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KINETIC STUDIES OF EXTRACELLULAR α - GALACTOSIDASE FROM *CITROBACTER FREUNDII*

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Abstract: Extracellular a-galactosidase producing bacteria were isolated from soil. Bacteria that showed a-galactosidase activity were identified as *Escherichia coli, Klebsiella pneumoniae* and *Citrobacter freundii* by morphological and biochemical tests. *Citrobacter freundii* showed the highest enzyme production of 19 milliunits/ml after 36 hrs of cultivation in pH 8 phosphate buffer containing peptone. a-Galactosidase from *Citrobacter freundii* was purified by ammonium sulphate fractionation and DEAE ion exchange chromatography. One a-galactosidase activity peak was observed indicating the presence of a single enzyme form. A 164 fold purification was obtained with a yield of 8%. Polyacrylamide gel electrophoresis of the enzyme showed 2 protein bands. The kinetic properties of the enzyme were studied using p-nitrophenyl a-D-galactopyranoside. The Michaelis constant and maximum reaction velocity obtained were 2.85 x 10^{-3} M and 14/mmol/min/mg of protein respectively. Studies on the effect of pH on enzyme activity showed a broad pH optimum from 6.0 to 8.0 with maximum activity at pH 7.5 at 29°C. The enzyme was stable between pH 5.5 to 8.0. The optimum enzyme activity was observed at 40°C at pH 7.5. The enzyme was stable upto 40 °C. The enzyme preparation did not contain any invertase activity.

Key Words : *Citrobacter freundii,* Extracellular, α - Galactosidase, Kinetics.

INTRODUCTION

α-Galactosidase (α-D-galactoside galactohydrolase) hydrolyses α-1,4 galactosidic linkages of galactose containing polysaccharides. It has been reported to occur widely in microorganisms, plants and animals. This enzyme is used in industry and in medicine.

In Japan and in USA, the raffinose content in molasses of the beetsugar industry is reduced using α-galactosidase by the addition of *Mortierella vinacea* mycelial pellets.[1]

Partially purified α-galactosidase from *Aspergillus satoi*[2] and *Cladosporium cladosporides*[3] have been shown to be capable of removing flatulence causing oligosaccharides from soy milk.

In addition to these, this enzyme has been used in the pulp and paper industry[4] and in the manufacture of gelling agents.[5]

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Microorganisms are the most promising sources for large scale enzyme production. They can be easily grown and it is usually not difficult to scale up the production process. With microbes, it is possible to increase the production by changes in the growth conditions.

In this paper, we report the partial purification of extracellular α-galactosidase from *Citrobacter freundii* and kinetic studies carried out with the same enzyme preparation.

METHODS AND MATERIALS

Materials: Analytical grade Serva Fein Biochemicals and Sigma Chemicals were used. DEAE Sephadex A-25 was obtained from Pharmacia Fine Chemicals.

Absorbance measurements were carried out using a Shimadzu UV 120-02 spectrophotometer. Bench centrifuge (MSE) and high speed centrifuge (Beckman Model J2-21) were used for centrifugation.

Enzyme Assay: To 1 ml of culture supernatant, 1ml of 0.15M McIlvaine buffer (pH7.5) was added and mixed well. 1ml of this mixture was incubated with 0.5ml of 1mM p-nitrophenyl α-D-galactosidase (PNGP) solution for 30 minutes at 29 °C. The reaction was terminated by the addition of 5ml of 0.1N Na_2CO_3. Absorbance was measured at 405 nm.[6]

A unit of enzyme activity is defined as the amount that hydrolyses 1µmol of substrate per minute under specified conditions.

Protein Estimation: The protein determinations were done by the method of Lowry et al[7] using crystalline bovine serum albumin as standard.

Isolation of bacteria producing extracellular α-galactosidase: Soil was incubated in a culture medium containing raffinose, peptone, yeast extract and salt solution. After incubation the bacteria present in the medium were isolated in pure form. These pure bacterial cultures were grown in the same culture medium and the supernatant was tested for α-galactosidase activity. Three bacterial species that showed high enzyme production were identified as *Escherichia coli, Klebsiella pneumoniae* and *Citrobacter freundii* by morphological and biochemical tests.[8]

Citrobacter freundii gave the highest enzyme production of 14mu/ml after 18h of cultivation when grown in peptone culture medium with an initial pH of 8. Extracellular α-galactosidase production of *Citrobacter freundii* could be increased upto 19mu /ml when cultivated in pH 8 phosphate buffer culture medium for 36h.[8]

Purification of α-galactosidase

Extraction: The α-galactosidase enzyme present in the culture medium was obtained by centrifugation of the culture broth at 5000g at 4^0C for 20 minutes. The supernatant was retained.

Ammonium Sulphate Fractionation: The supernatant was brought upto 75% $(NH_4)_2SO_4$ saturation using solid $(NH_4)_2SO_4$. The 75% saturated solution was centrifuged at 25000g for 20 minutes at 4 ^0C. The precipitate was dissolved in a minimum volume of 0.001M McIlvaine buffer (pH7) and dialysed with the same buffer.

DEAE Sephadex A-25 Ion Exchange Chromatography: A column of DEAE A-25 (1.6cm x 40cm) was prepared as described by Andrews.[9] The dialysed $(NH_4)_2SO_4$ fraction was applied and eluted with a 2:1 McIlvaine buffer containing NaCl. The mixing chambers contain 100ml of 0.001M McIlvaine buffer (pH 7) (starting buffer) and 50ml of 0.1M McIlvaine buffer (pH 7) containing 10% NaCl. Fractions (10ml) were collected in a refrigerated fraction collector at a flow rate of 1ml/min. (Figure 1) The fractions containing enzyme activity were pooled, dialysed and retained.

Polyacrylamide gel electrophoresis: (PAGE): PAGE was carried out by the method described by Weber & Osborne[10] using the Shandon apparatus. Polyacrylamide gels were prepared and loaded with 40μl of the enzyme preparation. 0.01M Phosphate buffer pH 8.0 was used as the reservoir buffer. Gels were stained for proteins using Coomassie blue.

The enzyme preparation was tested for invertase-activity using sucrose.[2]

Kinetic studies were carried out using PNGP as the substrate.

RESULTS

Purification

Purification of α-galactosidase from *Citrobacter freundii* is summarized in Table 1. The multistep purification gave an overall yield of 8% and the α-galactosidase was purified 164 fold. (Table 1). A high decrease in activity is observed in DEAE chromatography (Figure 1) indicating that the enzyme could be inactivated in solutions with a low protein concentration.

Test for purity: PAGE of the enzyme preparation gave two protein bands and one α-galactosidase activity band.

Table 1: Purification of α - Galactosidase from *Citrobacter freundii* spent culture medium.

Purification stage	Total Volume (ml)	Total Activity (mU)	Total Protein (mg)	Specific Activity (mU/mg Protein)	Recovery (%)	Purification (fold)
5000g supernatant- SI	2100	11603	2241	5	100	1
0-75% $(NH_4)_2SO_4$ pellet-P11	260	9900	43.5	228	85	44
Ion Exchange Chromatography DEAE-25	10	968	1.1	848	8	164

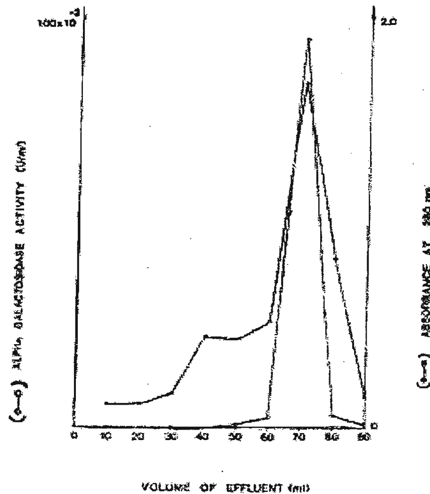

Figure 1: Fractionation of the partially purified alpha- galactosidase preparation (0-75% ammonium sulphate fraction) from *Citrobacter freundii* on DEAE sephadex A-25.

The enzyme preparation did not display invertase activity

Effect of subtract concentration on enzyme activity: α-Galactosidase was incubated at 29 °C in (0.15M) McIlvaine buffer (pH 7.5) containing PNGP for 30mins. The Km and Vmax values of α-galactosidase for the substract determined from the linear part of the Lineweaver-Burk plot were 2.85 x 10^{-3} M and 14 μmol/mg of protein (Figure 2). No significant difference in Km and Vmax values were observed when Km and Vmax values were calculated by the Hofstee plot.

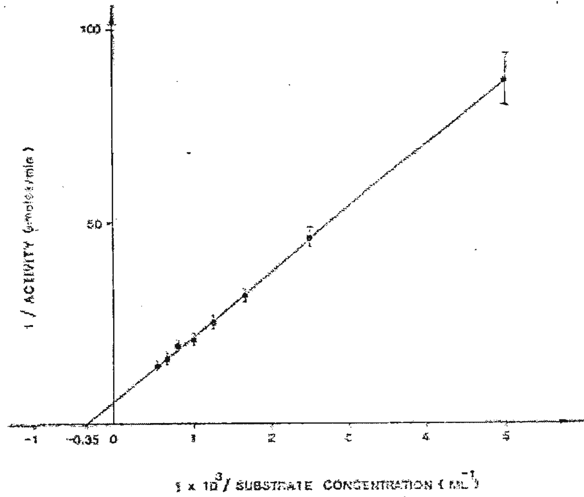

Figure 2: Lineweaver Burk double reciprocal plot for α-galactosidase from *Citrobacter freundii*

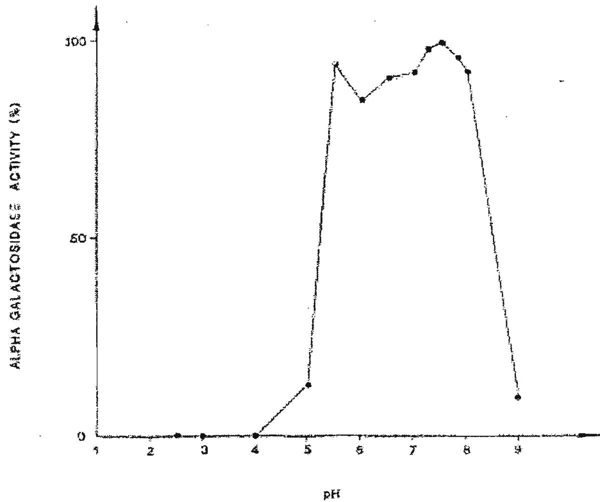

Figure 3: Effect of pH on the activity of alpha-galactosidase from *Citrobacter freundii.*

Effect of pH on enzyme activity: α-Galactosidase in (0.15M) McIlvaine buffer incubated for 30 minutes at 29 °C showed high activity between pH 5.5 to 8.0 with a maximum activity at pH 7.5 (Figure 3).

Stability of the enzyme at different pH values : The enzyme was incubated in 0.01M McIlvaine buffer at pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 at 29 °C and assayed for activity at 1 hour intervals using 0.1M McIlvaine buffer (pH7.5) at 29 °C.

The enzyme is stable over a broad pH range of 6.0 - 8.0. The enzyme is relatively less stable at low pH values than at high pH values (Figure 4).

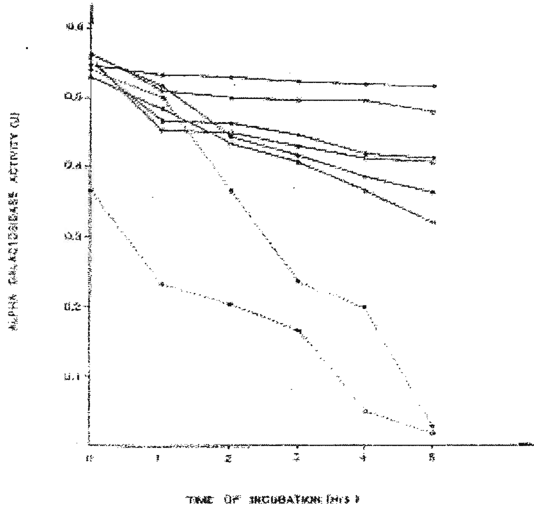

Figure 4: Effect of pH on the stability of alpha- galactosidase from *Citrobacter freundii*.

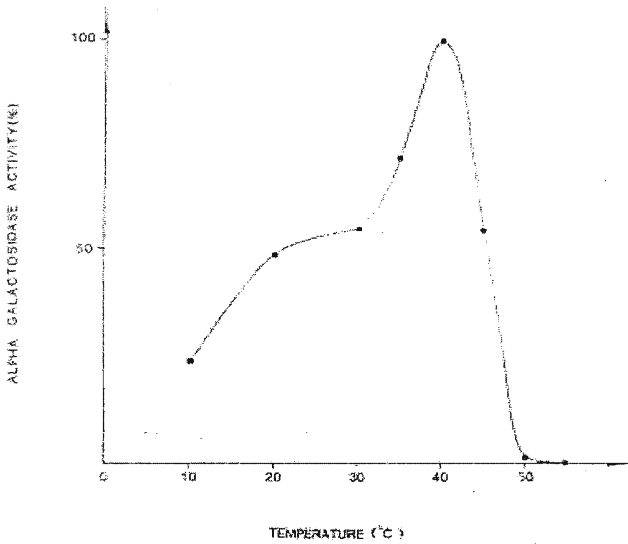

Figure 5: Effect of temperature on the stability of alpha- galactosidase from *Citrobacter freundii*.

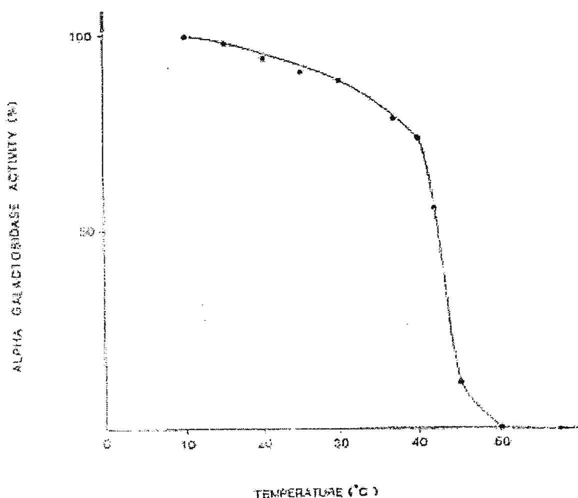

Figure 6: Effect of temperature on the stability of alpha- galactosidase from *Citrobacter freundii*.(incubated for 2h.)

Effect of temperature on enzyme activity: The enzyme when incubated at temperatures from 10 °C to 50 °C in 0.15M McIlvaine buffer (pH 7.5) for 30 minutes has an optimum activity at 40 °C. The activity of the enzyme increases with temperature from 10 °C to 40 °C, thereafter it decreases with further increase in temperature. (Figure 5).

Stability of the enzyme at different temperatures: When incubated for 2hours at temperatures varying from 10 °C to 50 °C in 0.15M McIlvaine buffer (pH 7.5) and assayed for enzyme activity at 29 °C it was observed that the thermal stability of the enzyme gradually decreases upto 40 °C. It retained about 60% activity at 40 °C (Figure 6). The enzyme lost almost 90% activity when incubated at 50°C for 30mins.

DISCUSSION

α-Galactosidase has been isolated from several bacterial species. *Klebsiella* sp.No.PG-2[11],E.coli sub sp.Communior IAM 1272[12] and *Bacillus stearothermophilus*.[13]

Michaelis constant (Km) for extracellular α-galactosidase of *C. freundii* was less than the Km value obtained for intracellular α-galactosidase of *Klebsiella* sp No. PG.2[11] for the same substrate indicating a higher affinity for the substrate.

α-Galactosidase from plant[14] and animal tissues[15] have shown optimum pH in the acidic range 2.5-6.0. In the present investigation, α-galactosidase from *C. freundii* appears to have a broad pH range from 5.5 - 8.0 with a maximum activity at pH 7.5. A neutral α-galactosidase activity has been reported in *Klebsiella* sp. No. PG 2[11], *E. coli*, sub sp, communior IAM 1272[12] & *B. stearothermophilus*.[13]

α-Galactosidase from *Aspergillus niger*[16] is stable at pH 3.5-5.8 α-galactosidase from *Trichoderma reesei* RUTC-30[17] is stable at pH 4.5-6.5. Thus α-galactosidase from *C.freundii* is stable over a broader pH range.

The optimum temperature of 40 ^{0}C, observed in the present study is higher than the optimum temperature reported from α-galactosidase of *E.coli*[12], *Klebsiella* sp. No. PG.2.[11]

The thermal stability of this enzyme is higher than α-galactosidase from *E.coli* sp. Communior IAM 1272[12], *Klebsiella* sp.[11] and is relatively less than α-galactosidase from *B.stearothermophilus*.[13]

α-Galactosidase from *C. freundii* is extracellular and inducible in nature. In industry, extracellular enzymes are preferred. Most of the α-galactosidase is optimally active at acidic pH values. In the sugar beet industry, low pH tends to cause inversion of sucrose or precipitation of proteins. Thus, it is important to have an enzyme with the optimum activity at pH 7.5. α-Galactosidase from *C. freundii* is stable at room temperature and the enzyme solution does not contain invertase activity. A combination of α-galactosidase and invertase activity results in an undesirable hydrolysis of raffinose to galactose.

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