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# Enzymatic characteristics of quercetinases from some indigenous *Aspergillus flavus* strains

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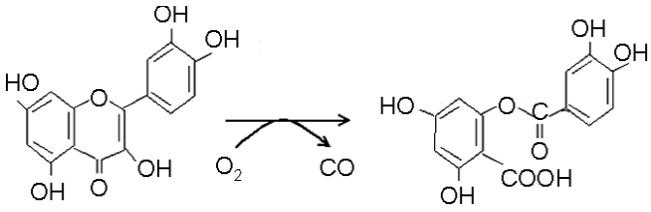
Five indigenous *Aspergillus flavus* strains MTCC-2206, 1884, 1883, 1783 and 2456 were screened for the secretion of quercetinase. Fungal strains MTCC-2206, 1884, 1883, and 1783 were found to secrete the quercetinase in the range of 0.24-0.36 enzyme unit/mL of the culture medium, while MTCC-2456 secreted only 0.04 enzyme unit/mL. The enzymatic characteristics of quercetinase were determined. The $K_m$ values using quercetin as the substrate were 12.5 μ*M*, 14.0 μ*M*, 12.5 μ*M* and 13.0 μ*M* for the quercetinase produced by MTCC-2206, 1884, 1883 and 1783, respectively. The *p*H optima for the above enzymes were 6.5, 6.5, 6.0 and 6.0 and temperature optima were 45, 40, 45 and 50ºC, respectively. The partial purification from only one strain MTCC-2206 was achieved (nearly 3-fold purification).

**Keywords:** Quercetinase**,** *Aspergillus flavus*, Dioxygenase, Copper enzyme

Quercetinase [E.C. 1.13. 11. 24] is a copper containing dioxygenase[1,2], different from Fe containing dioxygenases[3]. It catalyzes the cleavage of quercetin to carbonmonooxide and 2-protocatechuoyl phloroglucinol carboxylic acid[1-6].




Quercetin — 2-protocatechuoyl phloroglucinol carboxylic acid

Dioxygenases play a key role in the complex degradation pathway of aromatic and hetero aromatic compounds[2]. Most of the enzymes that catalyze dioxygen incorporation in aromatic substrates are nonheme, iron-containing dioxygenases. Among them, the intradiol-type catechol dioxygenases utilize mononuclear $Fe^{3+}$ centers for enzymatic activity, while the extradiol-type enzymes use $Fe^{2+}$ ions[7].

Since quercetinase contains copper, it is unique in dioxygenases and there is special interest in its studies. Although the enzyme was reported in 1971[5], but so far, it has not been studied extensively. It is only recently[1,2,8] that quercetinase from *Aspergillus niger* DSM 821[1] and *A. japonicus*[2,3] has been purified. Several differences have been observed between the quercetinases from the two sources[3] and also *A. flavus*[5]. Thus, for structural and functional studies, quercetinases from different sources are required. Also, quercetin 2,3-dioxygenase contains only $Cu^{2+}$ and no other additional co-factors[1]. The catalysis of the breakage of two carbon-carbon bonds makes the study of this enzyme interesting. Moreover, the studies on quercetinase are also important from the point of view of role of copper in metallo enzymes.

In the present study, we have screened five indigenous strains of *Aspergillus flavus* MTCC-2206, 1884, 1883, 1783 and 2456 for the extracellular production of quercetinase in their liquid culture medium. The fungal strains have been isolated, purified and deposited at the Microbial Type Culture Collection Center and Gene Bank, Institute of Microbial Technology, Chandigarh[9]. All the five strains secrete quercetinase, but the MTCC-2456 secretes enzyme in very low quantity. The enzymatic characteristics $K_m$, *p*H and temperature optima of quercetinase from the fungal strains *A. flavus* MTCC-2206, 1884, 1883 and 1783 have been reported. Partial purification of quercetinase from MTCC-2206 strain has also been done.

## Materials and Methods

Rutin, quercetin, Sephadex G-100 and 2-[N-morpholino] ethanesulfonic acid (MES) were obtained from Sigma Chemical Co., St. Louis, USA. The chemicals used in the gel electrophoresis of protein samples were from Genei Pvt. Ltd., Bangalore. All other chemicals were either from S. d. Fine Chem. Ltd, Mumbai, Qualigens Fine Chemical, a division of Glaxo India Ltd. Mumbai, CDH Pvt., New Delhi or Ranbaxy Laboratories Ltd., and were used without further purification.

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The fungal strains were procured from the Microbial Type Culture Collection Center and Gene Bank, Institute of Microbial Technology, Chandigarh and were maintained on agar slants. The growth medium[9] for agar slants for the strains MTCC- 2206, 1884 and 1883 consisted of Czapek concentrate 10.0 mL, $K_2HPO_4$ 1.0 g, yeast extract 5.0 g, sucrose 30.0 g and agar 15.0 g in 1.0 L double-distilled water. The Czapek concentrate consisted of $NaNO_3$ 30.0 g, KCl 5.0 g, $MgSO_4.7H_2O$ 5.0 g and $FeSO_4.7H_2O$ 0.1 g in 100 mL double-distilled water. Similarly, growth medium[4] for agar slants for the fungal strains MTCC-1783 and 2456 consisted of 50 mL of each stock Czapek solutions A and B and 2 mL each of $ZnSO_4.7H_2O$ 1.0 g in 100 mL and $CuSO_4.H_2O$ 0.5 g in 100 mL and made up to 1.0 L with double-distilled water. Czapek stock solution A contained $NaNO_3$ 40.0 g, KCl 10.0 g, $MgSO_4.7H_2O$ and $FeSO_4.7H_2O$ 0.2 g dissolved in 1.0 L of double-distilled water and Czapek solution B consisted of $K_2HPO_4$ 20.0 g dissolved in 1.0 L of double-distilled water.

The liquid culture medium[10] consisted of rutin 4.0 g, $(NH_4)_2HPO_4$ 3.0 g, $K_2HPO_4$ 1.0 g, $MgCl_2.6H_2O$ 1.0 g, $(NH_4)_2SO_4$ 8.0 g, $CuCl_2.5H_2O$ 0.02 g, $ZnSO_4.7H_2O$ 0.001 g, $FeSO_4.7H_2O$ 0.001 g and $MnSO_4.H_2O$ 0.001 g in 1.0 L of double-distilled water was used for screening the fungal strains for secretion of quercetinase in the culture medium. For starter cultures, 100 mL of the liquid culture media in 250 mL culture flask were sterilized and inoculated with 1.0 mL of spores suspension (spore density $1.2 \times 10^6$ spores/mL) under aseptic conditions. The cultures in the flasks were grown in the Orbital Shaker model Orbitek–LE (Scigenics, India) at 30ºC temperature and 150 rpm.

On 3rd day, 10 mL of mycelia suspension from the starter culture was withdrawn and inoculated in 1.0 L sterilized liquid culture media in 2.0 L flask. The harvest cultures were grown in the same orbital shaker at 30ºC and 150 rpm. To monitor secretion of quercetinase in the liquid culture medium, 1.0 mL aliquots of growth medium were withdrawn at 1-day intervals and filtered through Whatman filter paper (no. 42). 50 µL of the filtrate was added to 1.0 mL of 0.05 *M* MES buffer *p*H 6.0 containing 0.06 m*M* quercetin contained in 1.0 mL cuvette maintained at 25ºC. Decrease in quercetin concentration with time was monitored using UV/vis Spectrophotometer (Hitachi, Japan, model U-2000) at $\lambda$ = 367 nm. The molar extinction coefficient value of $1.57 \times 10^4$ $M^{-1}$ $cm^{-1}$ was used for calculating the enzyme unit, which was taken as 1 µ*M* decrease in the quercetin per min under the conditions specified above. The least count of the absorbance measurement was 0.001 absorbance unit. The maximum activity of the enzyme appeared on the 6th day of the inoculation of spores.

The enzymes for the reported experiments were prepared by growing the fungal strains in harvest liquid culture growth medium as mentioned above. On the 6th day of inoculation of fungal spores when the enzymatic activity reached the maximum, the liquid culture medium was filtered through four layers of cheese cloth to remove the fungal mycelia. The filtered enzyme had activity in the range 0.24 to 0.36 enzyme units per mL of the filtrate .The enzyme samples were kept in fridge at 4ºC and did not loose appreciable activity for 3 months.

The culture filtrates were concentrated nearly 30-times using Amicon concentration cell with YM-10 ultrafiltration membrane having molecular weight cut-off value 30,000 Da. The concentrated enzyme samples were dialyzed against 0.1 *M* MES buffer, *p*H 7.0 in volume ratio 1:1000 and dialyzed sample was loaded on a Sephadex G-100 gel filtration column (column size 2.6 cm x 60 cm) equilibrated with 0.1 *M* MES buffer, *p*H 7.0. The protein was eluted using the same buffer. 3.0 mL of the dialyzed enzyme samples were loaded on the column and eluted with the same buffer at a flow rate 14 mL/h. Fractions of 3.5 mL size were collected and analyzed for quercetinase activity and protein concentration. The active quercetinase fractions were pooled and concentrated using Amicon concentration cell with YM-10 ultrafiltration membrane.

The purity of enzyme was checked by SDS-PAGE gel electrophoresis[11]. The gel was prepared using routine technique with help of minivertical gel electrophoresis equipment (Techno Source, Mumbai). Standard staining and destaining procedure were adopted.

The enzymatic characteristics $K_m$, *p*H and temperature optima were determined using quercetin as the substrate and following the reaction spectrophotometrically at $\lambda$ = 367 nm. The oxygen concentration was kept fixed at the atmospheric oxygen saturation.

A broad screening test[12] based on vapour diffusion in sitting drops was used to find out the condition under which quercetinase could be crystallized. The experiment was performed in two culture plates containing 24 wells each. 1.0 mL of precipitant solution was placed in one well. One microbridge

with a grove was put in each well. 10 µl of protein solution was placed in the grove of the microbridge and 10 µl of precipitant solution was added to the each protein droplet. The solutions along with microbridges were sealed with cover slip using cedarwood oil. The appearance of microcrystals was observed at the regular intervals of two days using optical microscope.

## Results and Discussion

Fig. 1 shows that all the strains, except MTCC-2456 secreted quercetinase and the maximum activity appeared on 6$^{th}$ day after the inoculation of the fungal spores. The peak value were in the range 0.24 to 0.36 enzyme units per mL.


Fig. 1—Secretion of quercetinase in the liquid culture media containing different *A. flavus* strains [The enzyme secretion shown for 100 mL starter liquid culture media in 250 mL culture flasks, (o) MTCC-2206; (●) MTCC-1884; (▲) MTCC-1883; (Δ) MTCC-1783; and (□) MTCC- 2456]

Fig. 2(a) shows the Michaelis-Menten curve for the quercetinase secreted by the MTCC-2206 using quercetin as the substrate. The corresponding double-reciprocal plot is shown in Fig. 2(b) as an insert. The calculated $K_m$ value was 12.5 µ*M*. The Michaelis-Menten curves and the double-reciprocal plots for quercetinase of the other strains were similar (data not shown) to Fig. 2. The calculated $K_m$ values for the

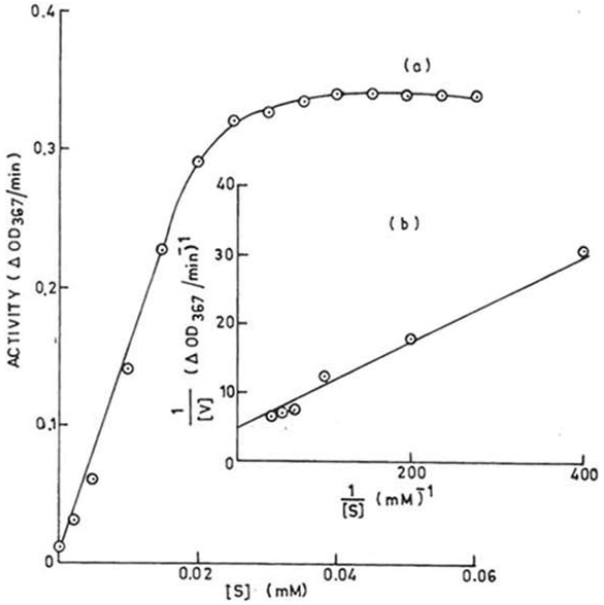


Fig. 2—Michaelis-Menten and double-reciprocal plot for the quercetinase of *A. flavus* MTCC-2206 [1 mL of 0.05 *M* MES buffer *p*H 6.0 containing variable concentrations of quercetin in 1 mL cuvette was maintained at 25°C and reaction was initiated by the addition of 0.05 mL of the enzyme stock with activity 0.24 enzyme U/mL]

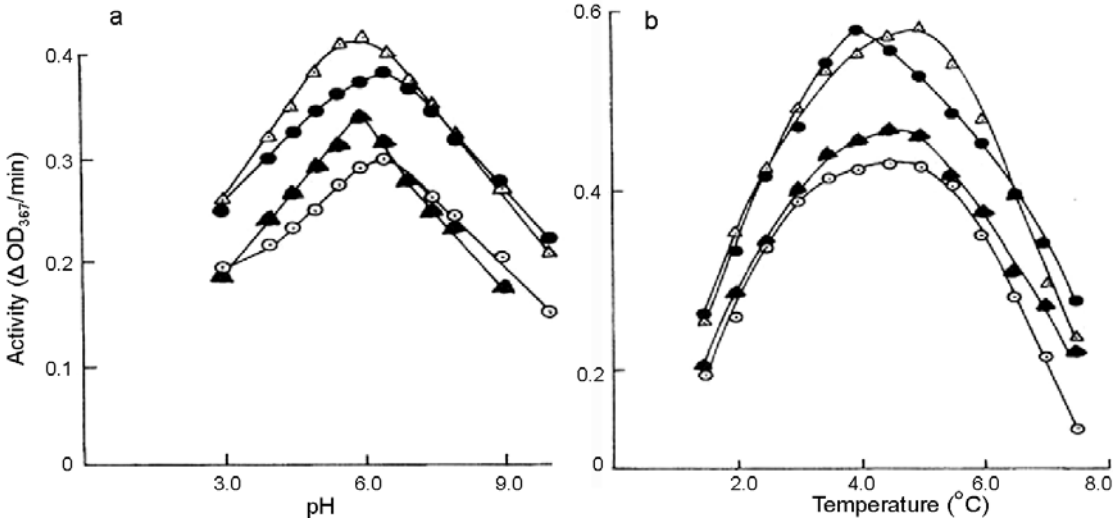


Fig. 3—Variation of quercetinase activity with *p*H (a) and temperature (b) [The reaction conditions were the same as mentioned in the legend to Fig. 2, except for the fixed concentration of quercetin at 0.06 m*M* and *p*H (a) and temperature (b) was varied, (O) MTCC-2206; (●) MTCC-1884; (▲) MTCC-1883; (Δ) MTCC-1783; and (□) MTCC- 2456

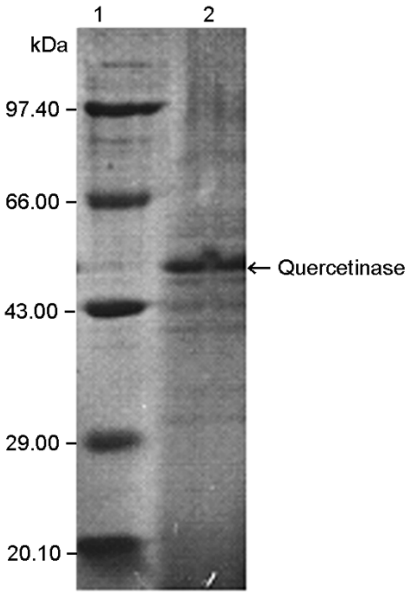


Fig. 4—SDS-PAGE of quercetinase of *A. flavus* MTCC-2206 [Lane 1, protein marker; and lane 2, pure quercetinase]

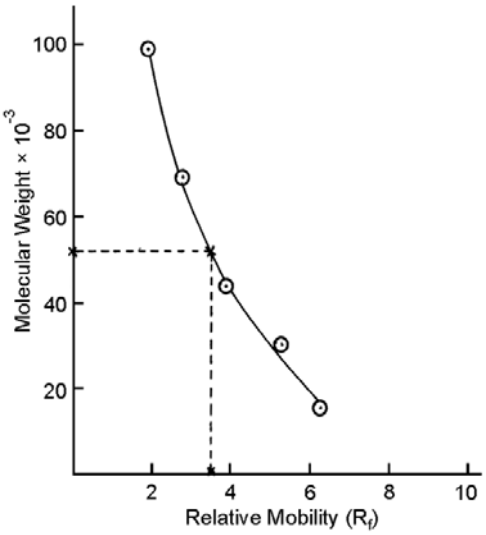


Fig. 5—Molecular weight determination of quercetinase using SDS-PAGE

quercetinase of strains MTCC-1884, 1883 and 1783, which were found to be 14.0 μ*M*, 12.5 μ*M* and 13.0 μ*M*, respectively. The $K_m$ value reported for the quercetinase purified from *A. niger* DSM 821 was 6.6 μ*M*[1], which was lower than the $K_m$ values of the quercetinases of *A. flavus* strains, indicating that the substrate affinity of the quercetinases of *A. flavus* strains for quercetin was lower than the *A. niger* DSM 821.

The variation of activities of the quercetinases from different *A. flavus* strains with *p*H and temperature are

Fig. 6—Microcrystals of quercetinase from *A. flavus* MTCC-2206 under the condition of 0.1 *M* HEPES buffer, *p*H 7.5, 2% polyethylene glycol 400 and 2.0 *M* ammonium sulphate in a single drop of protein and precipitant solution [One small division of the scale equals to 30 μm]

shown in Fig. 3a and b, respectively. The *p*H optima of the enzymes was in the range 6.0 to 6.5, which was near to *p*H optimum of the *A. niger* quercetinase (ranged 5.7 to 6.7)[1]. The temperature optima of the quercetinases were in the range 40 to 50°C (Fig. 3b), which was similar to the temperature optimum of 40°C reported for the *A. niger* quercetinase.

SDS-PAGE showed single major protein band, indicating the purity of the enzyme solution (Fig. 4). The molecular weight as determined by SDS-PAGE method was found to 51,000 Da (Fig. 5). However, this value cannot be taken as the correct, since during the preparation of sample for SDS-PAGE, the molecule of the enzyme might dissociate into its subunits. As the molecular weight reported for quercetinase from *A. flavus* PRL 1805 was 11,1000 ± 4000 Da and the enzyme was found to be a dimer[5], molecular weight of the quercetinase reported here also might be a dimer, each of subunit of 51,000 Da.

The partially purified enzyme sample gave microcrystals (Fig. 6) under the condition of 0.1 *M* HEPES buffer (*p*H 7.5), 2% polyethylene glycol 400 and 2.0 *M* ammonium sulphate, indicating that the enzyme was crystallizable.

In conclusion, all the five indigenous strains of *A. flavus* secreted quercetinase in good quantity, except MTCC-2456. The partial purification of quercetinase from only one strain MTCC-2206 was achieved.

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