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Characterization of pectin degrading polygalacturonase produced by *Bacillus licheniformis* KIBGE-IB21

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Citrus pectin
Mono galacturonic acid
Dinitrosalicylic acid (DNS)
Sodium potassium tartrate
Sodium hydroxide
Glycine
Acrylamide
Bisacrylamide
TEMED
Ammonium per sulfate
Ammonium sulfate

ABSTRACT

A commercially available pectin degrading enzyme preparation is not usually pure and may contain several other auxiliary enzymes including pectin lyase and pectin methyl esterase with reasonably trace quantities of cellulases as well. Current work is focused to characterize an industrially important pectin degrading enzyme known as polygalacturonase produced by *Bacillus licheniformis* KIBGE-IB21 in term of its catalytic activity. The enzyme showed maximum activity when it was incubated for 05 min at 45 °C in an alkaline pH environment of pH-10.0. This enzyme is stable at a broad pH range and retained almost 100% of its initial activity in between pH 8.0 and 10.0 after 60 min. It also exhibited high stability against different temperatures and 100% residual activity was measured at 30 °C and 40 °C up to 1 h. The apparent K_m and V_{max} values for pectin degradation were 1.017 mg ml^{-1} and 23,800 μM min^{-1}, respectively with an approximate molecular weight of 153 kDa. Polygalacturonase also demonstrated exceptional storage stability at →20 °C even after 30 days.

1. Introduction

Pectin is a complex hetero-polysaccharides found in middle lamella of higher plants and composed of linear chain of α-1, 4 glycosidic linked galacturonic acid residues (Gadre, Driessche, Beeumen, & Bhat, 2003; Kashyap, Vohra, Chopra, & Tewari, 2001; Nakagawa, Nagaoka, Taniguchi, Miyaji, & Tomizuka, 2004). The synergistic actions of pectinolytic enzymes degrade the complex structure of pectin polymer which has different drawbacks in food industries such as fruits and vegetables juices. The pectinolytic enzymes or pectinases are classified into pectin methyl esterase, pectin lyase and polygalacturonase on the basis of their mechanism of attack on pectin substances (Esquivel & Vogel, 2004; Gummadi & Panda, 2003; Soares, Da Silva, Carmona, & Gomes, 2001). Pectin methyl esterase catalyzes the de-esterification of pectin by breaking ester bond between the methyl group and carboxylic acid of galacturonic residues. Pectin lyases break the α-1, 4 glycosidic bonds between galacturonic residues by trans-elimination reaction. While, polygalacturonase catalyze the hydrolysis α-1, 4 glycosidic bonds of pectin polymer and depolymerized it into its monomer units of galacturonic acids.

Polygalacturonase has different applications in industries such as clarification and filtration of fruits juices, winemaking process for the increasing of the quality of wine, coffee and tea fermentation, degumming of plants fibers, oils extraction, textile, papermaking, protoplasm isolation and purification of plant virus as well as pretreatment of pectin containing wastewaters (Hoondal, Tiwari, Tewari, Dahiya, & Beg, 2002; Jayani, Saxena, & Gupta, 2005; Kashyap et al., 2001). The catalytic properties and stability of enzymes against different physio-chemical conditions are very

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important for their commercialization. The biochemical characterization of enzymes can help for enhancing the stability and maintaining the catalytic activity of enzyme over long period of time (Celestino, Freitas, Medranoc, Sousa, & Filho, 2006; Gummadi & Panda, 2003). Various microorganisms are known to produce pectinase with different molecular mass and catalytic properties (Esquivel & Vogel, 2004; Gummadi & Panda, 2003; Kaur, Kumar, & Satyanarayana, 2004). *Aspergillus niger* is mostly used for the industrial production of pectinase (Maldonado, Cáceres, Galli, & Navarro, 2002), but the pectinase from *A. niger* is acidic in nature and only can work in acidic preparation not in alkaline. Only few reports are available on the production of alkaline pectinase by bacterial strains (Beg, Bhushan, Kapoor, & Hoondal, 2000; Cao, Zheng, & Chen, 1992; Hayashi et al., 1997). Exploration of an enzyme with novel catalytic properties from microbial sources and cost effective production method is necessary in order to meet the escalating demand for the commercialization of pectinase for different industrial applications. The current study was designed to describe the biochemical characterization of novel polygalacturonase produced from *Bacillus licheniformis* KIBGE-IB21. Moreover, effect of different metal ions, detergents and organic solvents on polygalacturonase activity was also analyzed. Finally, the storage stability of the enzyme was investigated at different temperatures (−20 °C, 4 °C and 30 °C) for different time intervals.

2. Material and methods

2.1. Production of polygalacturonase

B. licheniformis KIBGE-IB21 was previously screened and isolated for the production of polygalacturonase and the enzyme was produced using pectin containing medium (Rehman, Qader, & Aman, 2012). Cells were harvested after 48 h by centrifugation at 10,000 rpm for 15 min and the cell free supernatant (CFF) was subjected to further purification.

2.2. Partial purification of polygalacturonase

CFF was partially purified by using salt precipitation method with 50.0% ammonium sulfate saturation. The mixture was equilibrated at 4 °C for 20 h. Precipitates were solubilized in glycine-NaOH buffer (50.0 mM, pH-10.0) and dialyzed against the same buffer for 03 h at 4 °C along with multiple changes of the buffer after every 30 min. The partially purified dialyzed protein was used for further characterization.

2.3. Determination of polygalacturonase activity

Polygalacturonase activity was measured by estimating the quantity of amount of galacturonic acids through DNS method (Miller, 1959) using 1.0% citrus pectin as a substrate and mono-D-galacturonic acid as a standard. One unit of polygalacturonase is defined as the "*amount of enzyme required to generate 1.0 μmole of galacturonic acid under standard assay conditions*".

2.4. Effect of temperature on the activity and stability of polygalacturonase

Determination of optimum temperature for maximum enzymatic activity of polygalacturonase was carried out by performing the pectin hydrolysis reaction at various incubation temperatures ranging from 30 °C to 60 °C at a constant pH (50 mM glycine-NaOH, pH-10.0) and a substrate concentration of 1.0%. The stability of polygalacturonase against different temperatures was determined by pre-incubation of partially purified enzyme without substrate at different temperatures (30 °C–60 °C) for 1 h. After 1 h aliquots were taken for the enzyme assay.

2.5. Influence of different pH on the activity and stability of polygalacturonase

Effect of pH on the activity of polygalacturonase was determined by performing the reaction in different pH levels starting from pH-5.0 to highly alkaline side of 12.0 and keeping the temperature (45 °C) and substrate concentrations (1.0%) constant. For the determination of pH stability the partially purified polygalacturonase was pre-incubated in various buffers having different pH values for 1 h at 37 °C. The residual activity was determined by performing the enzyme assay. The buffers tested include acetate buffer (50.0 mM, pH 5.0–6.0), phosphate buffer (50.0 mM, pH 7.0–8.0) and glycine-NaOH buffer (50.0 mM, pH 9.0–12.0).

2.6. Effect of various buffers on polygalacturonase activity

Effect of different buffers on polygalacturonase activity was investigated by individually performing the pectinolytic activity in glycine-NaOH, Tris–HCl and sodium bicarbonate-NaOH buffers with a constant pH (pH-10.0) and ionic strength (50.0 mM).

2.7. Effect of ionic strength of buffer on the polygalacturonase activity

In order to study the impact of ionic strength of buffer on the activity of polygalacturonase, the enzyme assay was performed in various concentrations of the selected glycine-NaOH buffer from 10.0 mM to 100 mM with a constant pH-10.0.

2.8. Determination of kinetic parameters of polygalacturonase

The Michaelis–Mentent constants (K_m) and maximum velocity (V_{max}) of polygalacturonase were determined by performing the enzyme assay at different concentration of substrate ranging from 1.0 to 20.0 mg ml^{-1}. Both the K_m and V_{max} values of polygalacturonase were calculated using Lineweaver–Burk plot ($1/[S]$ versus $1/[v]$).

2.9. Substrate specificity

Substrate specificity of polygalacturonase was determined in 1.0% solution of different substrates including citrus pectin, apple pectin, xylan and carboxymethylcellulose.

2.10. Influence of metal ions and detergents on the activity of polygalacturonase

The effect of different metal ions such as Co^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Ca^{2+} Ni^{2+}, Hg^{2+} and Fe^{2+} on the activity of polygalacturonase was determined by performing the enzyme assay in the presence of various metal ions (1:1) at 37 °C for 30 min. All the metal ions used were in the form of chloride salts. Enzyme assay was performed as described earlier. In the same way, the influence of different detergents including Tween-80, Triton X-100 and SDS on the polygalacturonase activity was analyzed.

2.11. Molecular weight determination by SDS-PAGE and in-situ electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and *in-situ* electrophoresis were used for the determination of molecular mass of polygalacturonase using BSA