International Journal of Science and Research (IJSR)
ISSN (Online): 2319-7064
Index Copernicus Value (2016): 79.57 | Impact Factor (2015): 6.391

Optimization of Pectinase Production from *Bacilluslechniformis* FH4-IRQ

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Abstract: ***Bacillus licheniformis FH4-IRQ, strain isolated from soil of Iraq (submitted in NCBI since October 2013 with accession no.KF531930) was examined for pectinase production in pectinase production media with optimization of different conditions including incubation period,carbon source, nitrogen source, incubation temperature and pH. Twenty four hour incubation period seems with higher production yield(12.67 U/ml) when comparing with 48 hr (10.73U/ml) and 72 hr(4.1 U/ml). Glucose (71.5 U/ml) and lactose (64.6 U/ml) seems better as a carbon source for pectinase production than starch (19.46 U/ml) and mannitol (15.47 U/ml).Different nitrogen sources were examined (peptone , ureae , ammonium chloride and sodium nitrat, urea was accompanied with higher yield (15.49 U/ml) than others. Temperature and pH value were also optimized for pectinase production , 37C gave higher production (12.67 U/ml) than other incubation temperatures(25 , 30 , 35 , 40 and 45 C) , pH 9 was the best(34.2 U/ml) comparing with other pH value tested(5, 6, 7, 8, 9, and 10).***

Keywords: Pectinase, Optimizationand*Bacillus lechniformis*FH4-IRQ

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], micro-organism 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 alkakine pectinase to degrade pectin residues, the textile processing of fibers such as flax, jute and hemp,, 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 ***Bacilluslechniformis*** **FH4-IRQ** and pectinase production optimization.

2. Materials and Methods

2.1 Bacterial strain

Bacterial strain *Bacilluslechniformis* 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, (NH_4)$_2PO_4$—3, KH_2PO_4—2, K_2HPO_4—3 , $MgSO_4$ _0.1 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 transfered 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 37ºC 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 37ºC 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 37ºC 24 hr.

2.7 Nitrogen sources Optimization:

Different nitrogen sources (Na_2No_3, NH_4Cl_2, 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 37ºC 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) ºC 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 37ºC 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 ***Bacillu* ,** 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.

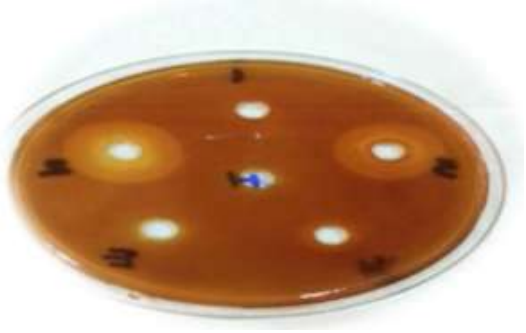

Figure 1: The diameter of pectin hydrolysis on PSAM

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).

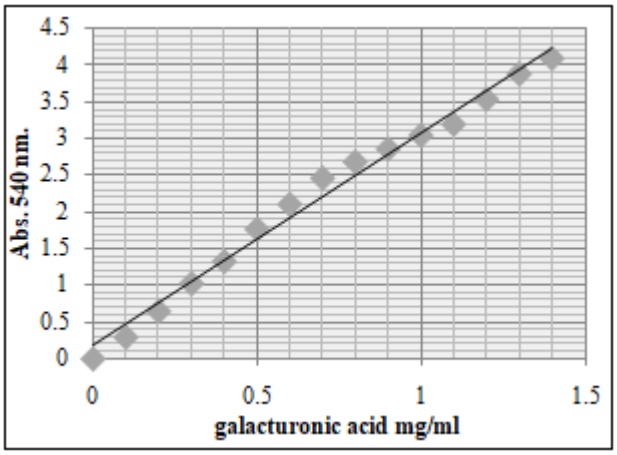

Figure 2: Standard curve of galacturonic acid

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).

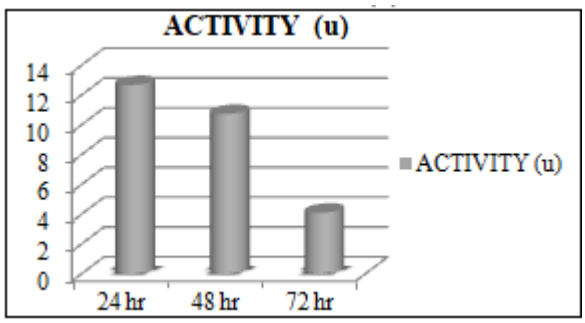

Figure 3: Effect of incubation period

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 48hr pectinase activity reach to maximum. [12],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. [13]Kumar and coworkers had different results, the maximum production of pectinase by different isolates of ***Bacillus sp.*** was found at 96hr of incubation period. Decreasing of pectinase value will be with increasing of incubation time. [14]. 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 mannitol 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 greatlyin 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].

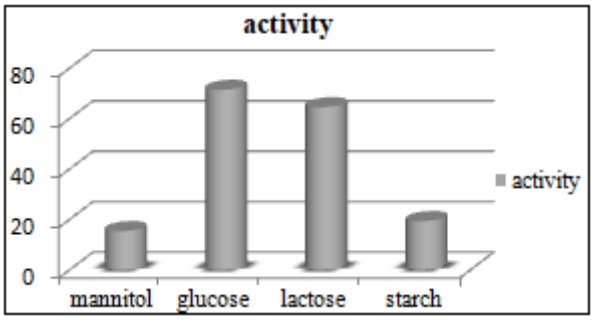

Figure 4: Effect of carbon source

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).

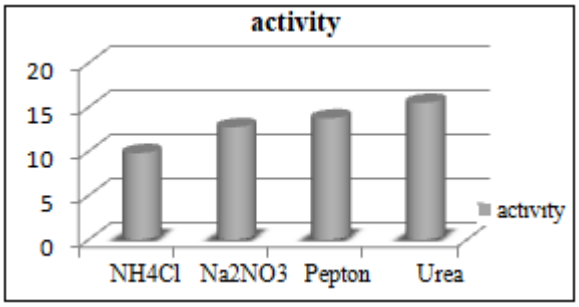

Figure 5: Effect of nitrogen source

The influence of organic nitrogen sources such as tryptone, yeast extract, peptone and inorganic nitrogen sources such as urea, ammonium nitrate and ammonium chloride on amylase production was determined by several researchers. Kumar found that ammonium nitrate be better among inorganic nitrogen sources, this result was identical with ours. While according to organic nitrogen sources Yeast extract was the better. [14]. Prakash *et al*. showed that pectinase production stimulated by peptone and yeast extract better than other nitrogen sources [18]. Karmakar and Ray revealed that tryptonerepresnt as better nitrogen source for pectinase production [20].Sukhumsirichart*et al* (2007) , Murad, and Azzaz (2011) and Phutela*et al* (2005) referred that Addition of Ammonium sulphate, peptone, yeast extract, soya bean as a nitrogen sources in the production media improves yield of PLs. [21,22,16].

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 optimam 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 thermophillic 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

increase.while the productivity decreased when the temperature increased above 40 °C .(fig 6).

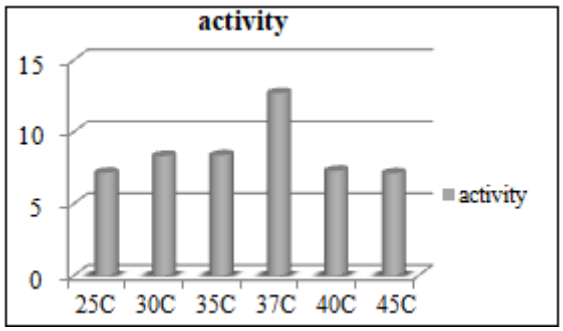

Figure 6: Effect of incubation temperature

Dey et al (2011) found that at 37° ***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 optimal fermentation temperature and is clearly much lower than the optimal temperature of 50°C reported for ***Bacillus pumilus***dcsr1 [24]. Jansiran*iet al* (2014) revealed that 35°C was the best incubation temperature and it represent the optimum to Bacillus species for the production and activity of the pectinase enzyme[25].

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 effect on the stability of proteins by altering their charge therefore adjusted of pH of media is important parameter for good bacterial growth and enzyme production ,the pH value determine the concentration of H ions and OH ions that control the metabolic activity of bacterial cell especially in Redox reactions (Oxidation and Reduction reactions),media with different pH values were examined for pectinase production, generally , media with alkaline pH value are better than acidic value for pectinase production, the highest value was when media pH adjusted at 9, it gave 34.2 U/ml . in contrast , the acidic pH 5 gave 8.93 U/ml and 6gave 9.6 U/ml and it represent very low when it compare with neutral and alkaline pH (fig 7).

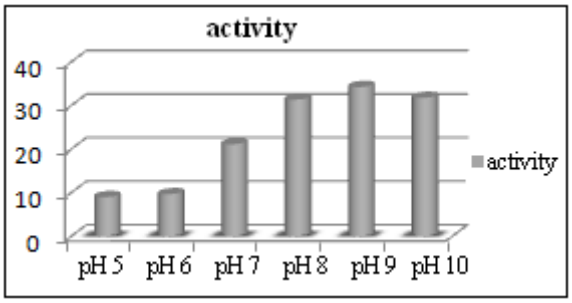

Figure 7: Effect of pH value

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] 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 PPB5 showed maximum activity at this pH6[20]. The same result was obtained by Reda*et 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 produced 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].

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