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Polygalacturonase: Production of pectin depolymerising enzyme from *Bacillus licheniformis* KIBGE IB-21

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ABSTRACT

Polygalacturonase is an enzyme that hydrolyzes external and internal α (1–4) glycosidic bonds of pectin to decrease the viscosity of fruits juices and vegetable purees. Several bacterial strains were isolated from soil and rotten vegetables and screened for polygalacturonase production. The strain which produced maximum polygalacturonase was identified *Bacillus licheniformis* on the basis of taxonomic studies and 16S rDNA analysis. The isolated bacterial strain produced maximum polygalacturonase at 37 °C after 48 h of fermentation. Among various carbon sources apple pectin (1.0%) showed maximum enzyme production. Different agro industrial wastes were also used as substrate in batch fermentation and it was found that wheat bran is capable of producing high yield of enzyme. Maximum polygalacturonase production was obtained by using yeast extract (0.3%) as a nitrogen source. It was observed that *B. licheniformis* KIBGE IB-21 is capable of producing 1015 U/mg of polygalacturonase at neutral pH.

1. Introduction

Pectin is a polysaccharide present in the primary cell wall and middle lamella of higher plants which contribute to the firmness and structural integrity of plant tissues (Gummadi & Panda, 2003). On the basis of their mode of action, pectinases are classified into polygalacturonase, pectin lyase and pectin esterase. Polygalacturonase are further classified into endopolygalacturonase (EC 3.2.1.15) and exopolygalacturonase (EC 3.2.167) which hydrolyze the internal and external α-(1,4)glycosidic linkages of pectin, respectively. Pectin lyase (EC 4.2.2.10) split α-(1,4)glycosidic bonds by transelimination, which results in galacturonide with double bond between C-4 and C-5 at the non-reducing end, while pectin esterase (EC 3.1.1.11) catalyzes the hydrolysis of methyl group to produce pectin and methanol. Among these three pectinases, polygalacturonases are widely used in food industries and facilitate maceration, liquefaction and extraction as well as filtration process of fruits and vegetables juices (Dosanjh & Hoondal, 1996). Polygalacturonases are also used in the industrial processing of wine, coffee and tea fermentation (Hoondal, Tiwari, Tiwari, Dahiya, & Beg, 2002). On industrial scale, *Aspergillus neiger* is usually use for the commercial production of polygalacturonase (Maldonado, Cáceres, Galli, & Navarro, 2002) and only few studies are available for the production of polygalacturonase from bacterial sources (Ahlawat, Mandhan, Dhiman, Kumar, & Sharma, 2008; Jayani, Shukla, & Gupta, 2010; Kashyap, Chandra, Kaul, & Tewari, 2000). It was reported that fungal polygalacturonases required optimum pH (3.0–6.0) for activity this pH suits the processing of fruit juices which have almost same pH, while vegetable juices and other preparation require neutral pH (Soares, da Silva, & Gomes, 1999). The aim of this studied to isolate *Bacillus* strain capable of producing polygalacturonase from indigenous sources (rotten vegetables and soil) and optimize fermentation conditions for maximum polygalacturonase production using batch fermentation. In addition, pectin containing agro industrial wastes such as orange bagasse, sugarcane bagasse, rice bran and wheat bran were also tested as substrates for the polygalacturonase production.

2. Materials and methods

2.1. Organism identification

Several bacterial strains were isolated from rotten vegetables and soil samples collected from different vegetative fields and after purification all isolates were screened for pectinolytic activity on pectin agar medium. Clear zone around the colony was detected by overlying with potassium–iodide solution (Fernandes-Salomao et al., 1996). Among these isolates three strains showed maximum zone of hydrolysis were selected and identified on the basis of

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morphological and biochemical characteristics of *Bacillus* species (Holt, Krieg, Sneath, Staley, & William, 1994).

2.2. Medium preparation

These strains were further screened for polygalacturonase production using submerged fermentation technique in the medium containing citrus pectin (1%), ammonium sulfate (0.14%), dipotassium hydrogen phosphate (0.6%), potassium dihydrogen phosphate (0.20%) and magnesium sulfate (0.01%) and pH of the medium was adjusted to 6.0 before sterilization (Soares et al., 1999). Further identification was performed by 16S rDNA sequence analysis as described by Ansari, Aman, Siddiqui, Iqbal, and Qader (2012).

2.3. Enzyme assay

Polygalacturonase activity was determined by colorimetric method using citrus pectin as substrate. Partial purified enzyme (0.1 ml) was added to 1.0 ml of substrate (0.5% citrus pectin in 0.05 M potassium phosphate buffer, pH 7.0) and incubated at 40 °C for 30 min. The reducing sugar released as galacturonic acid was determined by 3′ 5′ dinitrosalicylic acid method (Miller, 1959). One unit of enzyme activity is defined as the amount of polygalacturonase required to release 1 μmol of galacturonic acid per minute at 40 °C in phosphate buffer, pH 7.0.

2.4. Estimation of total protein

Total protein was estimated using bovine serum albumin as a standard (Lowry, Rosembrough, Farr, & Randall, 1951).

2.5. Optimization of polygalacturonase production by one variable at a time approach

Bacillus licheniformis KIBGE IB-21 was used for the production of polygalacturonase by batch fermentation. The optimization of fermentation conditions was carried out using one variable at a time approach for the maximum production of enzyme.

2.5.1. Effect of different media on polygalacturonase production

The effect of different previously reported media on polygalacturonase production was studied for *B. licheniformis* KIBGE IB-21. These media include medium-1 (Soares et al., 1999), medium-2 (Bayoumi, Yassin, Swelim, & Abdel-All, 2008), medium-3 (Kashyap et al., 2000) and medium-4 (Jacob & Prema, 2006). The cultures were incubated at 37 °C for 24 h and cells were harvested by centrifugation at 15,000 rpm for 15 min at 4 °C and supernatant was used for further enzyme activity.

2.5.2. Time course for polygalacturonase production

The effect of fermentation time on polygalacturonase production was analyzed by incubating the bacterial isolate for different time interval ranging from 24 to 120 h.

2.5.3. Effect of temperature and pH

The impact of temperature and pH on enzyme production was determined by growing the bacterial strain at different temperatures and pH levels ranging from 20 °C to 60 °C and pH 5.0 to 9.0 respectively.

2.5.4. Effect of different carbon sources

The effect of various carbon source polygalacturonase production were studied by growing the *B. licheniformis* KIBGE IB-21 in medium containing different soluble carbon sources such as apple pectin, citrus pectin, glucose, galacturonic acid, lactose, sucrose, maltose, fructose, glucose and glycerol and agro industrial wastes having different complex carbon sources (wheat bran, rice bran, orange bagasse and sugarcane bagasse).

2.5.5. Effect of substrate concentration

The effect of substrate concentration on enzyme production was studied by using different concentrations of apple pectin ranging from 0 to 2.5 g% in fermentation medium.

2.5.6. Effect of different nitrogen source on polygalacturonase production

In order to study the impact of nitrogen source on enzyme production, bacterial culture was grown in fermentation medium containing various nitrogen sources including yeast extract, peptone, tryptone, urea, ammonium sulfate, ammonium chloride, sodium nitrate and potassium nitrate.

2.5.7. Effect of yeast extract on polygalacturonase production

The effect of yeast extract on enzyme production by bacterial strain was studied by using different concentration of yeast extract ranging from 0.1 to 0.5 g%.

All the experimental work was run in duplicates.

3. Results and discussion

In the current study several bacterial strains were isolated from various sources. For screening purpose these isolates were grown on pectin agar plate and pectinolytic activity was detected using plate assay method. The results indicated that three (03) isolates showed maximum pectinolytic activity on pectin agar plates and were identified as *B. licheniformis*, *Bacillus megaterium* and *Bacillus subtilis* on the basis of morphological, physiological and biochemical characteristics. These strains were further screened for polygalacturonase production using batch fermentation and the results showed that different polygalacturonase production was achieved from *B. licheniformis* (341 U/mg), *B. megaterium* (160 U/mg) and *B. subtilis* (140 U/mg) isolated from rotten vegetable, soil and environment respectively. *B. licheniformis* which showed highest polygalacturonase activity was further identified on the basis of 16S rDNA sequences analysis. This strain assigned NCBI GenBank accession # JQ411812 and was designated *as B. licheniformis* KIBGE IB-21. On the basis of maximum polygalacturonase production further work on standardization of culture conditions for polygalacturonase production was carried out using this selected strain.

Growth factors for microbial enzyme are of prime importance in industrial production, which resulted in the high production of enzyme for different applications. Several optimization methodologies have been applied for maximum production of polygalacturonase from different strains (Bayoumi et al., 2008; Soares et al., 1999). On the basis of previous reported studies optimization of fermentation conditions for maximum polygalacturonase production form *B. licheniformis* KIBGE IB-21 was designed using conventional methods (one variable at a time).

Different reported media were used for maximum polygalacturonase production by *B. licheniformis* KIBGE IB-21. It was observed that maximum enzyme production was found in medium-4 (591.0 U/mg). On the basis of nutrients presents in medium-4, a new medium-5 was designed from medium-4 by varying its components one at a time in order to observe variation in enzyme production. It was found that polygalacturonase production was increased 1.7 times (1015 U/mg) as compared to medium-4 (Table 1).

The pattern of polygalacturonase production with reference to fermentation time was monitored by incubating the *B. licheniformis* KIBGE IB-21 for different time intervals. The results indicated that *B. licheniformis* KIBGE IB-21 started polygalacturonase production

Table 1
Polygalacturonase production on various reported and newly formulated media.

Media	Enzyme activity (U/mg)
Medium-1	212.0
Medium-2	100.0
Medium-3	14.0
Medium-4	591.0
Medium-5	1015.0

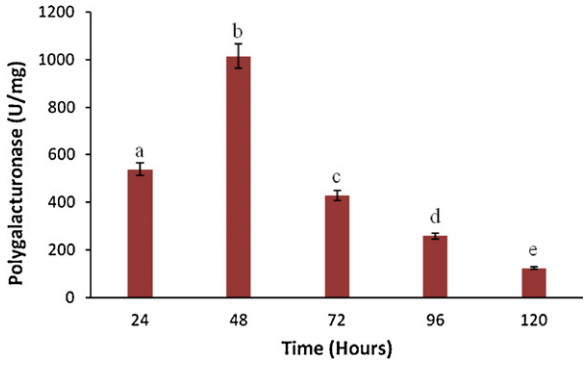

Fig. 1. Effect of fermentation period on polygalacturonase production by *B. licheniformis* KIBGE IB-21. Symbols (means ± S.E., $n = 6$) having similar letters are not significantly different from each other (Bonferroni test, $P < 0.05$).

after 24 h and reached its maxima at 48 h (Fig. 1). Further increased in fermentation time decreased polygalacturonase production and it was observed that after 72 and 120 h of incubation, 50% and 15% enzyme production was achieved respectively as compared to 48 h. However, maximum polygalacturonase production from *Bacillus sphacricus* MTCC (7542) was achieved in 72 h (Jayani et al., 2010). These results indicated that the *B. licheniformis* KIBGE IB-21 maintained log phase within 24–48 h and the variation of log phase is dependent on the nutrients present in the medium and culture conditions.

The effect of temperature on polygalacturonase production by *B. licheniformis* KIBGE IB-21 was studied at different temperatures ranging from 20 to 60 °C. An increase in polygalacturonase production was observed from 20 °C to 37 °C and maximum polygalacturonase production (1015 U/mg) was achieved at 37 °C (Fig. 2). It has been reported that maximum polygalacturonase production was found at temperature between 32 and 37 °C (Soriano, Diaz, & Pastor, 2005). As the temperature increased from 37 °C to 60 °C, decreased in enzyme production was observed and 42% enzyme production was lost at 50 °C as compared to at 37 °C, indicating that the indigenous isolate from rotten vegetable is

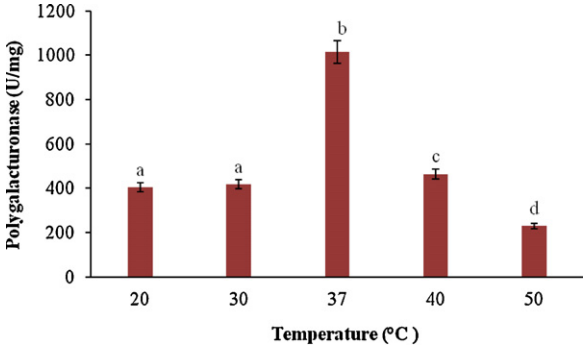

Fig. 2. Effect of temperature on polygalacturonase production by *B. licheniformis* KIBGE IB-21. Symbols (means ± S.E., $n = 6$) having similar letters are not significantly different from each other (Bonferroni test, $P < 0.05$).

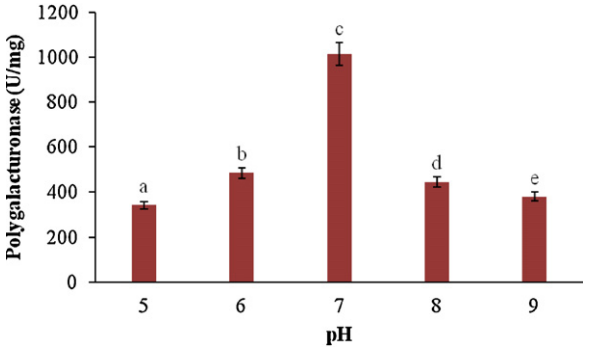

Fig. 3. Effect of initial pH of medium on polygalacturonase production by *Bacillus licheniformis* KIBGE IB-21. Symbols (means ± S.E., $n = 6$) having similar letters are not significantly different from each other (Bonferroni test, $P < 0.05$).

mesophilic in nature and at 60 °C no growth was observed which ultimately causes no enzyme production.

The initial pH of fermentation medium has a significant effect on various factors including growth of bacterial culture, enzyme production and also on stability of enzymes (Murad, 1998; Murad & Saleem, 2001). Most of the *Bacillus* species were found to produce maximum polygalacturonase at different pHs ranging from 7.0 to 9.0 (Kobayashi et al., 1999). However, some research workers also reported optimum pH for growth and polygalacturonase production from 7.0 to 10.0 (Horikoshi, 1972; Kelly & Fogarty, 1978). In order to study the effect of pH on polygalacturonase production from *B. licheniformis* KIBGE IB-21, culture was grown in production medium having different pH levels (5.0–9.0) and it was observed that polygalacturonase production was found at broad pH range. Maximum polygalacturonase production (1015 U/mg) was observed at neutral pH 7.0 and it was also found that any variation of extreme pH value toward the acidic (7.0–5.0) or alkaline (7.0–9.0) side resulted in approximately 42% decreased in polygalacturonase production as compared to pH 7.0 (Fig. 3).

The carbon source in fermentation medium had a profound effect on the functioning of enzyme production by *B. licheniformis* KIBGE IB-21. Various sugars and agro industrial wastes were used as sole source of carbon for polygalacturonase production. Maximum enzyme production was attained in medium containing apple pectin (1015 U/mg) which was nearly followed by citrus pectin (778 U/mg) and wheat bran (755 U/mg) (Table 2). Lowest enzyme production was observed in fermentation medium supplemented with fructose (50 U/mg), glucose (52 U/mg) and glycerol (62 U/mg). Better culture growth but low enzyme secretion was observed by some carbon sources including sucrose, maltose, fructose, glucose, glycerol, lactose and galacturonic acid. It was reported that these sources may repress catabolic production of polygalacturonase

Table 2
Effect of various carbon sources and agro industrial wastes on polygalacturonase production by *Bacillus licheniformis* KIBGE IB-21.

Carbon source	Enzyme activity (U/mg)
Apple pectin	1015.0
Citrus pectin	778.0
Lactose	202.0
Galacturonic acid	197.0
Sucrose	145.0
Maltose	145.0
Fructose	50.0
Glucose	52.0
Glycerol	62.0
Orange bagasse	695.0
Sugarcane bagasse	549.0
Rice bran	557.0
Wheat bran	755.0

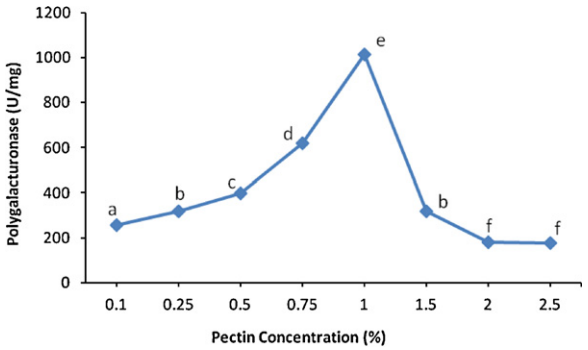

Fig. 4. Effect of substrate concentration on polygalacturonase production by *B. licheniformis* KIBGE IB-21. Symbols (means ± S.E., $n = 6$) having similar letters are not significantly different from each other (Bonferroni test, $P < 0.05$).

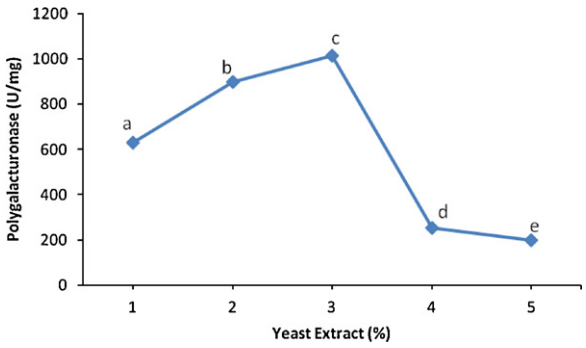

Fig. 5. Effect of yeast extract concentration on polygalacturonase production by *B. licheniformis* KIBGE IB-21. Symbols (means ± S.E., $n = 6$) having similar letters are not significantly different from each other (Bonferroni test, $P < 0.05$).

(Cavalitto, Areas, & Hours, 1996). So it was suggested that for maximum polygalacturonase production from *B. licheniformis* KIBGE IB-21 apple pectin can be used as a more efficient carbon source as compared to others on industrial scale.

Different concentrations of apple pectin were studied for polygalacturonase production and it was found that maximum enzyme production was achieved at 1.0% (Fig. 4). Further increase in concentration resulted in suppression of polygalacturonase production. When apple pectin was increased from 1.0% to 2.0% a sharp decline in polygalacturonase production was observed and only 19.0% enzyme production was detected as compared maxima. The decreased in polygalacturonase production at higher concentration of apple pectin might be due to increase in viscosity of fermentation medium which caused problem to maintain homogeneity of fermentation medium and oxygen transfer resulting in minimal cell multiplication and enzyme production. No activity was detected when the *B. licheniformis* KIBGE IB-21 was grown in the medium without apple pectin suggesting that the polygalacturonase production by *B. licheniformis* KIBGE IB-21 is inducible in nature. Similar finding was reported in case pectinase production from *Pencillium* SPC-F 20 (Mathew, Eldo, & Molly, 2008).

Maximum polygalacturonase production (1015 U/mg) was achieved when yeast extract was used in fermentation medium followed by peptone (700 U/mg) (Table 3). Yeast extract contains vitamins, minerals and amino acids which are necessary for bacterial growth and enzyme production thus facilitating cell growth and enzyme production. However, when inorganic nitrogen sources were used almost 40–50% decline in polygalacturonase production was observed as compared with yeast extract. It was reported that yeast extract as a nitrogen source is the best inducer for exo-pectinase production by *Aspergillus sp* (Aguilar, Trejo, Garcia, & Huitron, 1991). Different concentrations of yeast extract were used for enzyme production and it was found that polygalacturonase production was increased with an increased in yeast extract concentration and maximum production was achieved when 0.3% yeast extract was incorporated in the medium (1015 U/mg) (Fig. 5).

Table 3
Effect of different nitrogen sources on polygalacturonase production by *Bacillus licheniformis* KIBGE IB-21.

Nitrogen sources	Enzyme activity (U/mg)
Yeast extract	1015.0
Peptone	700.0
Casein	359.0
Tryptone	452.0
Urea	433.0
Ammonium sulfate	318.0
Ammonium chloride	394.0
Sodium nitrate	400.0
Potassium nitrate	536.0

4. Conclusion

In this study polygalacturonase producing newly isolated strain from an indigenous source was studied and this strain was identified as *B. licheniformis* KIBGE IB-21 after 16S rDNA analysis. New medium was developed for maximum polygalacturonase production which included apple pectin (1.0%), yeast extract (0.3%) and other minerals in fermentation medium (pH 7.0). Maximum production was achieved at 37 °C after 48 h of incubation.

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