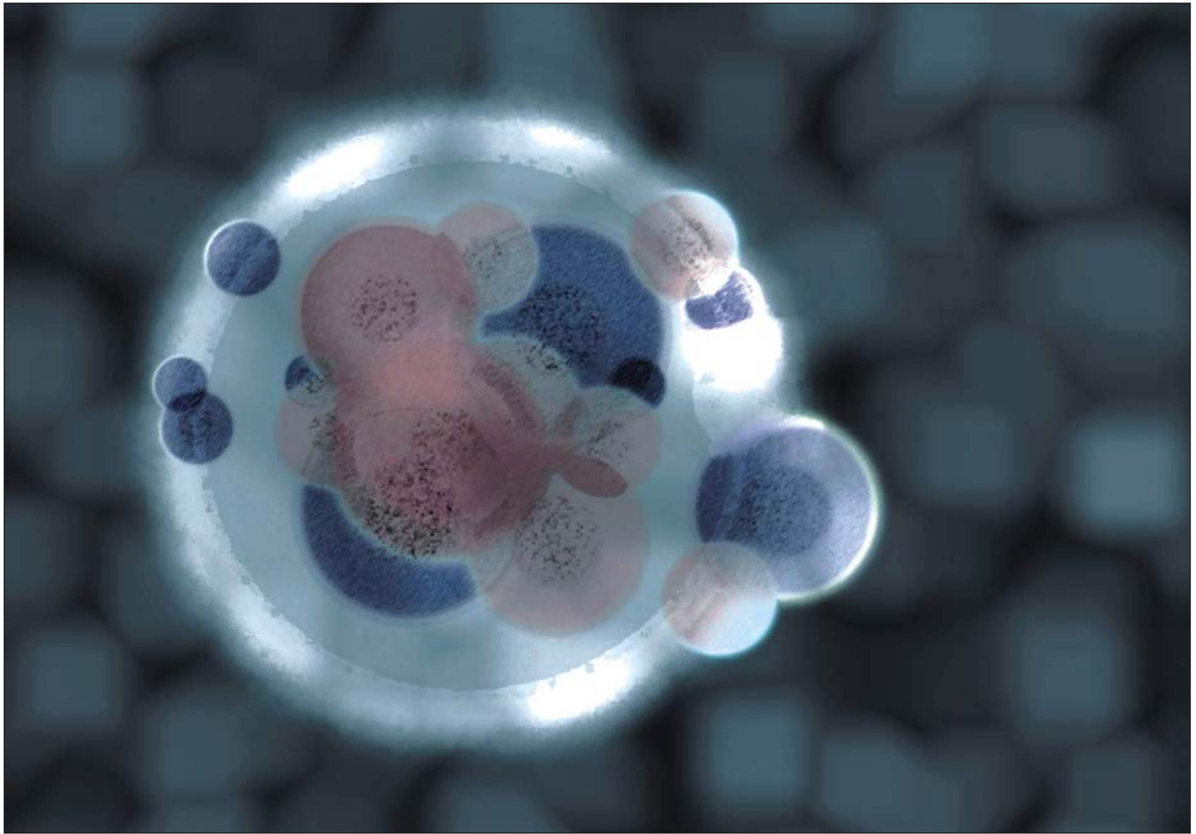

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ACC Deaminase from *Issatchenkia occidentalis*

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Abstract: In this study ACC deaminase from *Issatchenkia occidentalis* was isolated, partially purified and characterized. 1-Aminocyclopropane-1-carboxylic acid (ACC) deaminase catalyses the conversation of ACC to α-ketobutyric acid and ammonia. Most of the studies on this enzyme were done mainly from bacterial ACC deaminase; with only one reported nitrate utilizing yeast species (*Hansenula saturnus)***.** A yeast which exhibited high ACC deaminase activity was isolated and was identified as *Issatchenkia occidentalis*. Unlike *Hansenula saturnus*, it was non-nitrate utilizing and it did not utilize ammonium salts very well. The ACC deaminase from this yeast was inhibited by potassium cyanide, sodium borohydride and ACC-methyl ester all at 5 mM. The cofactor pyridoxal-5- phosphate was not needed for activity. The enzyme is very liable in nature, losing half of its activity after one week and losing almost all of its activity after 2 weeks when stored at 13°C. Storage of the crude homogenate at -15°C prolonged its shelf life beyond 2 weeks. The enzyme displayed an optimum temperature and pH of 40°C and 7.5, respectively.

Key words: Soil bacteria, ACC deaminase, *Issatchenkia occidentalis*

INTRODUCTION

It has been reported that a small number of soil bacteria contain ACC deaminase [EC 4.1.99.4] (Klee *et al*., 1991). The presence of this rather unusual enzyme is intriguing, since its role in the metabolism of its host bacterium is unknown. Jacobson *et al.* (1994), have demonstrated that *P. putida* GR12-2 contain ACC deaminase activity and that the enzyme from this organism has chemical and physical properties that are similar to the properties previously observed for ACC deaminase from other bacteria (Jacobson, 1993; Honma and Shinomura, 1978). To ascertain whether this enzyme plays a role in the stimulation of seedling root elongation by *P. putida* GR12-2, this organism was chemically mutagenized and three independent mutants that lack ACC deaminase activity were selected. Unlike the wild type, none of the selected acid mutants were able to promote the growth of canola seedling root under gnotobiotic conditions. This observation indicates that the enzyme ACC deaminase is involved in the mechanism that this bacterium (*P. putida*) uses to stimulate canola root elongation.

ACC is a simple compound in which the amino and carboxyl groups are attached to the same carbon in the cyclopropane ring. Deamination of ACC is catalyzed by ACC deaminase, a specific pyridoxal-5-phosphate enzyme. This enzyme is induced in microorganisms grown in media containing ACC as the sole source of nitrogen. Deamination proceeds together with opening of the cylcopropane ring to form α-ketobutyric acid and ammonia from ACC (Honma *et al.*, 1979). Enzymatic opening reactions of the cyclopropane ring were already observed in studies on cyclopropane carboxylate metabolism by *Rhodococcus rhodochrous* (Toraya *et al*., 2003), metabolism of cyclopropane fatty acid by *Tetrahymena pyriformis* (Tipton and Al-Shathir, 1974) and metabolism of cyclopropyl sterols by higher plants. A similar ring opening reaction in the metabolism of ACC can be catalyzed without addition of the co-factor (pyridoxal-5-phosphate) by the soluble enzyme and is regarded as γ-elimination by the deaminase, similar to that catalyzed by γ-cystathionase (Barber *et al.*, 1999) or methionase.

In recent genetic engineering experiments the ACC deaminase gene was placed under the control of the cauliflower mosaic virus (CaMV) 35S promoter and used to transform UC82B tomato plants (Klee *et al*., 1991). Reduction of ethylene throughout the plant did not have any significant effect on plant development since a number of phenotypically normal transgenic plants were obtained from the transformation. The plants exhibited no delay in the onset of flowering or ripening, however, they did show significant differences in the progression of

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ripening. Whereas control fruit passed from the breaker to fully red stage in 7 days and exhibited a marked degree of softening, the transgenetic plant produced fruits that ripened at a much slower rate (Klee *et al*., 1991).

This study was undertaken in light of the fact that the shelf life of many tropical fruits is relatively short and the successful use of ACC deaminase from soil bacteria to produce transgenic tomato plants may-in the not too distant future-also be accomplished using ACC deaminase from *Issatchenkia occidentalis.*

MATERIALS AND METHODS

Reagents: ACC, ACC methyl ester, gluconic acid, glucose, citric acid, Tris[hydroxymethyl] aminomethane, γ-keto butyric acid and DF -salts were obtained from Sigma (St Louis, MO, USA). 2, 4 Dinitrophenylhydrazine was obtained from BDH Chemicals (Poole, UK). All other reagents were analar in grade.

Equipment: A Beckman ultracentrifuge model J21 was obtained from Beckman Coulter (Fullerton, California, USA) and a Spectronic Genesys-5 spectrophotometer was from Milton Roy Company (Analytical Products Division) Rochester NewYork.

Isolation of *Issatchenkia occidentalis*: Forty five soil samples (0.01g each) obtained from different locations in Jamaica were incubated in 1 mL mineral medium with no source of nitrogen for 48 h. The liquid minimal medium contained DF salts (KH_2PO_4 - 4 g; Na_2HPO_4 - 6 g; KH_2PO_4 - 4 g; Na_2HPO_4 - 6 g; $MgSO_4.7H_2O$ - 0.2 g; $FeSO_4.7H_2O$ -1 mg; H_3BO_3 -10 mg; $MnSO_4$ - 10 mg; $ZnSO_4$ - 70 mg; $CuSO_4$ - 50 mg; MoO_3 - 10 mg, made up in 1 L) and 0.2% each of glucose, gluconic acid and citric acid. Samples from these liquid media were then streaked onto minimal media plates containing 3 mM ACC as the only source of nitrogen and were designated as: [+ ACC DF Plates] (Klee *et al*., 1991).

Test for assimilation of potassium nitrate by the yeast: The agar was washed for 8 days with running water and with frequent change of the water. In the final wash distilled water was used. The broth (1 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 20 g glucose and 15 g agar) was prepared with 1 litre distilled water and sterilised at 110°C for 15 min.

In a petri dish, 2 mL of concentrated yeast (later identified as *Issatchenkia occidentalis*) suspension was pipetted. The yeast cells were washed several times and bench centrifuged at 5,000 rpm for 5 min in order to concentrate. After allowing the broth to reach 40°C, about 20 mL was poured into the petri dishes containing the yeast suspension. After solidifying, the surfaces of the solidified gel in the petri dishes were divided into two equal parts (using a sharp sterilized needle) and a few crystals of potassium nitrate were sprinkled on one side, while some granules of peptone were sprinkled on the other side. The plates were left for 3 days at 25°C.

Culturing of yeast on liquid and solid medium 1: Fifty milliliters of liquid medium-I containing 2% glucose, 0.5% peptone and 0.5% of a commercial yeast extract which was used by Honma and Shimomura (1978) to culture ACC degrading bacteria, was inoculated with yeast (0.06 g mL^{-1}) under sterile conditions in a 250 mL conical flask. The yeast were allowed to grow overnight at 35°C.

Two hundred and fifty milliliters of the liquid medium-I (without yeast) that was supplemented with 1.5% Difco agar was poured into cake pans (17×17×4 cm). Each pan was covered with aluminium foil and autoclaved for 15 min at 15 psi. After the agar had cooled and solidified (solid medium-I), a small portion of the aluminium foil was raised at one corner of each pan and 5 mL of the cells (grown in liquid medium-I at 35°C) was pipetted onto the agar surface. These cells were spread evenly over the entire surface by gently tilting the pan from side to side. The inoculated pans were incubated at 35°C for 8-9 h. The cells were harvested by scraping them from the agar surface with a 7×12 cm index card into a sterile petri dish. One and a half to 2 g of cells (wet weight) per pan was routinely obtained. It was important to harvest the cells no longer than 10 h after incubation, as those which were harvested later proved difficult to be induced.

Enzyme extraction: The harvested cells from the solid medium-I were then placed in 50 mL of induction medium (as out lined under Isolation of *Issatchenkia occidentalis*) in a 500 mL flask at a cell density of no more than 0.06 g mL^{-1}. The yeast culture was then allowed to incubate for 9 h at 35°C with shaking at 30 rpm. The cells were then collected by centrifugation at 3,030 x g for 3 min and washed twice with 0.1 M potassium phosphate buffer pH 7.5. The pellet was then exposed to liquid nitrogen (approximately 10 mL) and then ruptured by grinding them in a mortar. Twenty milliliters of the phosphate buffer was added to the ruptured cells followed by centrifugation at 3,020 x g for 5 min. The pellet was discarded and the supernatant (crude extract) was used for the enzyme assay.

Purification steps:

Ammonium sulphate precipitation: For ammonium sulphate precipitation; 8 g of ammonium sulphate was dissolved in each 20 mL of the crude extract, with stirring.

The mixture was allowed to stand for a few hours (2-3 h) followed by centrifugation at 27,000 x g for 20 min at 0°C using a Beckman ultracentrifuge (model J21). The precipitate obtained was dissolved in 5.5 mL of a 0.1 M Potassium phosphate buffer pH 7.5 and then assayed for enzyme activity.

Ion exchange chromatography: Attempts to purify the enzyme was done using DEAE Sephadex A-50 (anion exchange resin) in a buffer medium (0.05M potassium phosphate buffer, pH 7.5, containing 1mM EDTA and 1mM mercaptoethanol) that was used to pack a column (34×1 cm).

Three milliliters of the ammonium sulphate precipitated enzyme was then loaded unto the column and allowed to equilibrate. The column was then eluted with buffer [0.05M potassium phosphate buffer (pH 7.5), containing 1 mM EDTA and 1mM mercaptoethanol] until 16 fractions were collected and then with buffer containing NaCl at concentrations that varied from 0- 0.4 M. Forty fractions were collected (1.3 mL each) and assayed for enzyme activity and protein content (Lowry *et al.*, 1951). Those fractions which showed enzyme activity were then pooled to give 16 mL total volume.

***In vitro* enzyme assay:** The enzyme activity (after ion-exchange chromatography) was assayed using a final reaction volume of 0.9 mL. The procedure applied was a modification of the method used by (Honma *et al.*, 1979). During an assay procedure, the following were added to the test tube: 0.2 mL buffer [0.1 M tris(hydroxymethyl) aminomethane buffer, pH 7.5], 0.5 mL H_2O, 0.1 mL ACC (100 mM) and 0.1 mL enzyme (2.89 mg protein mL^{-1}). The reaction was then incubated at 30°C for 120 min. The concentration of α-ketobutyric acid produced was determined by the method employed by Hatfield and Umbafger (1971). The reaction was stopped with 0.1 mL of 50% trichloroacetic acid. Three milliliters of 0.025% 2, 4 dinitrophenyl hydrazine was added and the mixture allowed to stand for 20 min. One millilitre of 40% NaOH was then added and the absorbance was read immediately at 540 nm using a Spectronic Genesys-5 spectrophotometer. One unit of enzyme activity is defined as one micromole (μM) of α-ketobutyric acid produced per min under the standard assay conditions.

Determination of the period in which α-ketobutyric acid synthesis is linear: Holding all parameters constant, except time, separate reactions were incubated for different time periods (30, 60, 90, 120, 150 and 180 min). The experiment was done in triplicate and the reaction was stopped by adding 0.1 mL of 50% trichloroacetic acid. The α-ketobutyric acid concentration in each tube was then determined as outlined above (*In vitro* enzyme assay).

Effect of ACC concentration on rate of reaction: The enzyme assay was carried out as usual except that the concentration of the ACC was varied from 0-2.5 mM. The final reaction volume of 1.0 mL was maintained by adjusting with distilled water.

Enzyme inhibition: For inhibition studies, 0.1 mL of the water was replaced by 0.1 mL of the inhibitors KCN, $NaBH_4$ and ACC-methylester all at a final concentration of 5 mM. The concentration of ACC varied from 0-2 mM.

The effect of pH: pH studies were carried out ranging from pH 2-10. For pH values between 2 and 5, 0.1 mM sodium acetate buffer was used. Between pH 5.5 and 8.5, 0.1 mM Tris buffer was used. Above pH 9, 0.1M $Na_2CO_3/NaHCO_3$ buffer was employed.

Effect of temperature: The enzyme and buffer were incubated at temperatures that ranged between -15 and 80°C once the desired temperature was reached, the substrate was then added and incubation was done for 30 min; after which test for enzyme activity was carried out as outlined above *(In vitro* enzyme assay).

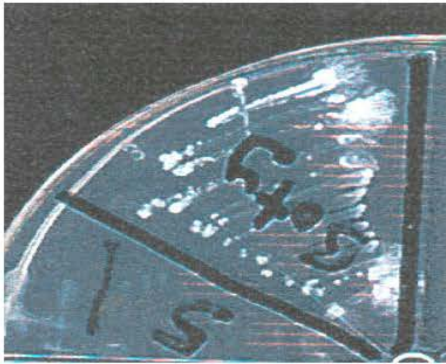
The storage of active ACC-Deaminase: Crude extracts of ACC deaminase were stored at -15 and 14°C and tested for initial enzyme activity. The enzyme activity of the extracts were then assayed every 24 h over a 14 day period

RESULTS AND DISCUSSION

An organism which grew diffusively on the +ACC DF plates was identified using methods similar to those of Kurtzman *et al.* (1980), as *Issatchenkia occidentalis* (Fig. 1). The presence of ACC deaminase was verified by assaying for α-ketobutyric acid. This confirmed that the yeast produced ACC deaminase.

There was diffusive growth of the yeast on the medium containing peptone, but no growth occurred on the medium which contained potassium nitrate. This proved that the yeast *(Issatchenkia occidentalis)* was unable to assimilate nitrates, as was shown by Kurtzman *et al.* (1980). This is quite unusual as most microorganisms of soil origin are able to utilize nitrates (Pancholy and Mallik, 2000); the yeast species *Hanseula saturns* is also able to utilize nitrates, as reported by Honma and Shimomura (1978). ACC deaminase from

A



B

Fig. 1: Growth of yeast (*Issatchenkia occidentalis*) on plates containing 3 mM ACC (A) and on plates without ACC (B)

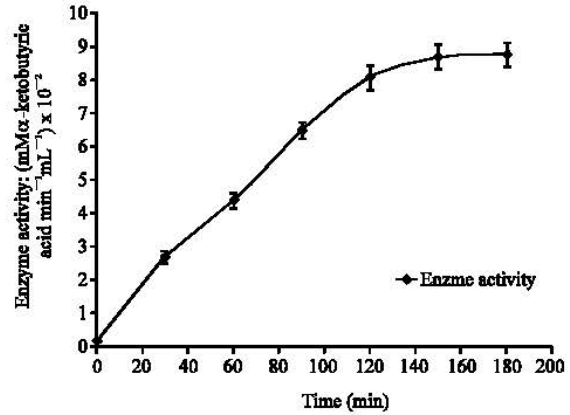


Fig. 2: Linearity in ACC deaminase activity over time

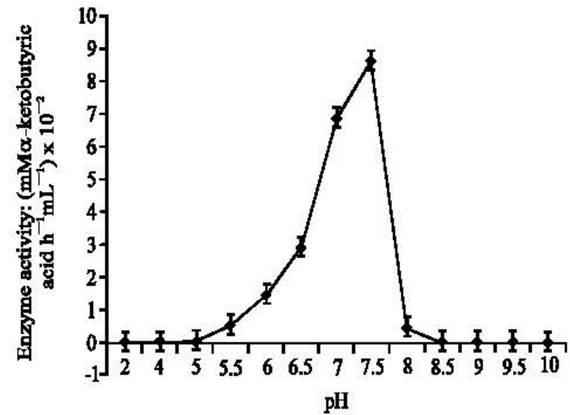


Fig. 3: Effect of pH on ACC deaminase activity

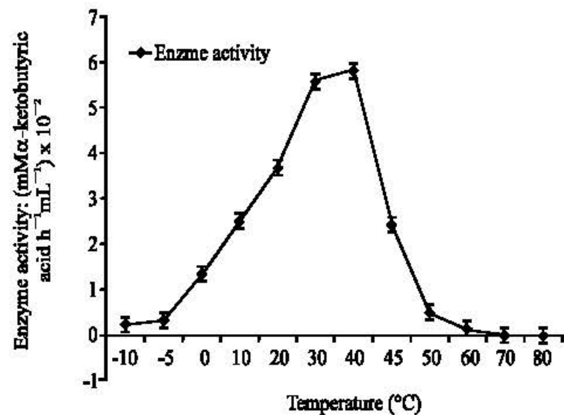


Fig. 4: Effect of temperature on ACC deaminase activity

Issatchenkia occidentalis did not need the cofactor pyridoxal-5-phosphate to be active and catalyses a reaction that is similar to that of γ-cystathionase (Barber *et al.*, 1999).

Characterization of ACC deaminase:

Optimum pH and temperature: Various studies were done using the ACC deaminase that was partially purified. The results, as shown in Fig. 2 indicated that beyond 120 min there was no significant increase in the activity of the enzyme. Linearity in enzyme activity was displayed from 0-120 min (Fig. 2). The results from the pH experiment shows that the enzyme acts within a narrow pH range (Fig. 3). Maximum activity (0.186×10^{-2} mM α-ketobutyric acid min^{-1} mL^{-1}) occurring at pH 7.3 (Fig. 3). Figure 4 shows that initially the activity of the enzyme increases as the temperature was increased. The enzyme activity dramatically fell upon reaching 45°C and activity was lost at 70°C and beyond, a temperature optimum was displayed at approximately 40°C ($8.73\times10\times^{2}$ mM α-ketobutyric acid min^{-1} mL^{-1}). Similar results were obtained by Honma and Shimomura (1978) for the yeast *Hanseula saturns* which exhibited an optimum pH of 8.0

and an optimum temperature of 37°C. Hontzeas *et al.* (2004) reported an optimum pH and temperature of 8.0 and 22°C, respectively for ACC deaminase isolated from the rhizobacterium *Pseudomonas putida* (UW4). A difference in pH and temperature optimum seems to be species related.

Enzyme inhibition: The enzyme was inhibited by 5mM each of ACC-methyl ester, KCN and $NaBH_4$ (Fig. 5). Inhibition by KCN and $NaBH_4$ was not affected significantly with increasing concentration of the substrate (ACC); this suggests noncompetitive inhibition. With increasing ACC concentration the inhibitory effect of ACC methyl ester was reduced, suggesting competitive inhibition.

Enzyme activity and ACC concentration: Table 1 shows the activity of the enzyme with respect to the concentration of ACC. The activity of the enzyme increases steadily until V_{max} is obtained; a plot of enzyme activity versus ACC concentration did yield a curve that was hyperbolic in nature.

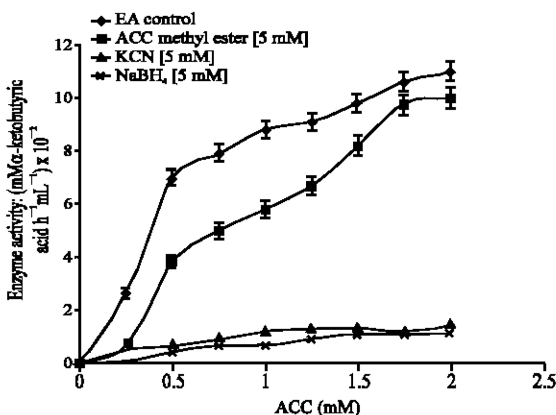


Fig. 5: Inhibition of ACC deaminase by ACC methylester, KCN and $NaBH_4$ each at 5 mM

Table 1: Effect of substrate (ACC) concentration on the rate of reaction

[ACC] (mM)	Enzyme activity: mM α-Ketobutyric acid min^{-1} mL^{-1} enzyme X10^{-2}	X10^{-2}	X10^{-2}	Average x10^{-2}	SE x10^{-2}
0.00	0.00	0.00	0.00	0.0	
0.25	2.75	2.83	2.20	2.6	±0.198*
0.50	6.75	7.25	7.00	7.0	±0.144
0.75	8.04	8.33	7.25	7.9	±0.300
1.0	9.00	8.33	9.20	8.8	±0.260
1.25	9.10	9.00	9.20	9.1	±0.060
1.50	10.60	9.60	9.25	9.8	±0.400
1.75	9.80	11.10	11.00	10.6	±0.420
2.0	11.30	10.70	11.50	11.0	±0.180

* Standard error of the mean for triplicate analyses

Enzyme activity and enzyme concentration: Initially there was a steady increase in activity with increase in enzyme concentration but no significant increase in the activity was observed with an enzyme concentration greater than 0.220 mM ACC, giving an activity of 7.7×10^{-2} mM α-keto butyric acid min^{-1} mL^{-1} enzyme (V_{max}). This is typical of enzymes that are not regulated allosterically (Table 2).

Storage and enzyme activity: With regards to storage of the enzyme, the results (Fig. 6) show that after 14 days the activity of the enzyme stored at -15 and 13°C was 6×10^{-2} and 1×10^{-2} mM α-ketobutyric acid min^{-1} mL^{-1} enzyme, respectively. As shown in Fig. 6, there is a dramatic decrease in enzyme activity after one week storage at 13°C. When stored at -15°C the enzyme activity was relatively unchanged. The shelf life of the enzyme was enhanced at temperatures below 0°C.

Figure 7 shows the elution profile of ACC deaminase from a column that was packed with DEAE sephadex (A-50). Two protein peaks are present, with only one showing enzyme activity (fractions 6-20). The second protein peak (Fractions 22-32) lacked enzyme activity.

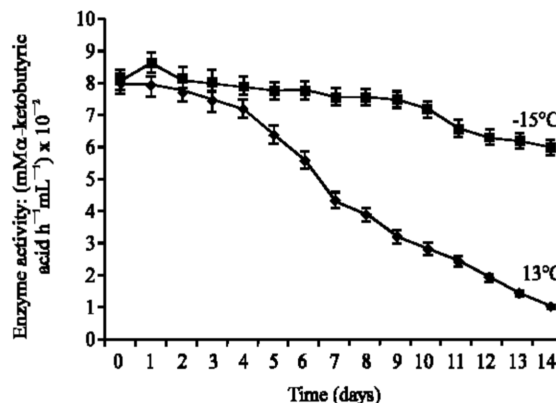


Fig. 6: Storage of ACC deaminase at 13 and -15°C

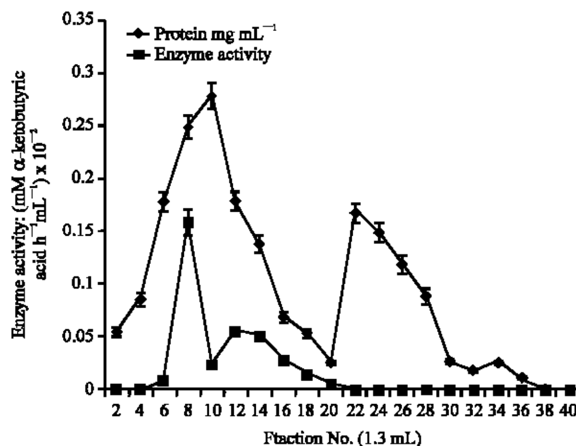


Fig. 7: Enzyme activity after ion-exchange chromatographym using DEAE Sephadex A-50

Table 2: Effect of enzyme concentration on rate of reaction

Conc. (mg mL^{-1} of Enzyme)	Enzyme activity: mM α-Ketobutyric acid min^{-1} mL^{-1} enzyme				
	X 10^{-2}	X 10^{-2}	X 10^{-2}	Average x 10^{-2}	SE x 10^{-2}
0.0	0.0	0.0	0.0	0.0	0.000
0.072	4.8	5.3	4.9	5.0	±0.153*
0.145	5.8	5.9	6.3	6.0	±0.153
0.220	7.2	8.1	7.8	7.7	±0.265
0.290	7.3	7.6	7.7	7.6	±0.175
0.360	7.9	8.4	8.0	8.1	±0.153
0.430	8.1	7.9	8.6	8.2	±0.208

* Standard error of the mean for triplicate analyses

CONCLUSIONS

This study isolated and partially purified ACC deaminase from the yeast *Issatchenkia occidentalis*. The enzyme displayed characteristics that are similar to ACC deaminases that were isolated from other bacterial sources. Unlike ACC deaminase from *Hansenula saturnus*, it was unable to use nitrates as a source of nitrogen.

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