Purification and Characterization of Extracellular *Penicillium brevicompactum* Uricase

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ABSTRACT

The uricase was purified to homogeneity from *Penicillium brevicompactum* grown on solid state fermentation. Different purification steps including ammonium sulfate fractionation followed by separation on DEAE-Spheadex A50 and Sephadex-G75 column were applied to the crude culture filtrate to obtain a pure enzyme. The enzyme was purified 64.59 fold and shoed a final specific activity of 3920 U/mg protein with 51.7 % yield. SDS-PAGE of the purified enzyme revealed it was one peptide chain with molecular weight of 43 kDa. Line Weaver-Burk analysis showed a km value of 0.5 mM and Vmax of 909 IU. Maximum enzyme activity was found at pH 9 and 30°C. The purified enzyme showed maximum stability over a wide rang of pH between 6-10and up to temperature of 70°C. The enzyme activity was stimulated greatly in presence Fe, Na, Ca, and Cu ions and inhibited greatly by addition of EDTA and KCN. The pure enzyme proved to be rich in methionine, glutamic acid and glycine

INTRODUCTION

Uricase or urate oxidase (urate: oxygen oxidoreductase, EC 1.7.3.3) is an enzyme that catalyze the oxidation of uric acid to allantoin and plays an important role in purine metabolism *(1)*. Uricase is widely found in animals, plants, fungi, yeasts and bacteria *(2)*. Higher primates (apes and humans) lake functional uricase and excrete uric acid as the end product of purine degradation *(3,4)*. In human increased level of blood uric acid over the normal value lead to a group of diseases, such as gout, chronic renal failure, some organic acidemias and Lesch–Nyhan syndrome *(5, 6)*. Uricase is used as a diagnostic reagent in medicine and clinical biochemistry, where it is implicated in the determination of uric acid in biological fluids *(7)* and in commercial formulations of hair coloring *(8)*. Uricase was originally isolated from mammalian organisms and recently interest was concentrated on several microbial sources which have been proposed for this clinical indication. But only one microbial uricase isolated and purified from *Aspergillus flavus* has actually been used commercially under trade mark of the uricozyme *(9)*. The microbial enzyme is inducible and therefore, the presence of uric acid or some other inducer in the medium is necessary for enzyme production *(7)*. The present work was aimed to produce, purify thermally stable and highly active extracellular uricase from *Penicillium brevicompactum* using a new waste-fungus combination (Banana skin) and characterizing its kinetic properties.

MATERIAL AND METHODS

Microorganism, Media and cultivation

A fungal strain of *Penicillium brevicompactum* used in this study was maintained on Potato-Dextrose-Agar slant medium. The slants were grown at 30°C for 7 days and stored at 4°C.

Enzyme production

The highest *Penicillium brevicompactum* uricase productivity was achieved on using an optimized modified fermentation medium containing (g/l): uric acid 1.0; K2HPO4 4.0; MgSO4 0.5; NaCl 0.2; FeSO4 0.01, sucrose 20.0, and banana waste 8.0 (10). The contents of 250 ml Erlenmeyer flask, were mixed thoroughly and autoclaved at 121°C for 20 min. SSF was carried at 30°C with substrate initial moisture content of 64% for 4 days using 0.5ml spore suspension ($6x10^7$spores/ml) as inoculum.

Extraction of crude enzyme

At the end of incubation period, the flasks were centrifuged at 8000 rpm for 45 min

at 4°C. The clear supernatant was considered as a source of enzyme and was analyzed for its protein content and uricase activity.

Enzyme assay

Uricase activity was measured at 30°C by following the decrease in the absorbance of uric acid at 293 nm *(7)*. One unit of uricase enzyme was equal to the amount of enzyme which converts 1 µmol of uric acid to allantoin per min at 30 °C. The protein was determined *(11)*.

For determination of kinetic parameters, substrate concentrations of 5–100 mM were used and the data collected were treated with the Line weaver–Burk plot. Thermal stability of *Penicillium brevicompactum* uricase was evaluated by heating the purified uricase for 30 min in closed vials at scheduled temperatures (20–90°C) and then cooled to room temperature. For the pH stability measurements, samples of *Penicillium brevicompactum* uricase were dissolved at room temperature in a buffer containing 0.05 M sodium acetate (pH 3–6),0.05 M potassium phosphate (pH 7),or 0.05 M sodium borate (pH 8–11). After 2 h of incubation, the enzyme activity was evaluated.

Purification of Uricase

Penicillium brevicompactum uricase was purified according to the method of ***Ma et al, (2)*** with some modifications. All purification steps were carried out at 4°C. Finely powdered ammonium sulfate was added to the culture supernatant up to 75% saturation. The mixture was allowed to precipitate for 3 h at 4°C, the precipitate was collected by centrifugation (8,000 rpm for 30 min at 4°C) and re-suspended in 0.05 M borate buffer (pH 9) and dialyzed overnight against the same buffer. The sample was loaded onto a DEAE-Sephadex A50 anion exchange column and eluted with 0.2 M borate buffer (pH 9) containing 0.0 - 0.5 M NaCI gradient at a flow rate of 0.5 ml/min. The most active fractions were pooled out, dialyzed, concentrated and applied to a Sephadex-G75 gel filtration column. The column was eluted with 0.1M borate buffer (pH 9) at a flow rate of 1ml/min. The active fractions were pooled, concentrated and lyophilized for further studies.

Molecular weight determination

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out *(12)* in 3 mm slab gel of 6% acrylamide in Tris borate buffer pH 7.1 containing 0.1% SDS. Gels were stained with 0.1% Coomassie brilliant blue R-250 and destained.

Amino acid analysis

The amino acid composition was determined in the acid hydrolysate of the purified uricase using a Beckmann Amino Acid Analyzer (Modell 119 GK) according to the method described by ***Speckmann et al. (13)***.

RESULTS

The partial purification of *Penicillium brevicompactum* uricase was affected by 75% ammonium sulfate as precipitant. The supernatant possessed a very minor uricase activity and most of the activity was concentrated in the precipitate. Results given in Table 1 indicates that the specific activity of ammonium sulfate fraction was 274.71 U/mg protein compared with that of the crude extract 60.69 U/mg protein, while the total protein decreased from 1218 mg to 195.75 mg. This step was associated with activity preservation of 72.75% and purification factor of 4.53 folds. The dialyzed ammonium sulfate fraction was applied to DEAE Sephadex A50 column. Fig 1 shows the complete profile of the DEAE-Shadex A50 anion exchange step. It was found that the ammonium sulfate fraction contained different protein molecules, only one of them had uricase activity. It seems that the uricase was firmly bound to the gel since it was eluted at high concentration (0.0-0.5M) of NaCl. Table 1 also shows that the uricase was enriched after the DEAE Sephadex A50 column and the specific activity became 1148.65 U/mg protein with purification factor 18.93. The protein was reduced by 96.96%. Further purification was conducted on the second DEAE Sephadex G75 gel filtration column. Fig. 2 reveals that this purification

step gave only one peak corresponding to uricase was raised up to 3920 U/mg protein with a purification factor of 64.59 fold and 51.7% yield. On the other hand the protein reduced by 99.20%. On using SDS-PAGE the final fraction showed that the purified uricase gave only one band indicating high purity of the purified enzyme. On using different standard proteins with known molecular weights, it was found that the apparent molecular weight of *P.brevicompactum* uricase was 43 kDa (Fig.3).

Table 1. Purification of extracellular *Penicillium brevicompactum* uricase

Purification steps	Total volume	Activity (U/ml)	Protein (mg/ml)	Total activity (Unit)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield %
Crude extract	840	88	1.45	73920	1218	60.69	1.00	100
Ammonium sulfate (75%)	225	239	0.87	53775	195.75	274.71	4.53	72.75
DEAE-Sephadex A50	100	425	0.37	42500	٣٧	1148.65	18.93	57.49
Sephadex G75	65	588	0.15	38220	9.75	3920	64.59	51.7

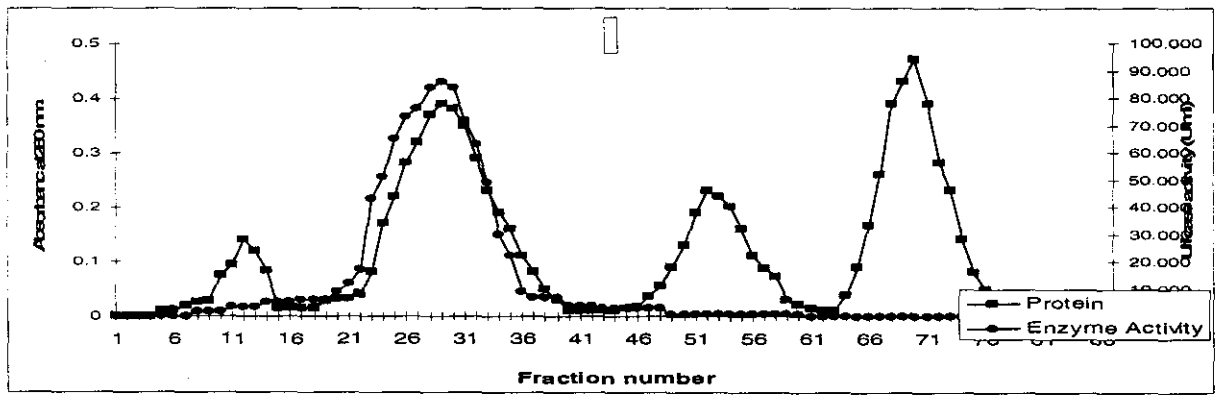

Fig. 1. First DEAE- Sephadex A50 anion exchange column chromatography of *P. brevicompactum* uricase.

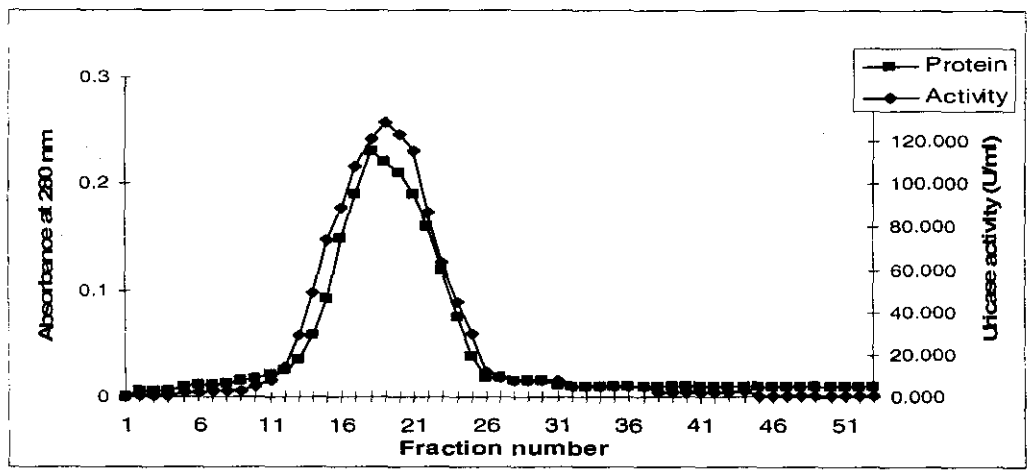

Fig. 2. Sephadex-G75 gel filtration column chromatography of *P. brevicompactum* uricase.

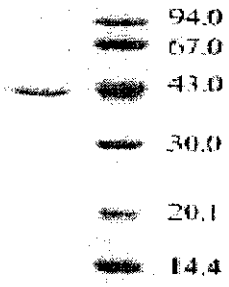

Fig. 3:SDS-PAGE of *P. brevicompactum* uricase.

The enzyme kinetic studies indicated that the enzyme activity depends on substrate concentration (Fig. 4). When the relation between the enzyme activity and the substrate concentration was treated by Line Weave-Burk analysis (Fig.5), it was found that the apparent Km and Vmax were 0.5 Mm and 909 IU respectively. The effect of pH on the stability and activity of *P. brevicompactum* uricase is indicated. (Fig. 6a). *P. brevicompactum* uricase was showed that it is maximally activated at pH 9. (Fig 6b) revealed that the uricase was mostly stable over a broad pH range of 6 to 9. The reaction rate of *P .brevicompactum* uricase was measured at various temperatures (15-70°C). Maximum activity was obtained at 30°C (Fig. 7a). At higher temperature the enzyme activity decreased sharply. The thermal stability of *P. brevicompactum* uricase was monitored by measuring its activity over a wide range of temperature (20-90°C). Fig 7b shows that *P. brevicompactum* uricase retained 100% of its activity at temperature up to 70°C, indicating that the enzyme is thermally stable for a wide rang of uses and different application.

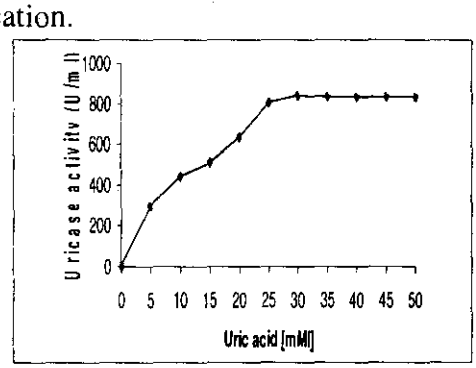

Fig. 4. Effect of substrate concentration on *P. brevicompactum* uricase activity

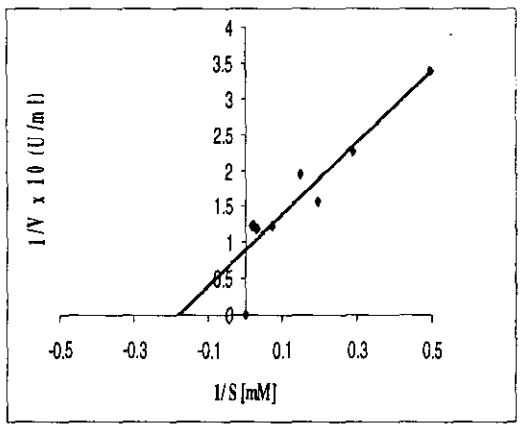

Fig. 5. Line weaver-Burk plot of *P. brevicompactum* uricase activity

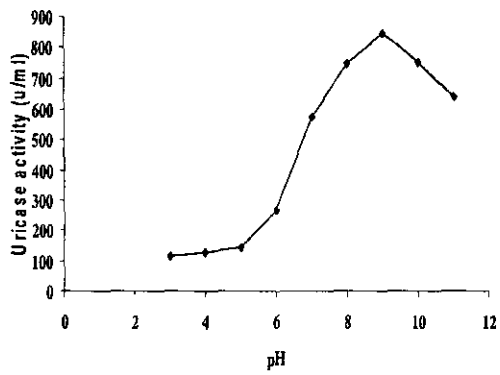

Fig. 6a. Effect of pH (B) on *P. brevicompactum* uricase activity

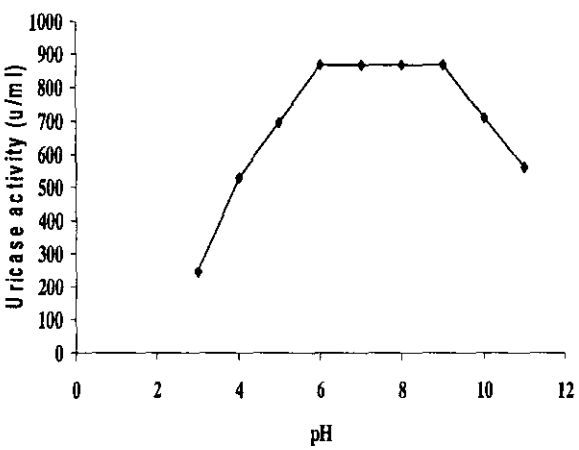

Fig. 6b. Effect of pH (B) on the stability of *P. brevicompactum* uricase activity

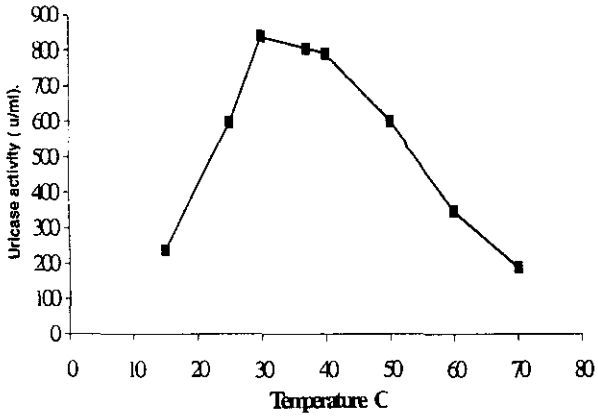

Fig. 7a. Effect of incubation temperature on *P. brevicompactum* uricase activity.

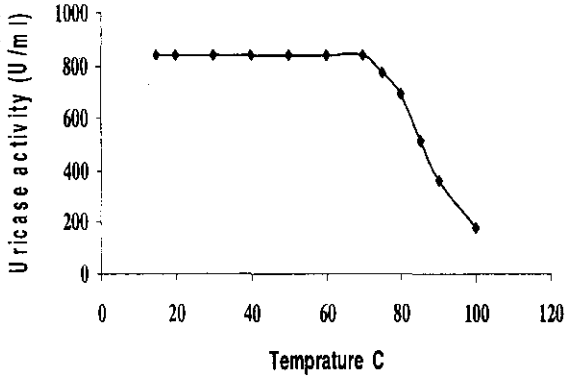

Fig. 7b. Thermal stability of *P. brevicompactum* uricase.

The effect of different metal ions (1mM) namely CaCl2, CoCl2, MgCl2, Bacl2, ZnCl2, FeCl2, MnCl2, NaCl, KCl, HgCl2 ,CuSO4 and some chemical reagents (5mM) as KCN, DDT,EDTA, mercaptoethanol and cysteine on *P. brevicompactum* uricase activity was determined. Table 2. showed that *P. brevicompactum* uricase activity was strongly activated obtained in presence of FeCl3, NaCl, CaCl2, CuSO4, DDT, mercaptoethanol and cysteine respectively while the other reagents inhibited the uricase activity by different degrees. The amino acid contents of purified *P.brevicompactum* uricase is shown in Table 3. The purified enzyme proved to be rich in methionine, glutamic acid and glycine. Also it had high quantities of tyrosine, alanine and serine while contains low amount of arginine and lysine.

Table 2. Effect of some metal ions and chemicals on *P. brevicompactum* uricase activity.

Metal ions (1mM) and son chemical reagents (5mM)	Uricase activity U/ml
Control	845
FeCl3	927
MnCl2	820
BaCl2	670
CuSO4	899
CaCl2	901
HgCl2	289
KCl	847
ZnCl2	790
CoCl2	869
NiCl2	448
MgCl2	845
NaCl2	920
EDTA	45.0
DDT	879
KCN	67.0
Cystein	897
B- mercaptoethano	907

Table 3. Amino acid composition (mole %) of the purified *P. brevicompactum* uricase.

Amino acids	Mole%
Aspartic	6.9
Threonine	9.8
Serine	15
Glutamic	23
Proline	2.9
Glycine	21
Alanine	19
Valine	9.5
Methionine	24
Isoleucine	4.2
Leucine	5.5
Tyrosine	15
Phenylalanine	3.9
Histidine	7.1
Lysine	3.2
Arginine	1.6

DISCUSSION

Purification of *P. brevicompactum* uricase was achieved by using 75% ammnonium sulfate saturation, DEAE Sephadex A50 ion exchange and Sephadex G75 gel filtration column respectively. The specific activity increased from 60.69 to 3920 U/mg protein from the crude extract and the final preparation respectively. The final preparation was examined using SDS-PAGE, which revealed that the purified uricase contained one protein band with molecular weight of 43 KDa. It has been reported that the native uricase purified from *Microbacterium* sp. strain ZZJ4-1 and *Candida. Utilis* composed of one subunit with 34 kDa *(2)*. Also ***Saeed et al (9)*** revealed that *Pseudomonas aeruginosa* uricase had a single subunit with molecular weight of 64 kDa. Moreover, Uricases purified from liver of pig, mouse and baboon composed of 4 subunits with molecular weight of 32-33 kDa for each subunit ***(14)***.

Line Weaver-Burk plot showed that, the Km and Vmax values of *P .brevicompactum* uricase were 0.5 mM and 909 IU respectively, indicating high affinity of the purified uricase to its substrate. Uricase from different microorganisms has different substrate affinities and probably plays different roles in the enzyme activity. ***Ma et al, (2)*** reported that *Microbacterium* sp. strain ZZJ4-1 had Km value of 0.31 mM. *Bacillus thermocatenulatus* uricase was found to have a km value of 0.25 mM ***(17)***.

Maximum *P .brevicompactum* uricase activity and stability obtained when it was incubated with optimum substrate concentration at PH 9. A similar pH values for from *Microbacterium* sp. strain ZZJ4-1 (2), *Candida utilis* ***(15)*** and caprin kidney (8) uricases. ***Huang & Wu (18)*** reported that both wild-type and mutant *Bacillus subtilis* uricase have 100% activity at a pH value from 6 to 10. Highest *P .brevicompactum* uricase activity was obtained at 30°C and the enzyme was thermally stable up to 70°C at which the enzyme retained 100% of its activity. These results agreed with uricase isolated from *Bacillus fastidiosus* ***(16)***. ***Ma et al. (2) and Lotfy (17)*** reported that uricase from *Microbacterium* Sp and *Bacillus thermocatenulatus* retained about 100% of initial activity after heat treatment at 70°C for 30min and 75°C for 45min respectively. On the other hand, both wild-type and mutant *Bacillus subtilis* uricase retained 45-60 % of activity at 70°C ***(18)***.

Salts of FeCl3, NaCl, CaCl2 & CuSO4 and some chemical reagents as DDT, cysteine & mercaptoethanol increased *P .brevicompactum* uricase activity significantly, while HgCl2, EDTA and KCN inhibited the enzyme activity greatly. These results agreed with those obtained by ***Ma et al, (2)*** who reported that, uricase activity of *Microbacterium* sp. strain ZZJ4-1 had increased greatly in presence of Fe. Also it has been indicated that metal ions like Na, Ca & Cu strongly enhanced the activity of *Pseudomonas aeruginosa* uricase by 182.2, 236 & 275 % respectively***(9)***. *Bacillus fastidiosus* uricase was inhibited by Zn, Co and cyanide ***(16)***. The activity of uricase isolated from poultry manure had greatly inhibited by Zn and Cu while it less inhibited in presence of Mg and Mn ***(19)***. The quality of *P. brevicompactum* uricase was assessed for its amino acid contents. The purified enzyme was rich in methionine, glutamic acid and glycine. Relatively higher amount of tyrosine, alanine and serine were present.

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الملخص العربى

تنقية وتوصيف انزيم اليوريكاز المتحمل للحرارة من فطر البينيسليم بريفيكومباكتم

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في محاولة لتنقية الأنزيم المتحمل للحرارة يوريكاز والذى تم انتاجه من فطر البينيسليم بريفيكومباكتم المعزول من التربة وذلك بعد زراعته باستخدام تقنية التخمر فى الحالة الصلبة على مخلف قشر الموز فانه تم معالجة محلول الأنزيم بمحلول٧٥% سلفات الأمونيوم وذلك عند درجة حرارة ٤ °م ولمدة ثلاث ساعات وبعد عملية الطرد المركزي تمت معاملة المحلول بواسطة كروماتوغرافيا التبادل الأنيوني (DEAE-Sephadex A50) ثم تم معالجتة بواسطة عمود الترشيح (Sephadex-G75). هذا و قد كان مجمل التنقية 64.59 مرة بانتاجية 51.7%. ثم بعد تركيز الأجزاء التي تحتوي على نشاط الأنزيم تم أختبارها بواسطة طرق التهجير الكهربي وجد انه عند استخدام (SDS/PAGE) الفصل الكهربي لمعرفة الوزن الجزيئي لجزيئات البروتين وجد ان وزنه الجزيئي 43000 دالتون ،وعند دراسة ديناميكية الأنزيم والعوامل التي تؤثر على وجد ان نشاط الأنزيم يعتمد على تركيز المادة الخاضعة لفعل الأنزيم حيث لوحظ ان نشاط الأنزيم يزداد بزيادة تركيز مادة التفاعل وعند دراسة هذه العلاقة بواسطة طريقة لينويفر – بروك وجد ان Km تساوي 0.5 مللى مول وان Vmax تساوي909 وحدة دولية ، وعند دراسة تأثير درجة الحرارة المثلى لنشاط الأنزيم وجد انها ٣٠ °م وعند دراسة تأثيرمدى تحمل الإنزيم للحرارة وجد انه قادر على تحمل درجة حرارة حتى ٧٠ °م وأتضح من الدراسة أن الأس الهيدروجيني الأمثل هو (٩) فى حين أنه قادر هلى التحمل فى المدى من ٦-٩، إضافة الى دراسة تاثير كاتيونات بعض فلزات الاملاح المختلفة ووجد ان ايونات الحديد والنحاس والصوديوم و الكالسيوم اعطوا زيادة ملحوظة في نشاط الانزيم عن ايونات الاملاح الأخرى التى كان لها تأثير مثبط. هذا وقد أثبت أن هذا الأنزيم النقى كان غنيا جدا بالأحماض الأمينية خاصةالميثيونين وحمض الجلوتاميك و الجليسين مقارنة بالكميات القياسية حسب مواصفات منظمة الأغذية.