Optimization of Pectinase Production from Bacillus lechniformis FH4-IRQ

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1. Introduction

Pectin substances are characterized as a long chain of galacturonic acid residues bounded with carboxyl group, which are sometimes been as methoxyl groups when it modified by the addition of methyl groups [1,2], plant cell wall contain pectin and it represent as important component of wall especially in the middle lamella, where it acts as a cementing material between adjacent cells [3,4]. Pectinolytic enzymes or pectinases are enzymes that hydrolyze pectin, these include: polygalacturonase (PGase), pectin esterase (PEase), pectin lyase (PLase). Bacteria, fungi, actinomycetes, yeasts, insects, nematodes, protozoan and plants all are known as pectinase producers [5,6], microorganism producing Pectinase are widely distributed in: soil, spoiled fruits, vegetables, decayed leaves and wood [7]. Pectinase accounts for about 10% of total enzyme production in the world market [8]. Processing of fruits and vegetables for juices and wine are all dependents on acidic pectinases. [7] The pectic substances account 0.5-4% of the weight of fresh material [9], that hindering the juice extraction process by pressing so, addition of pectinase in the process of extraction improves the yield of fruit juice by an easier process, juice viscosity decreased and gel structure will degrade, thus the juice concentration capacity will be improved. Yields of fruit juice, increase by more than 90% by extraction with enzymatic maceration when it compared to conventional mechanical juicing, besides improving nutritional (vitamins) properties and the organoleptic (color, flavor) and technological efficiency [6,7,9].

Alkaline pectinases are pectinases with higher activities in alkaline conditions, pre-treatment of waste water from vegetable food was processed by alkaliine pectinase to degrade pectin residues, the textile processing of fibers such as flax, jute and heme, coffee and tea fermentation, vegetable-oil extraction [5,6], the treatment of paper pulp and also in bioscouring of cotton fibers [5,6,10].

This study was designed to screen pectinase production from sporeformer bacteria Bacillus lechniformis FH4-IRQ and pectinase production optimization.

2. Materials and Methods

2.1 Bacterial strain

Bacterial strain Bacillus lechniformis was isolated from Iraq, identified by 16s rRNA sequenced in GTCA company /Germany and submitted in NCBI in October 2013 as Bacillus lechniformis FH4-IRQ with accession no. KF531930.

2.2 Pectinase screening assay

Pectinase screening agar assay medium (PSAM) was prepared as in the following [3]: [gm/l D.W.: pectin—1, (NH₄)₂PO₄—3, KH₂PO₄—2, K₂HPO₄—3, MgSO₄_0.01 and agar—25], pH was adjusted to 7. Using cork borer to make 2 wells (each well 6 mm in diameter), after that 50 μl was transferred from the subculture of bacteria [McFarland standard (no.0.5)], incubated at 37º C for 2 days. After incubation the plate was flooded with iodine solution (0.25%/iodine, 0.5% potassium iodide and31 ml of 20% ethanol) for 15 min at 30º C, a clear zone was measured in mm.

2.3 Pectinase production medium

Pectinase production broth was prepared as described by (4): [gm/l D.W.: pectin—5, (NH₄)₂SO₄—2, KH₂PO₄—2, K₂HPO₄—2 and yeast extract—3],pH was adjusted to 7. Medium was inoculated with 100 µl of subculture bacteria (McFarland turbidity standard (tube no. 0.5)).

2.4 Quantitative Assay

Galacturonic acid standard curve was done by dissolving 1g of galacturonic acid (GA) / 100 ml D.W. (stock), serial
concentrations were prepared. 2ml of each concentration mixed with 2 ml of Dinitrosalicylic acid (DNS) reagent, the mixtures then boiled for 5 min. absorbance is determined at 540 nm for each concentration, D.W. was used as a blank instead of pectin solution. The pectinase activity for bacterial pectinase production was done as the following:1ml of cell free supernatant was mixed with an equal volume of an aqueous pectin solution (1g/100ml), incubated at 37°C for 15 min. then 2 ml of DNS the mixtures then was boiled for 5 min, the absorbance is determined at 540 nm.

**One unit (U/ml) – the amount of enzyme required to release 1µg of GA/min under the assay condition.**

2.5 Incubation period Optimization:

Production medium were prepared 50ml/100ml Erlenmeyer flask, and inoculated as mention before. Each flask was incubated at 37°C for 24hrs, 48 hrs, 72 hrs and 96 hrs.

2.6 Carbon sources Optimization:

Different carbon sources (glucose, lactose, mannitol, and starch) at 1% concentration were used in preparation of production medium 50ml/100ml Erlenmeyer flask, and inoculated as mention before. Each flask was incubated at 37°C 24 hr.

2.7 Nitrogen sources Optimization:

Different nitrogen sources (Na₂NO₃, NH₄Cl₂, urea, and peptone) at 0.03% concentration were used in preparation of production medium 50ml/100ml Erlenmeyer flask, and inoculated as mention before. Each flask was incubated at 37°C 24 hr.

2.8 Temperature Optimization:

Production medium were prepared in several flasks, 50ml/100ml Erlenmeyer flask, and inoculated by 24 hr aged inoculums. Each flask was incubated at different temperatures (25, 30, 35, 40, 45) °C for 24 hr.

2.9 pH value Optimization

Production medium were prepared in several flasks 50ml/100ml Erlenmeyer flask, the pH were adjusted as the following (5, 6, 7, 8, 9 and 10), then autoclave, and inoculated as mention before incubated at 37°C for 24 hr.

3. Results and Discussion

3.1 Pectinase screening assay

After the plates were flooded with iodine solution the diameter of the Clear zones around wells were measured in mm , central well was filled with sterile broth as control, well 1 was filled with *E.coli* suspension, well 2 with our strain (*Bacillus lechniformis FH4-IRQ*), well 3 and4 with another strains of *Bacillus*, well 5 was filled with *Paenibacillus spp.* suspension. As it seems in figure 1, *Bacillus lechniformis FH4-IRQ* gave widest clear zone (23 mm) comparing with another strains.

**Figure 1:** The diameter of pectin hydrolysis on PSAM

3.2 The effect of physical and chemical value

To estimate the pectinase activity in unit/ml (U/ml), the standard curve of GA was used (fig 2).

![Standard curve of galacturonic acid](image)

**Figure 2:** Standard curve of galacturonic acid

3.3 The effect of incubation period

The productivity of pectinase was estimated against incubation period, 24 hr seems as preferable period with highest production rate of pectinase (12.67U/ml), the productivity continued decreased with prolonged incubation period to72 hr (4.1 U/ml). Generally growth curve of *Bacillus* species that was obtained by plotting the graph between time intervals (hours) and absorbance at 660nm exhibit that it reached stationary phase after 19hours. The reduction of pectinase production might be due to change in the pH of fermentation medium as a result to galacturonic acid that produced from pectin hydrolysis by pectinase (figure 3).

![Effect of incubation period](image)

**Figure 3:** Effect of incubation period
This is good explanation for decreasing in pectinase production that the bacteria need it during log phase in order to degrade the complex substrate in media and reduce it into a simpler form that act as a good nutrition for the bacteria. Change of pH during fermentation reduce the production of pectinase after 24 h and it may be as a result of denaturation or decomposition of enzyme due to interaction with other components of medium and medium exhausted from their nutrients [11]. Samriya and coworkers showed slightly prolonged result that after 48 hr pectinase activity reach to maximum. Bhardwaj and gara showed that Bacillus sp. MBRL576 reach maximal pectinase level of production at 24 hours of incubation period when compared with different incubation periods with intervals of 6 h, 12 h, 18 h, 24 h, 48 h, 72 h, 96 h, and 120 h,144 h, 168 h, 192 h and 216 h. [12]Kumar and coworkers had different results, the maximum production of pectinase by different isolates of Bacillus sp. was found at 96 hr of incubation period. Decreasing of pectinase value will be with increasing of incubation time. Metha and coworkers result agreed with Kumar result [15]. Phutela and coworkers revealed that incubation period of 48 hr was optimal for production in some fungal strains [16]. Rhizopusoryza produce polygalacturonase at maximum level within 72 hr of incubation [17].

### 3.4 The effect of carbon source

Carbon source have important role in cell growth , it represent the precursors for several molecules and it represent the energy source . Supplementation of carbon sources in the form of carbohydrates resulted increase in pectinase production by Bacillus sp. Monosaccharide more suitable for enzyme production than disaccharide and polysaccharide. Media supported with glucose gave higher enzyme production rate (71.5 U/ml) than others. Slightly difference appeared with lactose (64.5 U/ml) while mannanol supported media seems with lowest pectinase production (15.47 U/ml) (fig 4).

Prakash and coworkers revealed the same results that glucose followed by lactose observed with the highest production of pectinase[18].Starch supported media decreased the production of pectinase greatly in contrast, glucose supported media gave good pectinase production [14]. Ranveer and coworkers were reveals to different result about glucose, That pectinase synthesis was greatly suppressed when the bacterium was grown either on glucose, maltose, or sucrose, but lactose be good for pectinase production by Bacillus sphaericus (MTCC 7542). [19].

![Figure 4: Effect of carbon source](image)

3.5 The effect of nitrogen source

Nitrogen source is one of the essential components in media for bacterial growth and enzyme production because nitrogen represent as the most important components in amino acids and nucleic acid, so the selection of appropriate nitrogen source is very important role in enhancement of enzyme production. Urea that composed from two ammonia molecules exhibit better support for media to produce pectinase (15.49 U/ml) , in contrast with ammonium chloride supported media that gave the lowest production value (9.8 U/ml) comparing with other nitrogen sources that used in the experiment(sodium nitrate and pepton)(fig 5).

![Figure 5: Effect of nitrogen source](image)

3.6 The effect of incubation temperature

Temperature considered as crucial parameter in bacterial growth and enzyme production by their effect on enzymes of cell that controlling the metabolism and other cellular activities .The increasing of kinetic energy can lead to increasing of collisions between enzyme and substrates to form a complex of enzyme substrates (ES) and finally can increase the product .so, all organisms have three cardinal temperature ,optimum , maximum and minimum, and according to the optimum temperature we can classify the organism to psychrophilic(0 – 20 °C ), mesophilic (20-50°C) and thermophilic (above 50°C).Some of Bacillus sp. are thermophilic that prefer incubation temperature above 45°C for optimum growth and production of different enzymes. In our study the optimum temperature for pectinase production obviously seems at 37°C (12.67 U/ml)when it compared with other temperatures that used in the experiments, the range of incubation temperature between 25°C to 35°C exhibit slightly raising in enzyme productivity with temperature.
3.7 The effect of pH value

Value of pH effect on the three dimensional structure of different enzymes that control the cell growth, it also affect on the stability of proteins by altering their charge therefore effect on the metabolic activity of bacterial cell especially in Redox reactions (Oxidation and Reduction reactions). Media with different pH values were examined for activity of the pectinase enzyme [25]. Kashyap and coworkers referred that the Bacillus sp. DT7 produce maximum level of pectinase at pH 7.0 [23]. Sharma and Satyanarayana, showed that B. subtilis ERFL 01 reach to the best fermentation, the enzyme titer and the final biomass concentration at temperature 45°C, it was the optimum fermentation temperature and it represent the optimum to Bacillus species for the production and activity of the pectinase enzyme [25].

The effect of pH value

![Figure 6: Effect of incubation temperature](Image)

Dey et al (2011) found that at 37°C Bacillus subtilis produce maximum level of pectinase enzyme [20]. While Kumar et al. found it at 35°C by Bacillus sp. MFW7 and any increasing in the temperature results in the decrease of pectinolytic activity [14]. Kashyap and coworkers referred that the Bacillus sp. DT7 produce maximum level of pectinase at 37°C incubation temperature [23]. Sharma and Satyanarayana, showed that B. subtilis ERFL 01 reach to the best fermentation, the enzyme titer and the final biomass concentration at temperature 45°C. It was the optimum fermentation temperature and it represent the optimum to Bacillus species for the production and activity of the pectinase enzyme [25].

The optimal initial pH value for producing pectinases depends on the nature of microorganism, a pH 8.5 has been reported as the higher initial optimal pH for pectinase production by Bacillus pumilus dcsr1 [24]; At least some of genes are known to be pH regulated involved in the production of certain enzymes in microorganisms [26]. Jansirani found that pH 7.0 was the optimum for production and activity of the pectinase [25] and Dey et al (2011) observed that pH 6 gave maximum activity of enzyme was at for all Bacillus isolates and it considered as optimum pH for enzyme production. Isolate FPB5 showed maximum activity at this pH6 [20]. The same result was obtained by Redaet al (2008) and Mehta (2013) with Bacillus sp. FW2 [27, 15]. By using orange peel as substrate, Bacillus sp. produce pectinase in maximum value at pH 6.5 [20]. Bacillus firmus isolated from soil to produce maximum level of pectinase at pH 7-8 [28]. Uenojo and Pastore showed that the pH 8 was the optimal initial value for pectinase production even though the pH 7 considered as the optimal initial for biomass production. Initial pH values of >8 adversely affected microbial growth and enzyme production [29].

References


thermostable pectinase by enhancement in the production of a highly alkaline and


