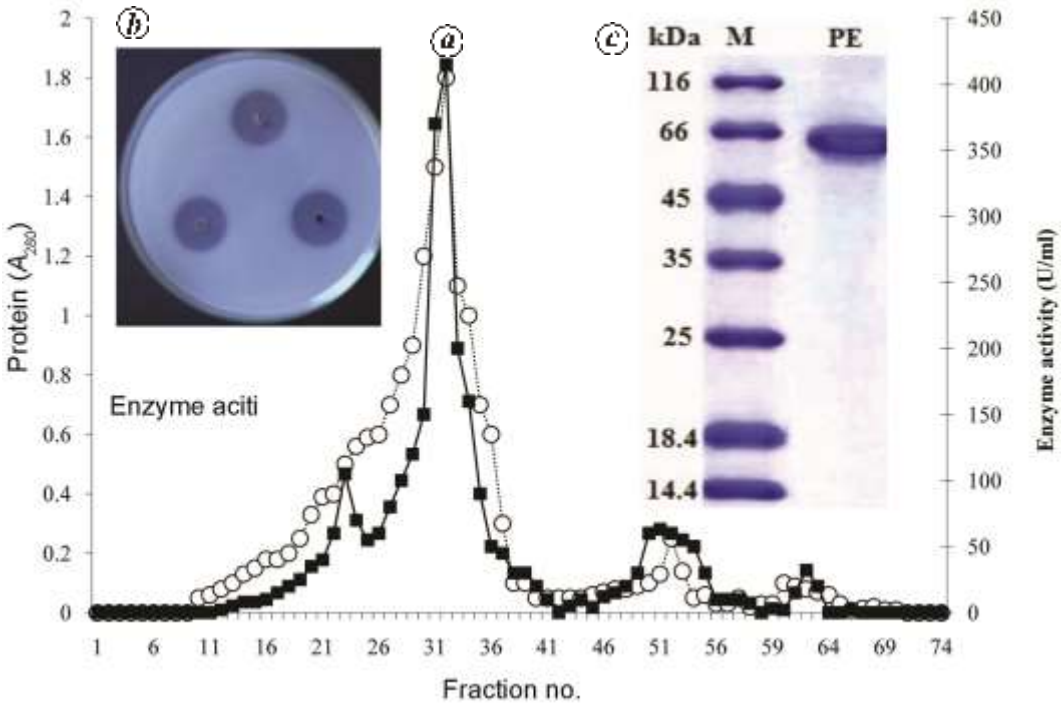


**Figure 3.** ***a***, Elution profile of OxDcase through Sephadex G-100 FF column (2.5 × 45 $cm^2$) using 20 mM sodium acetate buffer at pH 5.5: (-○-) protein content ($A_{280}$) and (-□-) enzyme activity (U/ml). ***b***, Plate showing OxDcase activity of Sephadex G-100 column active fractions after being collected. Medium containing 0.1% $KO_x$ and 1.5% agar with pH adjusted at 6.0. Five microliter enzyme solution was applied to each hole after being punched with a capillary tube and incubated at 35°C for 12 h. ***c***, SDS-PAGE pattern of OxDcase using 5 and 15% (w/v) stacking and separating gels, respectively. Lane M represents the standard protein markers; lane PE represents the purified OxDcase.

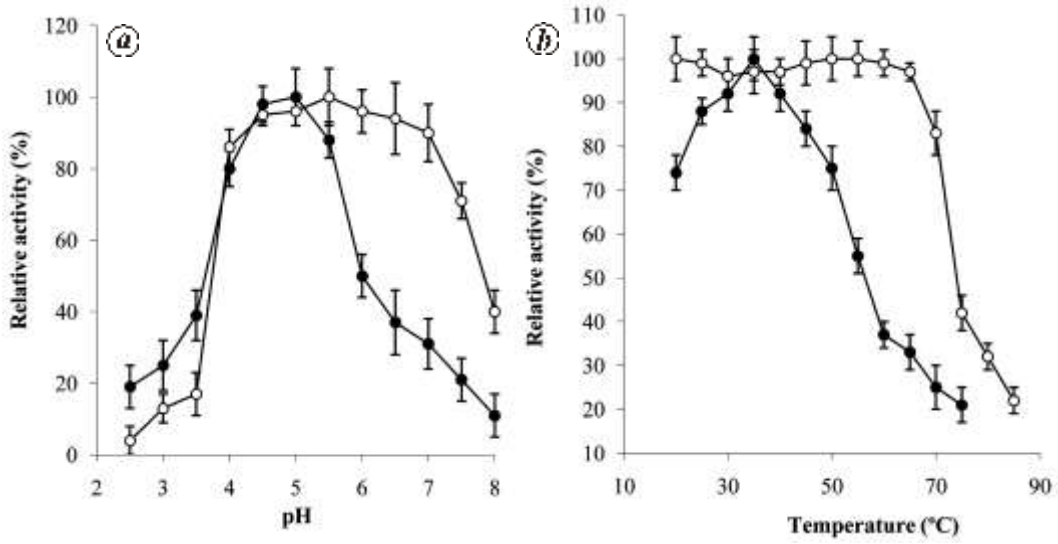


**Figure 4.** Effect of pH (***a***) and temperature (***b***) on the purified OxDcase activity (-•-) and stability (-○-).

**Table 3.** Effect of different intervening chemicals on OxDcase activity

| Chemical | Concentration (mM) | Relative activity (%) |
|---|---|---|
| Oxalic acid* | 5 | 100 ± 3 |
| *o*-hydroxylamine | 5 | 50 ± 1 |
| Sodium-L-ascorbate | 5 | 110 ± 4 |
| Sodium sulphite | 5 | 60 ± 2 |
| Sodium dithionite | 5 | 75 ± 3 |
| Potassium ferrocyanide | 5 | 112 ± 5 |
| *o*-PDA | 5 | 138 ± 4 |
| Triton X-100 | 5 | 115 ± 6 |
| Sodium chloride | 50 | 98 ± 3 |
| Sodium bromide | 50 | 71 ± 4 |
| Sodium fluoride | 50 | 68 ± 6 |
| Sodium iodide | 50 | 82 ± 5 |
| Maleic acid | 25 | 69 ± 1 |
| Oxaloacetic acid | 25 | 68 ± 2 |
| 2-ketoglutaric acid | 25 | 82 ± 2 |
| Succinic acid | 25 | 77 ± 1 |
| Fumaric acid | 25 | 73 ± 2 |
| Malonic acid | 25 | 68 ± 1 |
| Citric acid | 25 | 77 ± 2 |
| Phthalic acid | 25 | 97 ± 1 |

*The activity of OxDcase upon oxalic acid in the absence of intervening substances was taken as 100%. Enzyme activity in this experiment was assessed by measuring the remaining oxalate as formate may be produced from the tested carboxylic acids.

65°C for 1 h as it conserved more than 97% of its original activity. The higher the temperature, the higher the denaturation rate of the enzyme. The thermal stability is a characteristic feature of the cupin enzyme superfamily due to their structure which has large number of subunits, short loops, efficient packing, hydrophobic interactions, hydrogen bonding and fewer cavities[3]. The optimum temperature for our enzyme is similar to the OxDcases from *A. bisporus* (35°C)[10], *C. velutipes* (37°C)[27] and *A. niger* (37°C)[31]. However, the OxDcase of *A. niger* retains only 50% of its original activity after 10 min at 65°C (ref. 31) and the OxDcase of *A. bisporus* is inactivated at 45°C (ref. 10). Furthermore, the OxOxase from barley retains only 25% of its original activity after incubation for 30 min at 70°C (ref. 32). Therefore, they are not applicable for enzymatic treatments at temperatures higher than 37°C.

OxDcase complete inhibition was observed after bubbling of pure $O_2$ for 1 h through enzyme solution in the absence of substrate. In a parallel test, no enzyme inhibition was recorded after bubbling of both enzyme and substrate mixture. This agrees with the proposal that, in the presence of substrate, $O_2$ binds to the metal centre only after the substrate is already bound, to prevent oxidation of the metal centre by $O_2$ (ref. 33). In view of earlier findings, OxDcase of *A. niger* was irreversibly inactivated in the atmosphere of pure $O_2$ (refs 33, 34). For OxDcase of *C. versicolor*, all white-rot fungi showed inactivity in the absence of oxygen[8].

Inhibition and activation studies on OxDcase of strain KSK-II (Table 3) demonstrated that weak reducing agents (sodium-L-ascorbate, potassium ferrocyanide and *o*-PDA) activated the enzyme and stronger reducing agents (*o*-hydroxylamine, sodium dithionite and sodium sulphite) inhibited the enzyme. Earlier it was proposed that, reducing agents interfered with the ES–$O_2$ complex[4,33,34]. Strain KSK-II OxDcase was activated (1.4-fold) by *o*-PDA up to 5 mM concentrations. With reference to other studies, *B. subtilis* decarboxylase was also activated by *o*-PDA at concentrations ≤ 5 mM while it was inhibited at higher concentrations[33]. Both activity and tolerance to $O_2$ of OxDcase from *A. niger* were increased by *o*-PDA (ref. 5). This was explained on the basis that, during the reaction, ES–$O_2$ complex reduced to superoxide, further

reduced to peroxide and the active centre was left electron deficient. This hypothesis was supported by the observation that $O_2$ inhibition is followed by peroxide production[34]. In this case, the role of *o*-PDA might be to prevent superoxide reduction or to provide electrons to the electron-deficient active centre.

Reagents like non-ionic detergent, triton X-100 were found to enhance OxDcase activity. Activity was inhibited with sodium fluoride, bromide and iodide. However, sodium chloride did not have significant effect (Table 3). The inhibitor effect of halides (bromide, fluoride and iodide) was also reported for the OxDcase of *B. subtilis*[33] and the OxOxase of barley[35] and was attributed to the competition of halides with oxalate for the $Mn^{2+}$ binding at the active centre. The non-inhibitory activity of chloride is significant for the clinical application of *L. plantarum* KSK-II OxDcase in measuring the oxalate levels in biological fluids.

Carboxylic acids such as oxaloacetic, citric, succinic, maleic, 2-ketoglutaric, fumaric, malonic and phthalic were tested as competitive inhibitors or substrate analogs for OxDcase (Table 3 and Figure 5). They were weak competitive inhibitors and the maximum inhibition was reported for malonic acid and oxaloacetic acid, which may be attributed to the better association with enzyme and the less side oxidation–reduction reactions due to the presence of only one methylene group in their structure. Only association and dissociation with enzyme can occur[36]. Inhibition profile shown in Table 3 and Figure 5 declares the weak competitive inhibition between both substrate and inhibitors for the same active site. This characteristic specificity is also common to other OxDcases isolated from *B. subtilis*[33], *A. bisporus*[10], *A. niger*[5], *M. verrucaria*[6], *C. velutipes*[4,37] and all the white-rotting fungi[8]. Substrate specificity is not surprising because oxalate decarboxylation is a redox reaction in which the redox potential of all substrates is finely regulated with redox properties of the OxDcase.

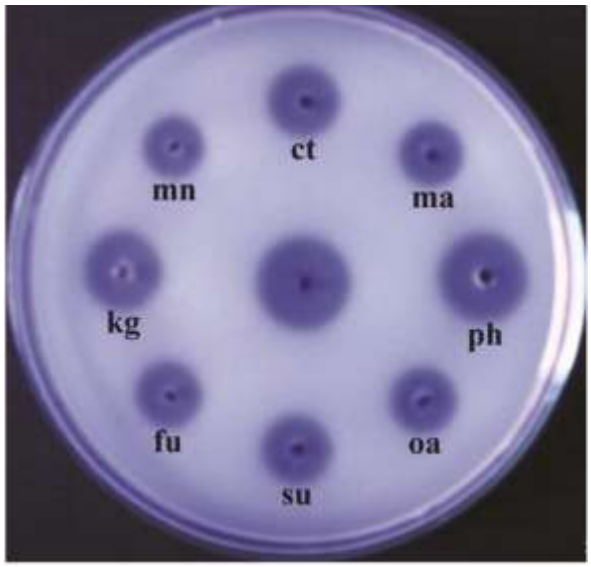


**Figure 5.** Effect of carboxylic acids such as oxaloacetic (oa), citric (ct), succinic (su), maleic (ma), 2-ketoglutaric (kg), fumaric (fu), malonic (mn) and phthalic (ph) as competitive inhibitors of OxDcase. The activity of OxDcase towards oxalate in absence of competitive inhibitors was taken as 100%. Medium containing 0.1% KOx and 1.5% agar with pH adjusted at 6.0. Five microlitre of enzyme and competitive inhibitor mixtures were applied to each hole after being punched by a capillary tube and incubated at 35°C for 6 h.

### *Kinetic parameters*

To determine the kinetic parameters for OxDcase, $KO_x$ was used as substrate. The Michaelis constants, $V_{max}$ and $K_m$ were determined from the intercepts and slopes of the regression curve of Lineweaver–Burk plot (Figure 6) by measuring OxDcase activity at 35°C. The apparent $K_m$ value of enzyme was 12.70 mM, the corresponding $V_{max}$ was 25.64 mM/min, the turnover number ($K_{cat}$) was 64.10 $s^{-1}$ and the catalytic efficiency ($K_{cat}/K_m$) was 5.05 $mM^{-1}$ $s^{-1}$. With reference to other studies, the $K_m$ values for OxDcases of different strains of *C. velutipes* were 2.05 mM (ref. 37), 4.5 mM (ref. 27) and 0.49 mM (ref. 38). For different strains of *B. subtilis* the values were 5 mM (ref. 33), 15.46 mM (ref. 39) and 15 mM (ref. 1). For *A. niger* and *M. verrucaria* the values were 4 mM (ref. 5) and 1.7 mM (ref. 6) respectively. Different $K_m$ values have been also reported for barley OxOxases, 1.3 mM (at pH 4.0 and 22°C)[40], 0.27 mM (at pH 3.8 and 37°C)[32] and 0.42 mM (at pH 3.5 and 22°C)[41]. The $K_{cat}$ and $K_{cat}/K_m$ values for the recombinant OxDcase of *B. subtilis*[33] were 56 $s^{-1}$ and 11 $mM^{-1}$ $s^{-1}$ respectively. Summary of enzyme productivity, purification and characterization is included in Supplementary Table 1.

### *In vivo study*

Oxaluria was induced in rats with oral administration of sodium glycolate for 10 days (Supplementary Table 2). This method of oxalate induction was successfully done in earlier studies[2]. Normal oxalate level in the control group was 0.54 mg/24 h urine and after induction of oxalate formation by glycolate, it reached 3.26 mg/24 h urine as reflected by group A. Supplementation of oxaluric rats with *L. plantarum* KSK-II and/or the prebiotic mixture (FOS + arabinogalactan) significantly decreased oxalate, calcium and phosphate levels.

The prebiotic mixture enhanced the growth and enzyme production by normal gut flora as reflected by group D where, oxalate levels dropped to 1.27 from 3.26 mg/24 h urine. Oxalate levels maximally dropped in the presence of *L. plantarum* KSK-II and the prebiotic mixture (0.72 mg/24 h urine) suggesting a successful synbiobtic system in the prevention of oxalate stones formation. KSK-II OxDcase may also be clinically significant from the perspective of its thermo-tolerance

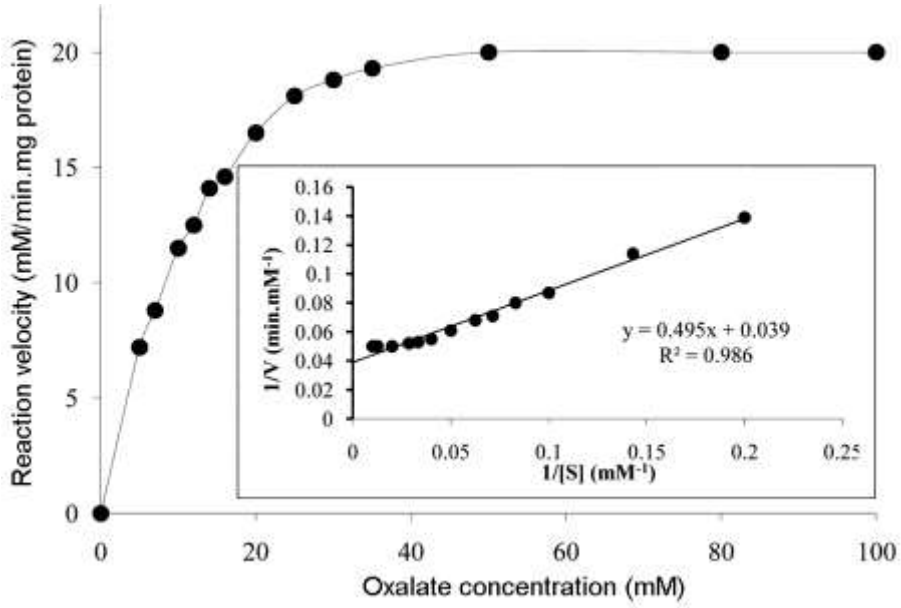


**Figure 6.** Determination of Michaelis–Menten constants for OxDcase from the hyperbolic and Lineweaver–Burk plots of the reaction rate versus substrate concentration.

and activation by triton X-100 and the reducing agents (sodium-L-ascorbate, potassium ferrocyanide and *o*-PDA). The non-inhibitory activity of chloride and the weak competitive inhibition exerted by carboxylic acids other than oxalate are also significant for clinical applications in measuring the oxalate levels in body fluids and tissue homogenates. Further *in vivo* studies are in progress.


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