

Production, Purification and Biochemical Characterization of Cyclodextrin Glucanotransferase from *Bacillus cereus* N1

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CYCLODEXTRIN glucanotransferase- (CGTase), (EC.2.4.1.19) producing bacteria were isolated from different sources of soils and identified as *Bacillus cereus* N1 and the best source was the soil of the National Research Centre. The maximum production of the crude CGTase enzyme was observed after 48hr of incubation at 37°C producing CGTase activity of 3.5 U/ml.

The effect of nutritional requirements on the CGTase production were carried out. Soluble starch and yeast extracts were found to be the best carbon and nitrogen sources, respectively.

The enzyme was successively purified by ammonium sulphate precipitation, DEAE-cellulose and sephadex G-100 column chromatography and the final specific activity of CGTase enzyme was increased by 24 fold. The SDS-PAGE showed that the purified CGTase enzyme was homogenous and the molecular weight of the purified enzyme was about 75 kDa.

The characterization of the enzyme exhibited optimum pH and temperature at 6.0 and 40°C, respectively. The enzyme was stable at pH 6.5 to 8.0 and retained its high activity up to 45°C.

Keywords: Cyclodextrin, Glucanotransferase, Cyclodextrins, *Bacillus cereus*, β -CD.

Cyclodextrin glucanotransferase (EC 2.4.1.19) is an extracellular enzyme, a member of the amylolytic glucosylase family (Zain *et al.*, 2007). This enzyme degrades starch to form cyclodextrins (CDs) via a cyclization reaction. In this specific reaction, starch is cleaved and the ends are joined to form closed circular structures. In addition to catalyzing this intramolecular reaction (cyclization), CGTase is also involved in intermolecular transglyco-sylation that involves coupling reactions, as well as the hydrolytic action on starch and CDs. Bacteria are still regarded as an important source of CGTase. *Bacillus macerans* is the first reported source of CGTases but a wide variety of bacteria have been determined now as CGTase producers, namely aerobic mesophilic bacteria, aerobic thermophilic, anaerobic thermophilic and aerobic alkalophilic bacteria (Tonkova, 1998).

CDs are able to form inclusion complex with various kinds of organic or inorganic compounds inside the cavity of the ring structure. The enzyme is divided into three main types, α -, β - and γ -, according to the major type of CD formed. CGTases from different sources show different biochemical characteristics and yield different ratios of CDs-products. Cyclodextrins are biochemical products having industrial importance, especially in foods, cosmetics and medicine (Szejtli, 1998).

The aim of this work is isolation of CGTase-producing bacteria from local media, in addition to study the factors affecting the CGTase production, purification of the isolated enzyme and finally measurement the constants of the purified enzyme.

Materials and Methods

Isolation and screening of bacteria

Bacteria were isolated from different sources of soils such as National Research Centre (NRC), Aswan, and Alexandria. The samples were suspended in sterile water and then subjected to serial dilution followed by inoculation in nutrient agar plate at 37°C for 48hr. After the examination under microscope for its purity, the pure strain was selected for the production of CGTase.

Characterization and identification of bacteria

Characterization and identification of bacteria was done by the method of Bergey's Manual Determinative Bacteriology (Holt *et al.*, 1984).

Preparation of crude enzyme

The chosen bacterial culture was inoculated on rotten potatoes which contained: soluble starch 1% (w/v), potatoes extract 2% (w/v), $(\text{NH}_4)_2\text{SO}_4$ 0.2% (w/v), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.02% (w/v) and phosphate buffer pH 7.5 (KH_2PO_4 , 0.1% ; K_2HPO_4 , 0.2%) and then incubated at 37°C with shaking at 180 rpm for 48 hr.

Assay of CGTase

The enzyme activity was determined using a phenolphthalein as indicator (Kaneko *et al.*, 1987). One unit of the enzyme activity is defined as the amount of enzyme that forms 1 μ mol of β -CD per minute.

Protein determination

The protein content was determined according to the method of Bradford (1976), using bovine serum albumin (BSA) as the standard protein. Absorbance at 280nm was used for monitoring proteins in column elutes.

Effect of carbon sources on CGTase production

Various types of carbon sources such as galactose, glucose, insoluble starch, lactose, maltose, mannose, soluble starch, soya bean, and sucrose (in a concentration of 1%).

As known that soluble starch is the best source of carbon for *Bacillus* sp. (Tonkova, 1998) therefore, different concentrations of soluble starch were used in the medium.

Effect of nitrogen source on CGTase production

Various types of nitrogen sources such as urea, yeast extract, peptone, NaNO₃, NH₄Cl, KNO₃, and (NH₄)₂SO₄ (in a concentration of 0.2%, original nitrogen source) were used as nitrogen source with equimolar nitrogen content.

Purification of the enzyme

After 2 days of growth, the culture medium was centrifuged at 4°C for 10 min at 10,000 rpm, to give a clear supernatant. Ammonium sulphate was added up to 20-80% saturation with gentle stirring at 4°C. Then the enzyme that precipitated by 80% ammonium sulphate was dissolved in phosphate buffer, 0.2M (pH 6.0) and dialyzed against of distilled water, still ammonium sulphate free using barium chloride.

The enzyme extract was then subjected to a column (1.5x40 cm) of DEAE-cellulose, then it was subjected to gel filtration process on a column (2.5x75cm) of Sephadex G-100 equilibrated at room temperature with 0.2M phosphate buffer (pH 6.0) and the fractions were collected at a flow rate of 30 ml/h in both columns.

Optimum pH and temperature

The optimum pH of the pure enzyme was determined by replacing the phosphate buffer (pH 6) in the CGTase assay method with the following buffers: sodium acetate buffer, 0.2 M (pH 3.5-5) or sodium phosphate buffer, 0.2 M (pH 6-9). The CGTase assay was done by the procedure mentioned above. The optimum temperature of the pure enzyme was determined by incubating the reaction mixture of the CGTase assay in different temperatures, ranging from 30 to 70 °C for 10 min.

pH and thermal stability

The pH stability of the enzyme was measured by incubating 0.1 ml of pure enzyme extract with 0.2 ml of 0.2 M sodium acetate buffer (pH 3.5-5), 0.2 M sodium phosphate buffer (pH 6-9) at 40°C, without substrate for 30 min then the enzyme activity was measured at pH 6.0. The temperature stability of the enzyme was measured by incubating 0.1 ml of pure enzyme extract with 0.2 ml buffer (0.2M sodium phosphate buffer, pH 6.0) without substrate at different temperatures (30-70°C) for 10 min and then the enzyme activity was measured at 40°C.

Electrophoretic techniques

SDS-PAGE was performed in Bio Rad Mini-Protein II Dual-Slab apparatus according to the method described by Laemmli (1970).

Results and Discussion

Identification and characterization of CGTase-producing bacteria

The isolated bacteria from rotten potato extract which gave the highest CGTase activity was rod-shaped bacteria and formed oval-shaped endospores. It could be suggested that this strain has been belonging to the genus *Bacillus* and therefore designated as *Bacillus cereus* N1, as illustrated in Table 1 (Holt *et al.*, 1984).

TABLE 1. Some characteristics of *B. cereus* N1.

Name of Test	Result
Shape	Rod
Gram staining	+
Sporulation	+
Motile	+
Growth temperature	25-40 C°
Growth pH	7.5
Hydrolysis of starch	+
Methyl red reaction	-
Production of indole	+
Utilization of citrate	+
Reduction of nitrate	-
Production of urease	-
Catalase	+
Growth on NaCl (% w/v) 7.5, 10	+ +
Acid produced from utilization of glucose arabinose, mannitol and xylose	-
Anaerobic growth	-
Voges test	+
Lecithinase active	+
Rhizoid growth	+
PCR	+

Effect of carbon sources on CGTase production

Bacillus cereus N1 could grow well on many of the carbon sources tested and maximum activity of 3.43 U/ml were obtained when soluble starch was used. Other carbon sources were also used such as galactose, glucose, insoluble starch, lactose, maltose, mannose, soya bean and sucrose with the same equivalent concentrations and number of carbons. The results of CGTase activity are shown in Fig. 1. The different concentrations of soluble starch were tried and 2% was found to be the optimum concentration, as shown in Fig. 2.

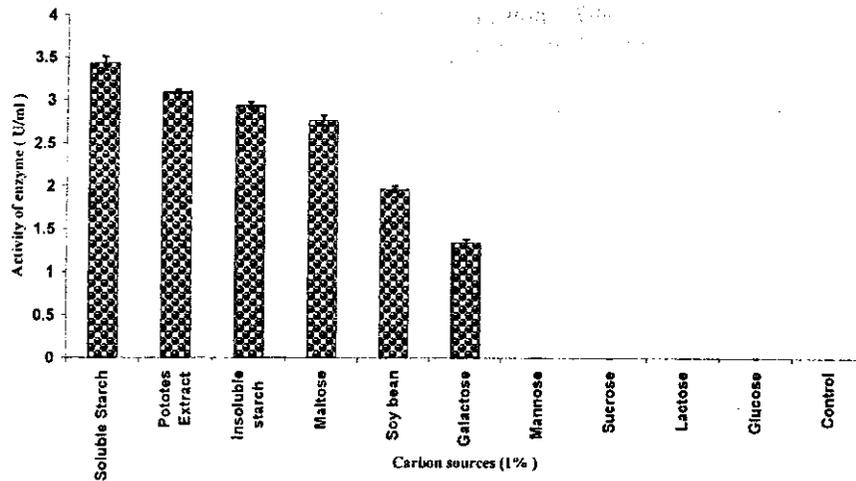


Fig.1. Effect of carbon sources on CGTase production from *Bacillus cereus* N1.

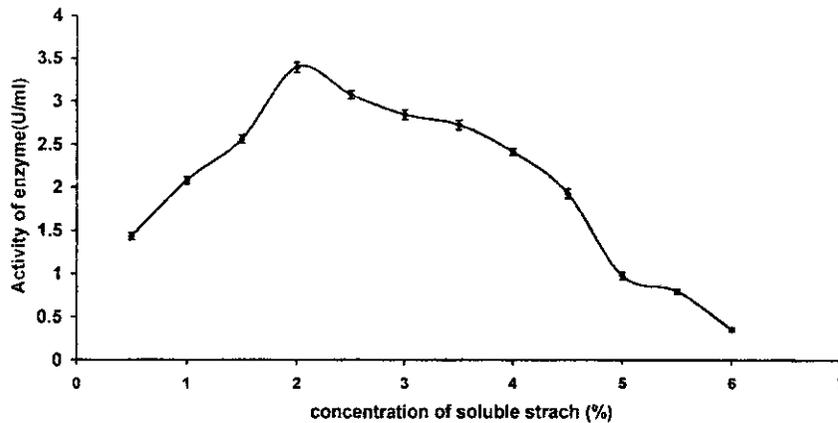


Fig.2. Effect of different concentrations of soluble starch on CGTase production from *Bacillus cereus* N1.

The differences in the enzyme activities obtained from different sources of carbon may be due to the difference in their chemical structures and the ability of *Bacillus cereus* N1 to hydrolyze specific glycosidic bonds for di-or polysaccharides .

Do *et al.* (1993) and Poci *et al.* (1998) found that soluble starch is the best carbon source for *Bacillus firmus* and *Bacillus macerans*, respectively, as shown in the present study on *Bacillus cereus*.

Varavinit *et al.* (1997) showed also that the CGTase production by *Bacillus* sp. MP523 was repressed significantly by glucose.

Effect of nitrogen sources on CGTase production

CGTase production was higher when yeast extract was present in the medium. CGTase production using inorganic nitrogen source was found to be low compared with organic nitrogen source, as shown in Fig. 3:

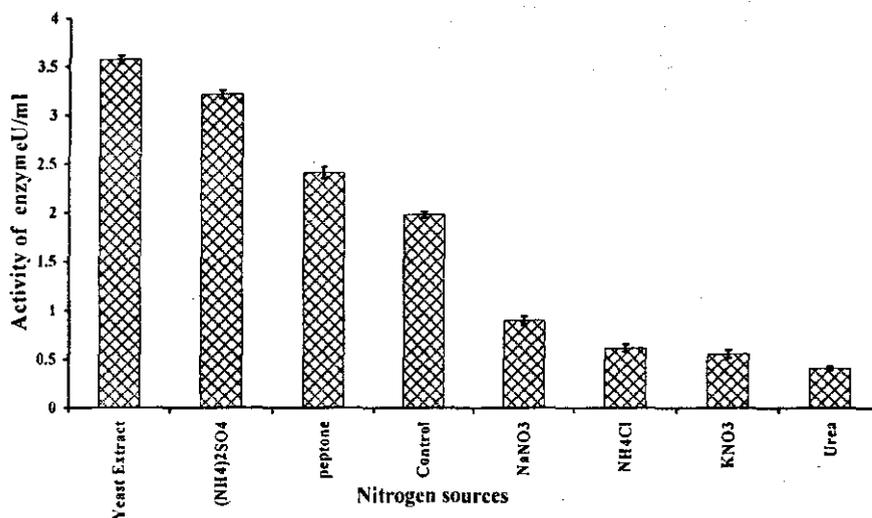


Fig.3. Effect of nitrogen sources on CGTase production from *Bacillus cereus* N1.

Gawande & Patkar (1999) observed maximum CGTase production when has been used pepton as nitrogen source for *Klebsiella pneumoniae* AS-22. Urea and NH₄NO₃ was found to induce the growth of *Bacillus circulans* and therefore, the enzyme was highly secreted.

Purification of the CGTase enzyme

After 2 days of growth, the culture medium was centrifuged at 4°C for 10 min at 10,000 rpm, to give a clear supernatant. The enzyme activity for each purification step is presented in Table 2 which showed that the crude CGTase was successfully purified in three steps. The enzyme eluted from DEAE-cellulose and sephadex G-100 columns showed 6.45 of 3.63% recovered activity with a purification fold 6.5 and 24, respectively, as shown in Table 2 and Fig.4 and 5.

Kitahata & Okada (1974) showed the purification processes of CGTase enzyme from *Bacillus macerans* IFO 3490 in four successive steps starting with the adsorption on corn starch followed by the above mentioned three steps. In addition, Stavn & Granum (1979) purified also the CGTase enzyme by the three above mentioned steps from *Bacillus macerans*.

TABLE 2. Summary of CGTase purification from *Bacillus cereus*.

Steps	Volume (ml)	Total protein content (mg)	Total CGTase Activity (U)	Yield (Recovered activity) %	Specific activity (U/mg)	Purification fold
Crude extract	1000	250	3100	100	12.4	1
(NH ₄) ₂ SO ₄ precipitation 80%	100	18	350	11.3	19.4	1.6
DEAE-Cellulose column	50	2.5	200	6.45	80	6.45
Sephadex G-100 column	25	0.38	112.5	3.63	296.1	23.9

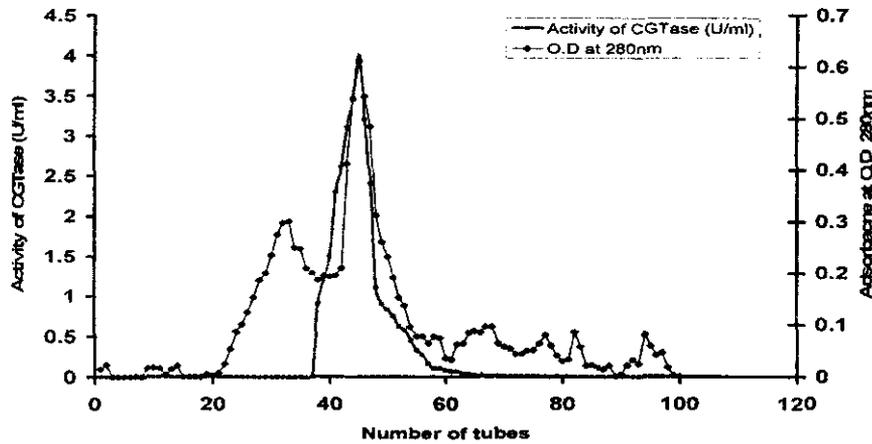


Fig.4. Elution profile of CGTase enzyme by DEAE cellulose column.

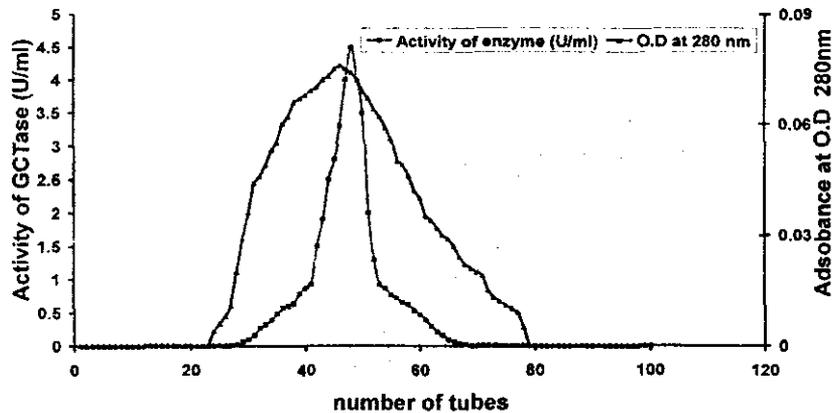


Fig.5. Elution profile of CGTase enzyme by sephadex G-100 column.

Characterization of purified CGTase enzyme

Molecular weight determination by SDS-PAGE

The crude CGTase was successfully purified to homogeneity and the purified enzyme gave a single protein band on a SDS-PAGE gel with molecular weight of 75 kDa, as shown in Fig. 6.

Stavn & Granum (1979) purified CGTase enzyme from *Bacillus macerans* and determined the molecular weight by dodecyl sulfate-gel electrophoresis in 9% polyacryl-amide gels in the presence of 2-mercaptoethanol and found that the molecular weight was 75 kDa and the protein consisted of one polypeptide chain only.

Also, Sian *et al.* (2005) showed that the molecular weight of CGTase enzyme from alkalophilic *Bacillus* species was 75 kDa using 15% Coomassie Brilliant Blue R-250 stained gel .

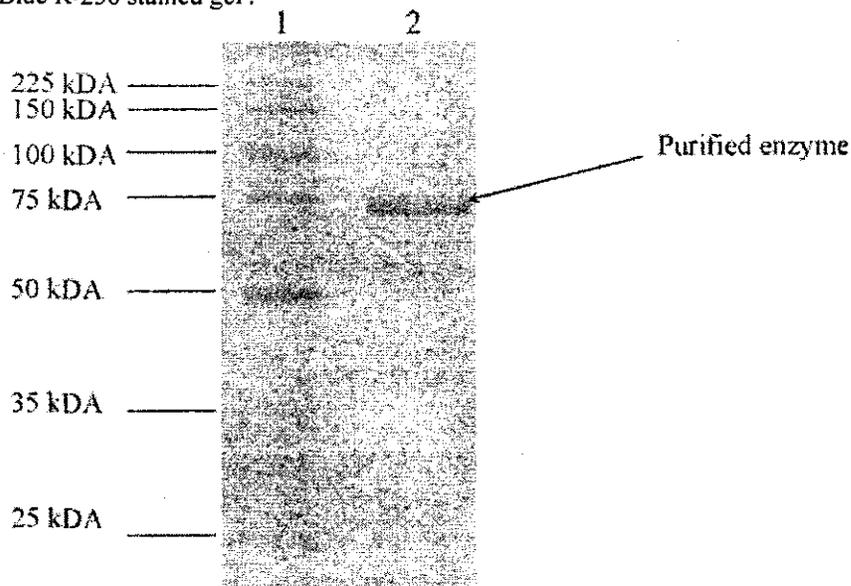


Fig. 6. Brilliant Blue analysis of purified CGTase .

Lane 1: broad range protein molecular weight markers

Lane 2: purified enzyme.

Effect of pH on the enzyme activity and pH stability

The enzyme activity was measured using the standard assay method by different pH values (3.5-9.0) at 40°C, and the optimum pH of the purified enzyme was determined as pH 6.0, as shown in Fig. 7. The enzyme did not show any activity at pH 3.5 and 9.0 indicating that the enzyme requires a near-neutral pH range to perform its reaction and the extreme pH values were not suitable for the enzyme activity. The activity of the purified enzyme showed activity percentages from 88% to 60% at pH 6.5 to 8.0, as shown in Fig. 8.

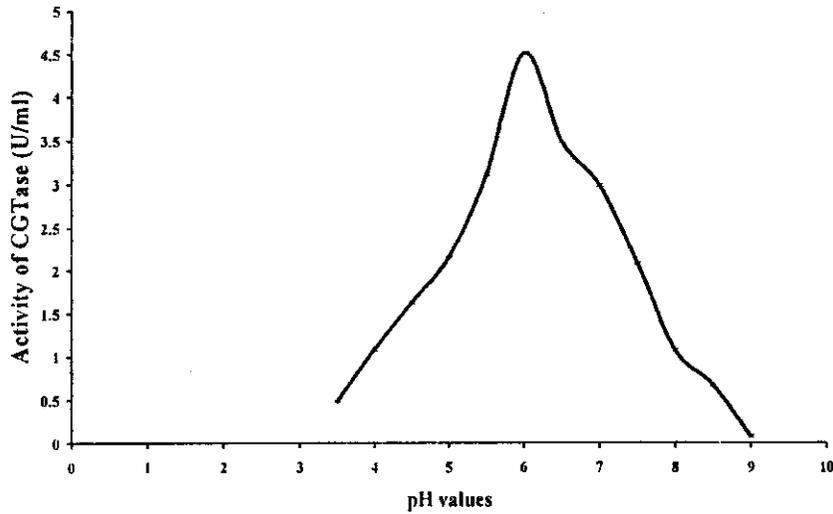


Fig. 7. Effect of different pH values on purified CGTase activity.

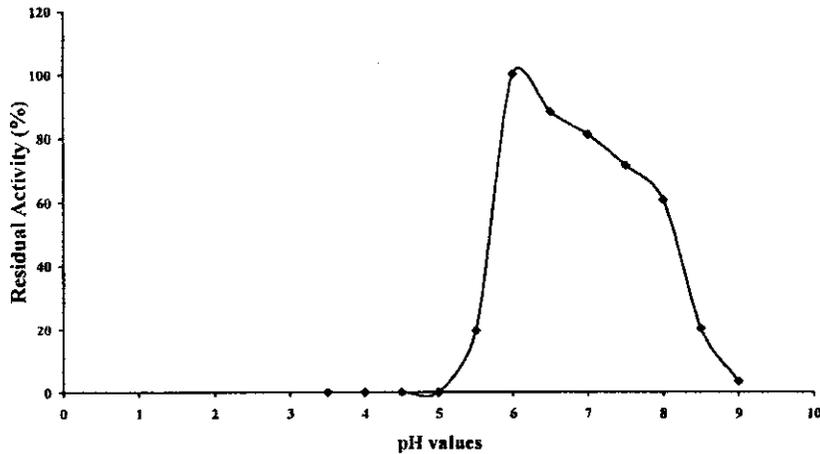


Fig. 8. Effect of pH stability on the purified CGTase activity.

Sian *et al.* (2005) showed that the optimum pH of CGTase enzyme from alkalophilic *Bacillus* sp. was 6.0 and it was stable from pH 7.0 to 9.0 at 60°C.

Effect of temperature on the enzyme activity and thermal stability

The activity of the purified enzyme was measured at different temperatures at pH 6.0 using the standard assay method. The optimum temperature of the purified enzyme was 40°C, as shown in Fig. 9.

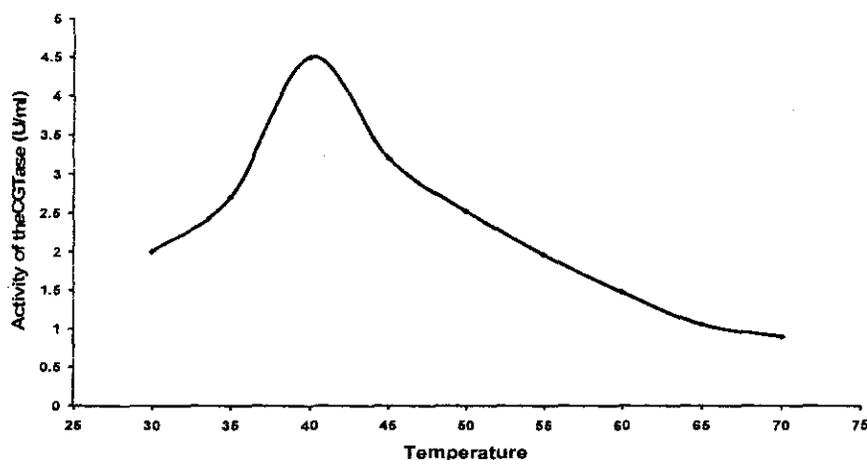


Fig.9. Effect of temperature on the purified CGTase activity.

The purified enzyme was stable up to 40°C at pH 6.0 for 10 min of incubation. However, it began to lose 23% of its total activity at 45°C and retained about 17% of its activity at 60°C, as shown in Fig. 10.

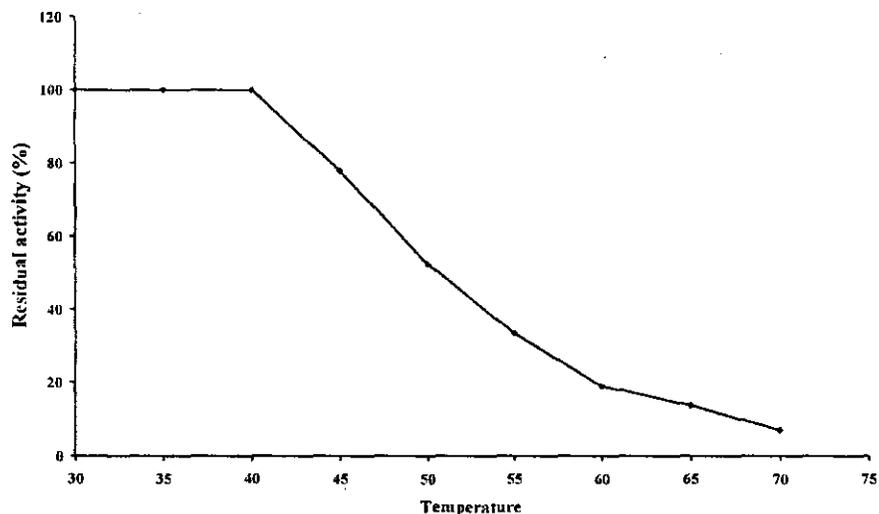


Fig.10. Effect of thermal stability on the purified CGTase activity.

Kitahata & Okada (1974) showed that the optimum temperature of the purified enzyme for *Bacillus macerans* IFO 3490 was 55°C. The authors checked the thermal stability of the purified CGTase enzyme and found that the enzyme was stable at temperature from 55 to 60°C.

Sian *et al.* (2005) showed also that the optimum temperature of CGTase enzyme for alkalophilic *Bacillus* sp. was 60°C and its stability was up to 60°C for 30 min.

Conclusion

Cyclodextrin glucanotransferase isolated from *Bacillus cereus* N1. From the above data we concluded that the enzyme CGTase was act upon starch at proper conditions producing cyclodextrins which is the target end product and we studied in details the proper experimental conditions its action and the kinetics of this enzyme.

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إنتاج وتنقية ودراسة خواص إنزيم سيكلو دكسترين جلوكانو ترانس فيريز من بكتيريا باسيليس سيرس

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إنزيم سيكلو دكسترين جلوكانو ترانس فيريز تم إنتاجه من البكتيريا المعزولة من التربة المحلية. وقد وجد أن أحسن إنتاج للإنزيم كان من البكتيريا المعزولة من تربة المركز القومي للبحوث وتم تعريف السلالة البكتيرية المنتجة للإنزيم *Bacillus cereus* NI ووجد أن الظروف المثلى للإنتاج تكون بعد ٤٨ ساعة عند درجة حرارة ٣٧°م وتلك الظروف أعطت ٣,٥ وحدة إنزيم في الملى الواحد .

وكذلك وجد أن أفضل مصدر للكربون و النيتروجين هما النشا الذائب و مستخلص الخميرة على الترتيب.

وقد تم تنفيذ التنقية الجزئية للإنزيم الخام المنتج من البكتيريا المعزولة بعدة خطوات متلاحقة من الترسيب الجزئي بكبريتات الأمونيوم ثم التنقية الكروماتوجرافية (كروماتوجرافيا العمود) للأنزيم. المنقى جزئياً باستخدام DEAE- cellulose chromatography حيث حملت الأجزاء الأنزيمية الراسبة عند تركيز ٨٠٪ من كبريتات الأمونيوم على العمود ثم تم تمريره بعد ذلك على العمود sephadex G-100 مما ترتب على ذلك الحصول على درجة نقاوة قدرها حوالي ٢٤ مرة من الأنزيم الخام.

كما تم عمل SDS-PAGE للأنزيم المنقى جزئياً وظهر أنه متجانس وذو وزن جزئي حوالي ٧٥ kDa وكذلك تم دراسة الخواص البيوكيميائية للأنزيم حيث أظهر أحسن نشاط عند الأس الهيدروجيني ٦,٠ ودرجة حرارة ٤٠ °م وقد أظهر الأنزيم ثباتاً عند الأس الهيدروجيني من ٦,٥ إلى ٨,٠ واحتفظ بحوالي ٧٨٪ من فاعليته عند درجة حرارة ٤٥ °م .