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PRODUCTION AND CHARACTERIZATION OF EXTRACELLULAR POLYGALACTURONASE BY *Erwinia carotovora* MTCC 1428

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ABSTRACT:

Bacteria of the genus *Erwinia* produce and secrete different pectolytic enzymes that effectively depolymerize pectin substances causing soft rot and other diseases in plants. Pectolytic enzymes bring about degradation of plant cell walls and maceration of plant tissues. Polygalacturonase (PG) is one of most important pectinolytic enzymes that degrade polygalacturonan present in the cell walls of plants by hydrolysis of the glycosidic bonds linking galacturonic acid residues. Polygalacturonan is a significant carbohydrate component of the pectin network that comprises plant cell walls.
Erwinia carotovora MTCC 1428 was studied for the production of Polygalacturonase in liquid culture containing different carbon and nitrogen sources under different growth conditions. Polygalacturonase was purified by means of acetone precipitation, acetate buffer extraction, and two successive treatments by chromatography on carboxymethyl cellulose. Pectin as carbon source was the best source for the production of enzyme. Assay for Polygalctouronase was carried out by estimating amount of reducing sugar released under assay conditions. The purified enzyme was studied for its properties. Enzyme showed maximum activity at pH 5.2 and at temperature 27°C.

Keywords: Polygalacturonase, *Erwinia carotovora* MTCC1428

[I] INTRODUCTION

Pectinolytic enzymes or pectinases are a heterogeneous group of enzymes that hydrolyze the pectic substances present in plants. They include polygalacturonases, pectin lyase, and pectinmethyl esterase that hydrolyze the glycosidic bonds of pectic substances. Endopolygalacturonase (EC 3.2.1.15) and exopolygalacturonase (EC 3.2.1.67) are the enzymes of particular interest to industry because they act on pectin, hydrolyzing its internal and external glycosidic bonds, producing shorter pectin molecular structures, decreasing the viscosity, increasing the yield of juices, and determining the crystalline structure of the final product [1, 2, 3, 4, 5] They have a share of 25% in the global sale of the food enzymes.

Pectinases are one of the most widely distributed enzymes in bacteria, fungi, and plants. One of the most studied and widely used commercial pectinase is polygalacturonase [6, 7] .

Erwinia carotovora (Eca) is a member of the Enterobacteriaceae and is the causal agent of soft rot disease in vegetable crops in temperate regions [8]. Its pathogenicity is related to the production of a variety of extracellular pectolytic enzymes of which pectate lyase (PL) and polygalacturonase (PG) are considered to be the most important [9, 10]. The basic unit of pectic substances which bind plant cells together, poly-galacturonic acid (PGA), is cleaved by PL by trans- elimination yielding a 4-deoxy-5-oxo-D glucuronate and by PG by hydrolysis eventually yielding a monomer of D- galacturonic acid [11, 12] .

[II] MATERIALS AND METHODS

2.1. Organism and Chemicals

Erwinia carotovora (MTCC, 1428) was procured from the Institute of Microbial Technology; Chandigarh, India was used for production of polygalacturonase. The culture was maintained on Nutrient Agar Slants.

All the chemicals used were either procured from Sigma Aldrich (USA) or HiMedia (Mumbai, India) and were of high analytical grade.

2.2. Production Media

The basal medium contained 5 g of pectin, 2.4 g of KH_2PO_4, 0.8 g of Na_2HPO_4, 0.2 g of $MgSO_4$. $7H_20$, 3 g of $(NH_4)_2SO_4$, 5 g of monosodium glutamate, and 1 liter of H_2O, adjusted to pH 6.8 with 1 N NaOH. Pectin and salts were autoclaved separately [13]. Consecutive optimization of production media was carried out by altering, the cultivation conditions and the composition of the culture medium [10].

The initial pH of the medium was adjusted to 6.8 in Erlenmeyer flask (250 mL) containing 100 mL of culture medium were inoculated with 1000 µL (2%, v/v) of bacterial suspension with a 0.8 absorbance at 620 nm. For inoculums preparation, two days old slant culture was scraped from agar surface, added to 0.85% sterile saline solution, and mixed until a homogeneous suspension was obtained. The flasks were incubated at 35°C on a rotary shaker at 120 rpm for 48 to 96 hours [14] .

2.3. Enzyme Purification

The purification of Polygalacturonase was carried out by ammonium sulfate precipitation method. PG in culture filter was precipitated using 90% ammonium sulfate. The solution was then centrifuged at 5000x g for 20 minutes. The pellet was dissolved in minimum amount of buffer (0.2 M acetate buffer pH 4.5).The enzyme solution was dialyzed against 0.002 M acetate buffer pH of 4.5 for 24 hours. After 24 hours, the dialyzate obtained was then used as the partially purified Polygalacturonase. The dialyzed acetate extract was placed on a carboxymethyl cellulose column. Elution was obtained by a linear gradient of buffer strength starting with 0.05 M sodium acetate buffer at pH 5.0; 10-ml fractions were collected [13].

The relative molecular weight of the purified enzyme fraction was estimated by SDS-PAGE (12%) according to the method of Laemmli. [15] against medium range protein molecular weight marker of merck biosciences. The Proteins were stained with coomassie brilliant blue R-250 [16] .

2.4. Polygalacturonase activity assay

Polygalacturonase (EC 3.2.1.15) activity was assayed according to Miller (1959) [17]. The reaction mixture (0.5 ml) contained 1% polygalacturonic acid, 0.05 M sodium acetate buffer pH 5.5 and a suitable amount of enzyme. Assay was carried out at 37°C for 1 h. Then, 0.5 ml dinitrosalicylic acid reagent was added and heated in a boiling water bath for 10 min. After cooling to room temperature, the absorbance was measured at 560 nm. The amount of galacturonic acid released per mL per minute was calculated from standard curve of galacturonic acid. One Unit of PGase activity was defined as the amount of enzyme required to release one micromole of

galacturonic acid per mL per minute under standard assay conditions [16] [18] [19].

2.5. Protein Determination

Protein was determined according to Bradford (1976) and bovine serum albumin was used as standard [20] .

2.6. Effect of Temperature and pH:

Most favorable enzyme production temperature was studied by incubating the production medium at different temperatures (20°C, 25°C, 35°C, 40°C, and 45°C). For optimizing the pH of production, medium with varying pH were used, namely, 4.4, 5.2, 6.0, 6.8, and 7.6.

2.7. Effect of Carbon Sources:

Various carbon sources, dextrose, fructose, galactose, glycerol, lactose, maltose, pectin, sucrose and xylose were used in the production medium at a concentration of 1% w/v to check the effect of carbon source on enzyme production. The culture supernatants were assayed for polygalacturonase activity [10] .

2.8. Effect of temperature & pH on Enzyme Activity

Enzyme activity was determined at a temperature range of 04 °C to 70°C and Polygalacturonase activity was checked by using the standard assay method. Effect of pH on Enzyme activity was determined at various pH using 0.2M each of sodium acetate buffer at various pH (4, 4.5, 5.2, 5.8, 6.2, 6.8 and 7.4) and activity was checked by using the standard assay method [18].

[III] RESULTS AND DISCUSSION

3.1. Production of Enzyme

Maximum production of Enzyme was observed after incubation of 72 hrs at 35°C in liquid state submerged fermentation process (Fig-1). Extracellular enzyme protein production was confirmed by determination of protein in medium after certain time intervals by Bradford method [20] .

Enzyme assay was carried out for confirmation of production of polygalacturonase in the medium by method of Miller [17] .

The SDS PAGE results show that a prominent band of purified protein fraction with molecular weight ~ 42 kDa and confirms presence of Polygalacturonase Enzyme. (Fig- 2) [21, 22] .

Enzyme production by *Erwinia carotovora* (MTCC, 1428) at various temperature and pH conditions was studied and found that maximum production of enzyme was at 35°C and at pH 5.2 respectively.

Most suitable carbon source for production of Polygalacturonase was Pectin where maximum production was observed and minimum production of enzyme observed when Dextrose was used as the carbon source (Fig-3)

3.2. Study of Enzyme Kinetics

Purified enzyme show maximum activity at temperature lower 27°C and in acidic range at pH 5.2. (Fig 4 ,5)

Fig: 1. Effect of Incubation time on Production of Enzyme by *Erwinia carotovora* MTCC 1428

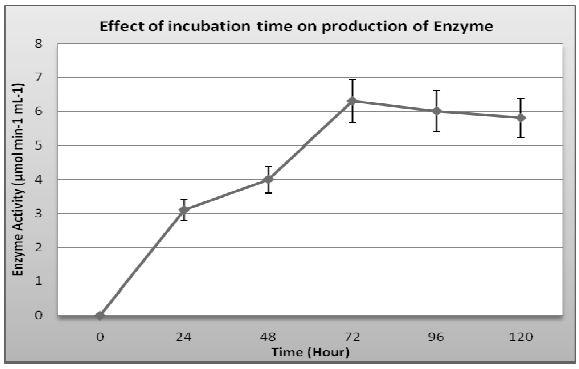


Fig: 2. SDS-PAGE of purified polygalacturonase Enzyme by *Erwinia carotovora* MTCC 1428

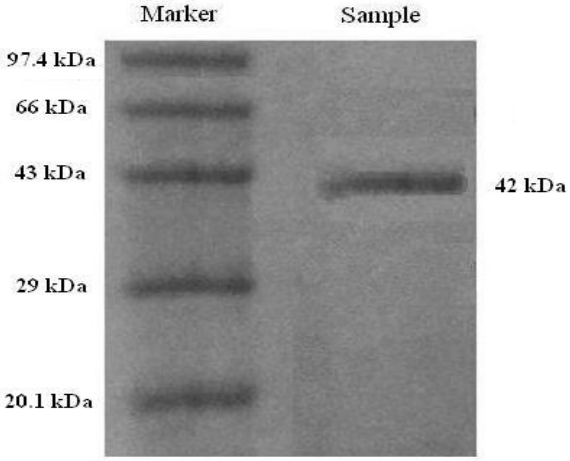


Fig: 3. Effect of Carbon source in medium on Production of Enzyme by *Erwinia carotovora* MTCC 1428

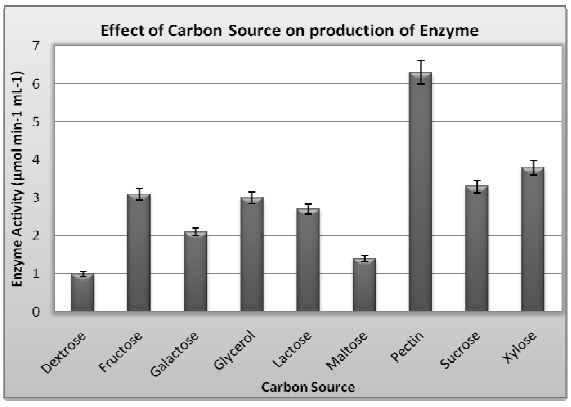


Fig: 4. Effect of temperature on purified Polygalacturonase Enzyme acivity

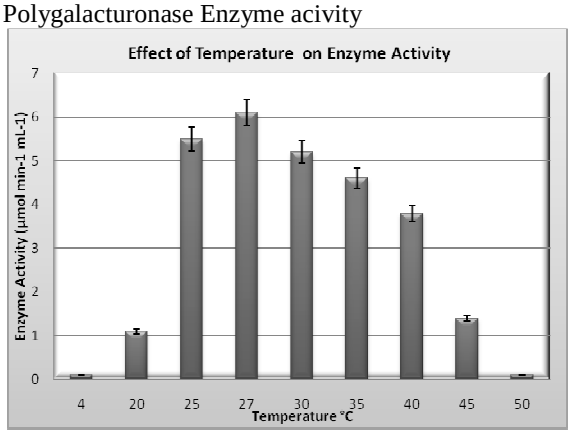
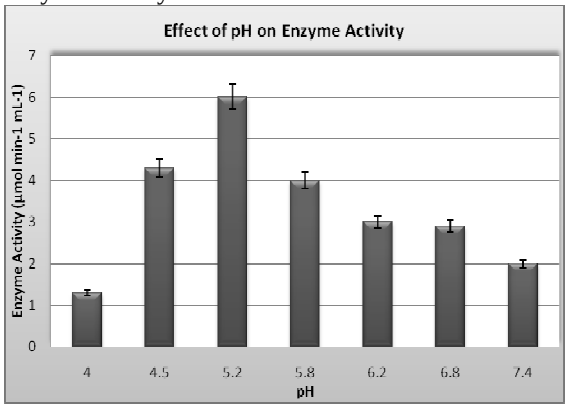


Fig: 5. Effect of pH on purified polygalacturonase Enzyme activity



[IV] CONCLUSION

Erwinia carotovora (MTCC, 1428) produced polygalacturonase enzyme when grown in submerged liquid fermentation at 35°C with pH 5.2 with pectin as the most suitable carbon source compared with other sugars. The enzyme was effectively been purified and characterized it showed its maximum activity in laboratory conditions at lower pH range and lower temperature hence this enzyme could find several industrial applications. Further studies required to reduced the cost of polygalacturonase production by using various pectin rich agriculture waste.

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