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# PURIFICATION AND PROPERTIES OF THE RAW STARCH-DEGRADING α-AMYLASE OF MUTANT STRAIN:BACILLUS CEREUS 1306

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## ABSTRACT

*The raw starch-degrading α-amylase Produced by Bacillus cereus 1306 was purified to homogeneity by acetone precipitation and gel filtration chromatography. The Molecular weight of α-amylase was estimated to be 58KDa. The enzyme displayed maximum activity 85 Units/ml at pH 7.0 and an incubation temperature of 37°C and Stable in the pH range of 5.0-9.0. Activity was inhibited in the presence of $Hg^{2+}$, $Cu^{2+}$, $Fe^{3+}$ but no inhibition was observed in the presence of $Zn^{2+}$. Medium containing $CaCl_2.2H_2O$ enhanced amylase production over that on $Ca^{2+}$-deficient medium. The detergent Tween-80 and Triton X-100 increased Biomass but Significantly Suppressed amylase production. The enzyme released large amount of glucose and maltose on hydrolysis of starch.*

## Introduction

The Enzymatic hydrolysis of starch is performed by different enzyme grouped in the α-amylase family (1,2). Some of the enzymes display specificity for either of the two

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types of bonds [α- (1,4) or α- (1,6)] and are classified as glycosidases or glycosyl hydrolase family 13(3,4). The others are referred to the tranferase class of enzymes (cyclomaltodextringlycanotransferase-EC2.4.1.19). α-Amylase (1,4-α-D-glycanohydrolase; EC 3.2.1.1) is endoamylases catalyzing the hydrolysis of internal α-1, 4-glucosidic linkages in polysaccharides (as starch, glycogen, dextrins, etc), containing three or more 1,4- α-Dlinked D-glucose units, in a random manner. They are found in various kinds of microorganisms, including bacteria, fungi, aswellas in higher plants, insects and mammalins tissues (5). The product of hydrolysis retains the α- configuration at the reducing glucose end. These amylases are used extensively in various industries particularly for the industrial bioconversion of starch to simple sugars. Bacillus cereus 1306 produces a raw starch-digesting α-amylase and the nature and mechanism of degradationof the insoluble substrate has been described. The purification and some properties of this unusual enzyme are described in this paper.

# Materials and methods

## Microorganism

*Bacillus cereus* 1306 was received from Institute of Microbial Technology, Chandigarh and was maintained at room temperature on 1% starch nutrient agar slopes (pH 7.0).

## Enzyme production

A standard inoculum medium was used (g/l): soluble starch 10.0, Bactopeptone 10.0, yeast extract 2.0, $KH_2PO_4$ 0.05, $MnCl_2$. $4H_2O$ .0015, $MgSO_4$. $7H_2O$ .25, $CaCl_2.2H_2O$ .05, $FeSO_4$ .$7H_2O$ .01, pH 7.0. After 24hr incubation at 37°C, a 1% (v/v) inoculum was transferred in to 250 ml erlenmeyer flasks containing 50 ml production medium. The production medium was (g/L): soluble starch 30.0, Yeatex 30.0, $KH_2PO_4$ 0.05, $MnCl_2.4H_2O$ .0015, $MgSO_4.7H_2O$ .25, $CaCl_2.2H_2O$ .05, $FeSO_4.7H_2O$ .01, pH 7.0. The cells were shaken at 220 rpm at 37°C. cells were removed from the medium by centrifugation at 3,000 rpm for 20 min at 4°C and enzyme activity in the cell free supernatant was measured. Biomass was determined turbometically at 650nm.

## Amylase Purification

*Bacillus cereus* 1306 α-amylase was purified by a combination of acetone precipitation and gel filration chromatography. A 1/2 volume of precooled Acetone (-18°C) was added dropwise to the CFS. After centrifugation at 3,000 rpm for 20

min, the precipitate was dissolved in a minimum volume of 10 ml of 0.1M phosphate buffer; pH 6.0 dialyzed overnight against the same buffer. The dialyzed enzyme preparation was applied to a di ethyl amino ethyl (DEAE)- Cellulose column equilibrated with 0.1M phosphate buffer pH 6.0. The bound enzyme was eluted at a Flow rate of 60-ml/ hour. Fractions containing amylase activity were pooled and concentrated.

### Enzyme assay

α-Amylase was assayed by adding 1 ml of enzyme to 1-ml soluble starch (1%w/v) for one minute at 37°C. The reaction was stopped and the reducing sugars determined with dinitro salicylic acid method [6]. An enzyme unit is defined as the amount of enzyme releasing 1mg of glucose equivalent from the substrate per minute at 37°C.

### Protein determination

The amount of protein was determined by using the bicinchonine acid method with bovine serum albumin as a standard [7].

### End production analysis

Sugars produced by the enzyme on hydrolysis of starch were identified and quantified by HPLC (waters, Millipore, USA) using a carbohydrate column, eluted with water and acetonitile (20& 80%)at 30°C as the mobile phase. Samples were deased and filtered through .22mm acro discs (Gelman, Mich, USA). Samples aliquotes were applied to the column in triplicate to each analysis using a waters marathon automous samples.

### Electrophoresis

To determine the homogeneity and molecular weight of the enzymes, the purified protein protein perparation was separated with 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) following standard procedures [8].

### Effect of detergents and metal ions

The effect of a no. of divalent cation such as $Ca^{+2}$, $Zn^{+2}$, $Cu^{+2}$, $Hg^{+2}$, $Mg^{+2}$,$Ba^{+2}$ and trivalent cations such as Fe+3 and detergents (Tween & Tritonx-100) were studied. A control without the addition of extraneous metal ions was maintained for comparison.

# Results

## Production and Purification of the α-amylase

*Bacillus cereus* 1306 produced high levels of extra cellular α-amylase (Figure 1). Amylase production was initiated in the early exponential growth phase and became maximum (85 units/ ml) during the stationary phase of growth (48 hr). After 80hr, both the biomass and amylase levels had started to decline each inciding with the steady increase in pH. The enzyme was purified by acetone precipitation and gel filtration chromatography (table1). The yielded an enzyme perparation with specific activity (1333 Units /mg protein) and a 14 fold increase in purification. The preparation was shown to be homogenous by SDS- PAGE.

## General Properties of the α-amylase

The enzyme was classified as an α-amylase based as its rapids endo attack of amylose azure and phadder starch, its low percentage bond hydrolysis (1.1%) relative to rapid reduction in viscosity (50%) and its rapid decrease in the Iodine staining power of starch. This together with the fact that the end- products release on hydrolysis of starch retained the α-anomeric for established the enzyme as an α-amylase. The optimum pH for starch hydrolysis by the amylase was 7.0 and the enzyme was most stable in the pH range 5.0-9.0. The enzyme displayed maximum hydrolysis at 37°C and was 100% stable for 1h to 37°C. Its molecular weight as determined by SDS-PAGE was 58KDa. The enzyme contained a carbohydrate moeity and had an isoelectric point of pH 3.0 suggesting the involvement of Carboxylic groups and tryptophan residues in the Catalytic Process.

## Substrate specificity

The α-amylase of *Bacillus cereus* 1306 was capable of hydrolysing insoluble substrates, in particular to corn and rice starch. Its ability to hydrolyze a range of soluble substrate is reported here. The enzyme displayed highest activity towarts the linear polymer, amylose (132%) followed by starch (105%) and amylopectin (95%) (Table 2). UP to 30% hydrolysis was achieved on the highly branched substrate, glycogen. Although the enzyme was incapable of hydrolysing the smaller cyclodextrins, the larger cyclodextrins, g- cyclodextrin was readily degraded.

## End product profile

The end- products produced by the α-amylase of *Bacillus cereus* 1306 initially produced large amount of glucose (86% and maltose (10%). Generally as the amounts of glucose and maltose increased significantly (Table 3).

**Table 1. Analysis of end-products produced on hydrolysis of starch**

| Time (h) Bacillus cereus 1306 Sugar (%, w/w) | | | | | | |
|---|---|---|---|---|---|---|
| | G1 | G2 | G3 | G4 | G5 | G6 |
| 1 | 86.66 | 10.385 | 2.876 | .079 | - | - |
| 5 | 86.831 | 7.587 | .202 | - | .751 | 4.629 |
| 24 | 89.029 | 8.446 | - | 2.525 | - | - |
| 72 | 100 | - | - | - | - | - |

Enzyme (2000Unit/g) was incubated with 1%(w/v) starch in 0.1M phosphate buffer, pH 6.0 for the time intervals indicated at 37°C . The concentration of Sugars (G1-G6) was analyzed by HPLC. G1 to G6 are glucose to other oligosaccharides, respectively.

## Effect of Metal Ions on activity

The activity of amylase was assayed in the presence of different metal ions. Strong inhibition of activity was observed in the presence of 5mM $Cu^{+2}$, $Hg^{+2}$ & $Fe^{+3}$ (Table 4). On the other hand, no significant inbition of activity was observed in the presence of 5mM $Zn^{+2}$ .

| Metal | Activity |
|---|---|
| None | 82 |
| $CaCl_2$ | 75 |
| $ZnCl_2$ | 80 |
| $CuSO_4$ | 16 |
| $HgCl_2$ | 0 |
| $FeCl_3$ | 12 |

**Table 2. Substrate specificity of the α-amylase**

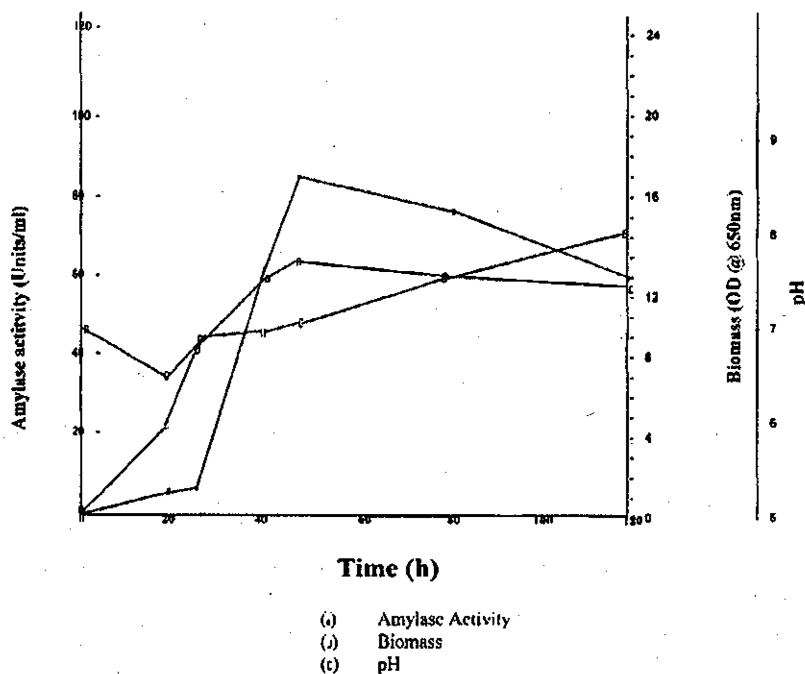
| Substrate (1%w/v) | % Relative activity |
|---|---|
| Amylose | 132 |
| Starch | 105 |
| Amylopectin | 95 |
| Glycogen | 32 |
| γ- Cyclo dextrin | 50 |

Substrate were incubated With Purified enzyme (2000Unit/g substrate) for 30 min at 37°C in 0.1M Phosphate buffer pH6.0 and the glucose equivalent released compared with that released from starch.

Table 3. Purification of the α-amylase of Bacillus cereus 1306

| Stage | Specific activity (Units /mg protein) | Purification (x-fold) | Recovery (%) |
|---|---|---|---|
| Cell free Supernatant | 94.7 | 1 | 100 |
| Acetone precipitation | 275 | 3.0 | 91.66 |
| DEAE-Cellulose | 1333 | 14.08 | 66.67 |

**Fig. 1.**
**Growth and amylase production by *Bacillus cereus 1306***



# Discussion

The extracellular amylase was purified from the cell free culture Supernatant of *Bacillus cereus* 1306. The enzyme released glucose, maltose and a range of other oligosaccharides from soluble starch, indicating that they are essentially α-amylase. Multiple amylase Production have been reported from many *Bacillus* strains [9,10]. The molecular weight of the α-amylase was estimated to be 58,000Da by sodium dodecyl sulfate- polyacryamide gel electrophoresis. At present, there is a growing interest in improving the efficiency of raw starch hydrolysis. Enhanced raw starch hydrolysis is reported from fungal strains that produce both glucoamylase and α-amylase [11,12]. This may indicate that better hydrolysis of raw starch can be brought about by the simultaneous use of raw- starch-digesting α-amylase and glycoamylase. From the technical and economic points of view, this is advantageous because it may allow a one- step conversion of raw starch to glucose. However, for efficient conversion, the temperature and pH optima of the glycoamylase and α-amylase used together must be compatible. The raw-starch-digesting bacterial α-amylase greatly varies in their ability to bind starch granules [13,14,15]. The α-amylase from *Bacillus cereus* 1306 completely adsorb to raw starch. This is a very interesting propery for it could offer the opportunity of developing an affinity Purification procedure for the isolation and concentration of the enzyme directly from the culture broth. Much has been studied on the appearance and composition of starch granules after enzymatic digestion [16,17,18,19]. On the other hand, little is known about the products that are released from granules. The end product profile of raw starch hydrolysis by *bacillus cereus* 1306 α-amylase showed the formation of glucose, maltose, and other oligosaccharides.

Most bacterial α-amylases are optimally active at slightly acidic to near neutral pH [20,21] concurring was this α-amylase of *bacillus cereus* 1306 displayed maximal activity at pH 7.0. Although it displays maximal activity at 37°C.The enzyme was not thermostable. Interestingly, the enzyme displayed significantly hydrolysis of g-cyclo dextrin. Although this proposal is not wide spread it has also been reported with few other amylases. The enzyme also hydrolysed the a-linked trisaccharide, maltotriose, a substrate not usually hydrolysed by α-amylase, yet, the trisaccharides containing α-1,6 bonds, and isomaltotriose was not hydrolysed. It was established that like other bacterial α-amylases Carboxylic groups and tryptophanyl residue were involved in catalytic activity [22,23]. This may be accounted for in that α-amylase capable of raw starch digestion often have a high molecular weight, possibly due to the presence of additional affinity site on such enzyme. The enzyme released glucose and maltose as the main end

products on hydrolysis of starch. Most α-amylase is inhibited by the metal ions $Cu^{+2}$, $Hg^{+2}$ , $Fe^{+3}$ $Zn^{+2}$ (24,25). This enzyme was also inhibitor $Cu^{+2}$, $Hg^{+2}$ , $Fe^{+3}$ However, $Zn^{+2}$ , Known inhibitor of amylase, did not inhibit the activity of *Bacillus cereus* 1306at a 5mM Concentration.

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