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Production, Purification and Characterization of Endopolygalacturonase by *Bacillus subtillus*

Nayab Munir*, Javaid Asad M and Haidri SH

Department of Biochemistry and Biotechnology, PMAS Arid Agriculture University Rawalpindi, Pakistan

Abstract

Plant cell wall is made up of complex carbohydrates like cellulose, hemicelluloses and pectin. Pectin is complex carbohydrates made up of galacturonic acid units linked by glycosidic linkage. For breakdown of this pectin microorganism require many different enzymes. Polygalacturonase is a pectinolytic enzyme catalyzed hydrolysis of α 1-4 glycosidic linkage in polygalacturonic acid by the addition of water. Polygalacturonase has two types; endopolygalacturonase and exopolygalacturonase. Endopolygalacturonase catalysis internal α 1-4 glycosidic linkage and exopolygalacturonase catalyze external α 1-4 glycosidic linkage in Pectate molecules. Endopolygalacturonase has various applications especially fruit juice industries used for clarification of juice as well as in food industry for maceration of vegetables. Endo-PG produced by fungus works in acidic pH and at low temperature but the application of endo-PG in food industry and in cloth industry needs an enzyme that works at high temperature and also in alkaline conditions. *Bacillus subtillus* was used as reference strain. By using different substrates like apple peels, radish peels and citrus peels, it was concluded that citrus peels produce maximum enzyme concentration. Further characterization showed that EPG produced by *Bacillus subtillus* have optimum temperature of 60°C and optimum PH 5. Maximum fold of purification was observing with Gel filtration. Molecular weight of enzyme was 67 kd. V_{max} for EPG produced by *Bacillus subtillus* was 1.21 mg/ml and Km was 2423 mol/min/mg.

Keywords: *Bacillus subtillus*; Galacturonic acid; Endopolygalacturonase; Pectate

Introduction

Enzymes are delicate proteins. A large no of bacteria and fungi produced Pectinolytic enzymes and mostly they are plant pathogenic. They uses these enzymes to invade host tissue, other than this these enzymes help in decay of dead organic matter [1]. These enzymes include depolymerizing enzymes and demethoxylating enzymes, Polygalacturonase (EC 3.2.1) is most common depolymerizing enzyme this enzyme catalyze The removal of α-1, 4 Glycosidic linkage among two galacturonic acid units and it coverts polygalacturonic acid into galacturonic acid, While pectin lyase (EC 4.2.2) enhance β-elimination reaction between methylated Units [2]. Similarly de-esterifying enzymes include pectin esterases, they remove methyl group from methylated pectin and help in production of methanol and pectin [3].

Naturally, microorganisms have been gifted with enormous ability to produce enzymes. They generate a group of enzymes, which have been oppressed commercially from many years. Cellulose and Pectic substances are most rich carbohydrates present inside plant. Pectin propectin and pectic acid contribute in firmness of plant, Pectin form a complex due to its composition and the other impurities like with some proteins and waxes are amalgamate through the back and side chain of Pectin. Pectin mortifying enzymes are formed in huge amount by plant related Microbes. Naturally, there are three main types of pectin mortifying enzymes: pectin Esterases, polygalacturonases and lyases. Pectin esterase (EC3.1.1.11) catalyzes removal of the methyl faction of pectin from pectic acid. Polygalacturonases are a set of biological catalyst which hydrolyze α-1, 4 glycosidic Linkage in pectin. Due to difference in their way of action pectinases are classified into different type'spolygalacturonase, pectinlyase and pectin esterase. The Lyase cleave polygalacturonate or pectin chains marks in the configuration of a double Bond linking carbon no 4 and carbon no 5 at the non-reducing site with eradication of carbon dioxide. Pectic substances are made up of D-galacturonic acid units connected by α-1, 4- glycosidic bond [4].

PG is further classified in to EPG and exopolygalacturonase. Endopolygalacturonase belongs to family 28 glycosyl hydrolase catalysis the internal α 1-4 glycosidic linkage and convert pectic substances into their monomeric form. PG is broadly used in food industries and help in grinding, liquefaction and mining out fruit juices [5]. In industries, Aspergillus neiger is mostly use for the profitable production of PG [6] and only some studies are existing for the manufacture of PG from bacteria [7-9]. Most of the pectinases produced by fungi have most favourable pH variety between acidic ranges. This pH range is appropriate for fruit juices, because fruit juices have same optimum pH range, but these enzymes cannot be used for the vegetable or the other measures for which neutral pH is required [9].

Endopolygalacturonase is broadly dispersed in fungi like yeast higher plants and Microorganisms [10]. The main source for the pectin degrading enzymes is yeast, bacteria and a large variety of filamentous fungi, for which the most common one is *Aspergillus.* Bacterial pectin degrading enzymes are present in different classes of *Bacillus* [11], *Amycolata* [12] *Aspergillus* Benen Decompose pectin randomly [10]. Even though the production of some pectin degrading enzymes is possible, at hand still production of enzymes is required with elevated activity and stability at basic environment. Pectin degrading enzymes were most common enzymes which were used at home. People were unaware of their importance on industrial scale. First of all their industrial uses was highlighted in 1930 for the

***Corresponding author:** Nayab Munir, Department of Biochemistry and Biotechnology, PMAS Arid Agriculture University Rawalpindi, Pakistan, Tel: +92-051-9290466; E-mail: nayab.munir@yahoo.com

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production of wines and fruit juices. After 1960 when scientist came to know about chemical composition of plant tissue requirement for production of these enzymes increases, scientists start to utilize a larger variety of enzymes more proficiently. As a consequence, pectinases are most popular enzymes of the profit-making. First and foremost, these enzymes were accountable for the breakdown of the extended and composite substances known as pectin that is polysaccharides present in middle lamella and the primary call walls of young plant. Pectin degrading enzymes are at this time an essential part of fruit juice and textile industries. The objectives of adding enzymes differ in different kinds of fruit and vegetable juices. If juice is dazzling clear enzymes are required to increase the juice quantity. In some cases enzyme is added throughout pressing and straining in order to eliminate floating substances and clear juice is obtained [13].

Apple juice is prepared as natural, unaltered and unclarified, juice consist of high quantity of mash it is unclear juice. We centrifuged it to take away suspended particles but not altered; and at the end filtered plain and yellowish-brown-colored juice form by adding enzyme [14]. Even though pectin degrading enzymes can depolymerize highly esterified pectin are the main types of enzymes required for production of clear apple juice [15].

Clarification of juice is dependent on pH, temperature and quantity of enzyme. We can easily clarify juice if pH is in acidic range. Similarly if temperature raises juice clarification become more and more easy but temperature cannot be higher than denaturation temperature of enzyme [14].

Clarification of apple juice possibly will be obtained by using mixture of pectin degrading enzymes like PG and PME [16]. Pectin enzymes are useful in wine making [17,18]. The improve deliberation of anthocyanin through red grapes into the juice by adding pectin enzymes is another benefit of these enzymes [13].

Agricultural resources of fermentable carbohydrates must be processed to get fermentable sugar like sugar beet; sugarcane, wheat and potatoes etc. Huge amount of plant cell-wall resources happen to accessible as by products in the beet-sugar and starch factories. Pectinases along with cellulase enzymes and hemicellulases is required for the manufacturing fermentable sugars from polysaccharides and for the breakdown of cell wall matrix, so liquefy the substances and the liberate carbohydrates present inside cell [19]. More emphasis is laid on production of high yielding strains [20]. *Bacillus subtilis* will be used for production of endopolygalacturonase it is sporullating, aerobic gram positive strain. This bacterium is non virulent. But this bacterial specie sometime may cause secondary infection and during storage they can produce soft-rot symptoms in some plant [21].

Material and Method

Selection of bacterial strain

Pure strain of the *Bacillus subtillus* was brought from; University of Waterloo Canada It was sub cultured after every three month on YUP medium and preserved it in refrigerator having temperature 4°C. New cultures were prepared every time whenever there was need for preparing inoculum.

Substrate preparation

Different agro-wastes like peels of citrus fruits, apple and radish were used as substrates for the production of the endo-PG. peels were collected from fruit juice shops from local market of Rawalpindi. The substrates was firstly sun dried and then in oven at 70°C and ground to a fine powder by using electric grinder. The ground substrates were passed through sieve to obtain mesh size of 40 mm and 80 mm. These ground substrates were used for the production of the endo-PG.

Preparation of Inoculum

Inoculum of the endo-PG positive bacteria was prepared in inoculum media with a Composition (0.05%KCl, 0.1% $MgSO_4.7H_2O$, 0.1% tri-sodium citrate dehydrate, 0.5% yeast extract, 0.5% tryptone) [9,22]. Inoculums was prepared by picking cultured bacterial colony with the help of Culturing loop, loop was dipped in inoculums media and the process was repeated In order to get enough concentration of bacterial colony the process was repeated

Medium was shaken for 6 hour for uniform suspension. Then through spreading and culturing inoculum was checked for presence of impurities.0.2 M HCl and 0.2 M NaOH was used to adjust pH of inoculum at 6.7.

Fermentation Chamber for Endopolygalacturonase

250 ml Erlenmeyer flasks, containing fermentation materials, placed in Incubator. Then all conditions were set like temperature, time and speed for shaking was adjusted at 170 rpm.

The Process of Fermentation

In each flask as a unit of Fermentation 5 gram substrate and 100 ml of distilled water was taken. At benchmark values of 121°C for 20 min reaction mixture containing substrate was autoclaved for sterilization. Autoclaved substrate was leave for some time to cool down at 37°Ctemperature; initial quantity of inoculum was poured in reaction mixture in Laminar flow in order to avoid contamination with other microbes and kept back inside incubator further for fermentation. After adding inoculum flasks were placed for shaking at 170 rpm at temperature of 35°C for 6 hour. Then after few hours reaction mixture was filtered by using filter paper. The crude extract was stored in refrigerator. Enzyme activity was determined within 24 Hours of the filtered extract.

Standard Curve for Galacturonic Acid

Standard curve for galacturonic acid was prepared by taking OD of different concentration of galacturonic acid at 640 nm. It was used to calculate the galacturonic acid produced by Endopolygalacturonase.

Enzyme Assay Protocol for Epg

The different amounts of endopolygalacturonase obtained after completion of fermentation process were approximate by measuring amount of galacturonic acid produced by endopolygalacturonase after reacting with Polygalacturonic acid. For that purpose polygalacturonic acid was added in reaction mixture along with crude enzyme. Crude enzyme was dissolved in phosphate buffer having pH of 4.8. Control was prepared by adding polygalacturonic acid D. H_2

O and buffer. After complete mixing of all these things test mixture and control both was placed in incubator at 37°C for 20 minutes then 0.1 ml DNS was added in all test tubes and keep it at 100°C or boiling water bath for few minutes [23]. After completing this process OD of control was measure by using spectrophotometer at 640 nm and converted in to zero after that OD of test mixture was measure at same wavelength.

Preparation of DNS Solution

2 ml of DNSA reagent solution A (Table 1) was poured in 2 mL solution of sugar in 20 ml flask. The flask was kept in boiling water for 15 minutes. After 15 minutes with gape of few minutes, 0.5 ml of solution B was poured in flask and kept for some time to cool down at room temperature.

Equations Used to Calculate Enzyme Activity

Enzyme activity was calculated in terms of the quantity of products formed by conversion of Substrate. The unit of enzyme activity was defining as micromole of galacturonic Acid released after 1 minute. It was calculated by the following formulas.

$$Enzyme\ activity = \frac{\text{Absorbance X standerd factor X dilution factor}}{\text{Time of incubation}}$$

Dilution factor=amount of water in ml which was added in flask.

$$Standard\ factor = \frac{\text{Concentration of Standard (µMol/ml)}}{\text{Absorbance of Standard}}$$

$$Specific\ activity\ of\ enzyme = \frac{\text{Enzyme activity}}{\text{Protein content(mg)}}$$

Optimization of Different Parameters for the Production of Endo-Pg

It is very important to check individual response of any condition on enzyme activity during process of fermentation for unbeaten optimization; for those purpose different experiments was conducted in order to study effect of different environmental conditions on enzyme production. At a time effect of two factors was studied, maintaining all others conditions at constant value by using Response surface methodology (RSM).

Optimization of inoculum size and age

Inoculum was prepared by the method of [22]. inoculum media will be placed for shaking for 8, 12, 16 hour inoculum of different sizes were used for the optimization of inoculum size 2.5%, 5%, 10% [9].

NO	Component	Quantity (g)
Solution A		
1	Distilled water	To make final volume up to 500ml
2	Dinitroisilicyclic acid	5mg
3	Phenol	1.5ml
4	Sodium Sulfite	0.25mg
5	Sodium Hydrooxide	5mg
Solution b		
1	Distilled water	To make final volume up to 100ml
2	Rochelle Salt	40mg

Table 1: Composition of DNS Solution.

Inoculum size (ml)	Inoculum age (hour)
0.95	16
6.25	16
10	8
6.25	27.31
10	24
6.25	4
2.50	8
11.55	16
2.50	24

Table 2: Effect of Inoculum Size and Inoculum Age on Enzyme production.

Temperature (°C)	Ph
9	5
30	3
19	5
55	7.8
80	3
55	2.17
30	**7**
55	5

Table 3: Effect of Temperature and pH on Enzyme production.

Loop containing bacterial colony was transferred to the inoculum media and the media was placed for shaking at 160 rpm for different time intervals (8, 12, and 16, 20 and 24 hours) to get an optimum time period. The time period giving the maximum endo-PG production will be used in next experiments [9]. Combine result for size and age of inoculum was determined by applying RSM. RSM provide different combinations of Inoculum size and age mentioned in Table 2.

Substrate water ratio and fermentation period

Substrate was used in different ratios (1%, 2%, 3%, 4% and 5%) with the distilled water in the production media to get an optimum substrate-water ratio. Growth media containing the inoculum and the substrate was placed on shaking for different time intervals (24, 48, and 72, 96 and 120 hours) to obtain the optimum fermentation period [9]. RSM was applied to get result.

Optimization of fermentation temperature and pH

The growth media having different pH values 2 to 7 was made and each was tested for the endo-PG production and the optimum growth pH was determined 2, 3, 5, 7 and 8 [9]. It was adjusted by adding 1 mM HCl and NaOH in crude extract. Other surroundings of the experiment were kept same as preliminary conditions. The pH of control was kept at 6.4 because inoculum was 6.4 without any adjustment. The growth media was placed for shaking at different temperatures (30, 40, 50, 60, 70 and 80) shown in Table 3.

Effect of Inducers/Inhibitors

Carbon sources and nitrogen sources

Fructose, galactose, lactose and sucrose will be used as carbon sources. They were used in different concentrations (0.5%, 1%, 1.5%, 2% and 2.5%) to find an optimum concentration [9]. Ammonium sulfate, ammonium nitrate, ammonium chloride and calcium nitrate will be used as nitrogen sources. Different concentrations (0.5%, 1%, 1.5%, 2% and 2.5%) of nitrogen will be prepared and the optimum concentration will be determined [9]. Different combinations of carbon and nitrogen sources were used along with different concentration by application of RSM (Tables 4-7).

Surfactants and mediator

Tween-20, tween-80 and SDS was used as source of surfactants. Their different concentrations were used (0.5%, 1%, 1.5%, 2% and 2.5%).

Urea, yeast extract, peptone and cane molasses will be used as mediators. Different concentrations (0.5%, 1%, 1.5%, 2% and 2.5%) of

Fructose (g)	Ammonium sulfate (g)
1.5	1.5
2.914	1.5
1.5	0.085
2.5	2.5
0.5	2.5
1.5	2.914
2.5	0.5
0.085	1.5

Table 4: Effect of Fructose and Ammonium Sulfate on Enzume production.

Lactose	Ammonium nitrate
1.5	1.5
2.914	1.5
1.5	0.085
2.5	2.5
0.5	2.5
1.5	2.914
2.5	0.5
0.085	1.5

Table 5: Effect of Lactose and Ammonium Nitrate on Enzyme production.

Galactose	Ammonium chloride
1.5	1.5
2.914	1.5
1.5	0.085
2.5	2.5
0.5	2.5
1.5	2.914
2.5	0.5
0.085	1.5

Table 6: Effect of Galactose and Ammonium Chloride on Enzyme production.

Sucrose	Calcium Nitrate
1.5	1.5
2.914	1.5
1.5	0.085
2.5	2.5
0.5	2.5
1.5	2.914
2.5	0.5
0.085	1.5

Table 7: Effect of Sucrose and Calcium Nitrate on Enzyme production.

Cane Molases (ml)	Tween 80 (ml)
2.5	2.5
0.5	2.5
0.5	0.5
1.5	0.085
1.5	1.5
1.5	2.914
0.085	1.5
2.914	1.5
2.5	0.5

Table 8: Effect ofCane molases and Tween 80 on Enzyme production.

mediators will be used. Combine effect of surfactant and mediator was studied by using RSM (Tables 8-10).

Metal ions

Calcium chloride, magnesium chloride and zinc chloride will be used as metal ion sources. Different concentrations (0.5, 1.0, 1.5, 2.0, 2.5 mM) of calcium chloride and magnesium chloride and different concentrations (1.0, 1.5, 2.0, 2.5, 3.0 µM) of zinc chloride will be used (Table 11).

Enzyme Purification

Partial purification of endopolygalacturonase

Ethanol, acetone and isopropanol were used as organic solvents in different ratios with the crude extract (10:90, 20:80; 30:70; 40:60; 50:50; 60:40). 10% organic solvent and 90% crude enzyme was added in 1st test tube then 20% organic solvent and 80% crude extract was added in 2nd test tube and so on up to 60% organic solvent and 40% crude extract in last test tube and then enzyme activity was done for both pallet and supernatant.

Partial purification by ammonium sulphate

Different concentrations taking start from 10% then 20%, 30%, 40%, 50%, 60% of ammonium sulfate with that of the crude enzyme

Peptone (g)	SDS (g)
2.5	2.5
0.5	2.5
0.5	0.5
1.5	0.085
1.5	1.5
1.5	2.914
0.085	1.5
2.914	1.5
2.5	0.5

Table 9: Effect of Peptone and SDS on Enzyme production.

Tween 20 (ml)	Yeast Extract (g)
2.5	2.5
0.5	2.5
0.5	0.5
1.5	0.085
1.5	1.5
1.5	2.914
0.085	1.5
2.914	1.5
2.5	0.5

Table 10: Effect of Tween 20 and yeast extract on enzyme production.

Calcium chloride	Magnesium chloride
2.5	2.5
0.5	2.5
0.5	0.5
1.5	0.085
1.5	1.5
1.5	2.914
0.085	1.5
2.914	1.5
2.5	0.5

Table 11: Effect of Calcium Chloride and Magnesium Chloride on E.A.

was used for the partial purification of the endo-PG and its activity for each concentration was determined.

Gel filtration

Gel filtration was carried-out to further purify the enzyme. Sephadex G-100 was used for gel purification. The column of 1.5× 60 dimension was used. The dry Sephadex powder was dissolved in phosphate buffer and we leave it for some time and wait for swelling of gel. After swelling of gel it was shacked for few minutes. After few hours supernatant was removed and same process was repeated for Several times, a burette was used as column it was 3 cm in diameter and 50 cm in Length, before packing of column burette was firstly washed with tape water and Then with phosphate buffer having same pH 4.8. A small piece of wool was placed at the end of column and funnel was placed at top of column. Firstly simple buffer was passed through column and then Sephadex swollen buffer was added in column. Bottom outlet was open and when suspended particles reach at bottom of column. After the complete packing of martial funnel was removed, few ml of buffer were Allowed to enter the column to avoid dryness of column, sample was added drop By drop and different frictions were collected and their OD was taken at 280 nm. The endo-PG after the gel filtration will be run on polyacrylamide gel electrophoresis (PAGE) to check the purity of the enzyme.

Enzyme Characterization

Effect of temperature and pH on EPG production

The outcome of temperature and pH on the production of enzyme was measure by carrying enzyme assay at different ranges of temperatures between 30 - 80°C and optimum temperature was determined for enzyme assay. By using buffer of diverse pH values from 5 to 9 was used for the enzyme assay to determine the optimum pH of the endo-PG [24].

Effect of substrate concentration

Substrate of different concentrations was used to find-out *Kmax* and *Vmax*. Line weaver Burk plot will be made to find-out the *Kmax* and *Vmax*.

Molecular weight determination

The purified endo-PG after the gel filtration will be run on SDS-PAGE to find-out its molecular weight.

Statistical analysis

All the data after complete optimization was arrange by applying ANOVA under completely randomized design (CRD) Treatment mean of different parameters was compared by Duncan's multiple range test (DMRT) (Steel *et al*., 1997).

Result and Discussion

Standard Curve for Galacturonic Acid

Standard curve of galacturonic acid was made by using its different concentrations. The regression equation, y= 0.004x - 0.005 was inserted by using Microsoft Excel 'y' was equal to concentration of Galacturonic acid in µg/mL and 'x' was OD at 640 nm (Figure 1).

Production of endopolygalacturonase from *Bacillus subtillus*

Bacillus subtillus is strain of bacteria which have great potential for production of endopolygalacturonase. According to previous research PG produced by *Bacillus subtillus* shows very high enzyme activity as compare to Aspergillus Niger.

EPG was produced by using citrus peels, apple peels and radish peels. Maximum enzyme was produced when citrus peels were used as carbon source. After citrus peels apple peels show high enzyme activity. Fur further optimization of different parameters citrus peels were selected as substrate. It was concluded that citrus peels are good substrate for production of EPG (Table 12 and Figure 2).

Optimization of Different Factors

Different parameters were optimized by using response surface methodology.

Optimization of F.P and substrate water ratio

Enzyme activity was done at different fermentation period and

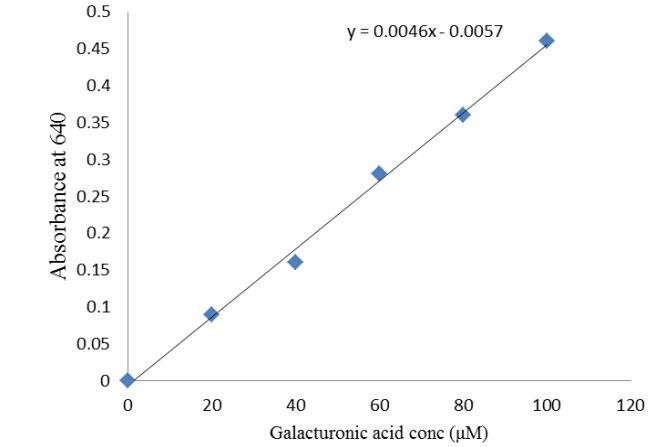

Figure 1: Standerd curve of galacturonic acid.

Source	DF	SS	MS	F-Value	P-Value
Concentration of substrate	1	15692	15691.8	24.54	0.00
OD	4	2557	639.4		
enzyme activity	5	18249			
S=25.2854 R^2=85.99%					

Table 12: ANOVA showing the effect of different substrates on enzyme production.

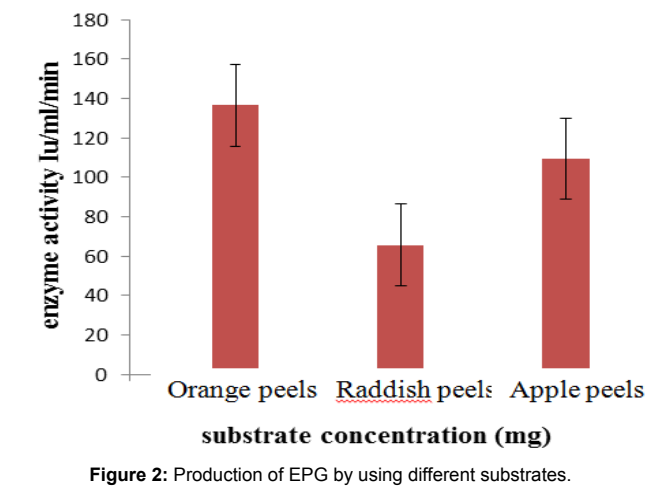

Figure 2: Production of EPG by using different substrates.

substrate water ratio. Maximum enzyme activity was observed at fermentation period of 24 hour and 5% substrate water ratio which is in accordance with previous results shown by Figures 3 and 4.

Optimization of inoculum size and age

Enzyme activity was measure by adding inoculum of different size and age. Maximum enzyme activity was observed at inoculum size of 11.9 ml and inoculum age of 16 hour as shown by Figures 4 and 5.

Temperature and pH

When fermentation was done at different temperature pronounced effect of temperature was observed at enzyme activity. With the increase of temperature enzyme activity also increases and maximum enzyme concentration was measure at temperature of 60°C and 80°C. Similarly effect of pH was also observed and maximum enzyme production occurs when pH of crude extract was 7.8. Figure 6 shows combine effect of temperature and pH.

Effect of inducers/inhibitors

Combine effect of inducers and inhibitors was study by using response surface methodology.

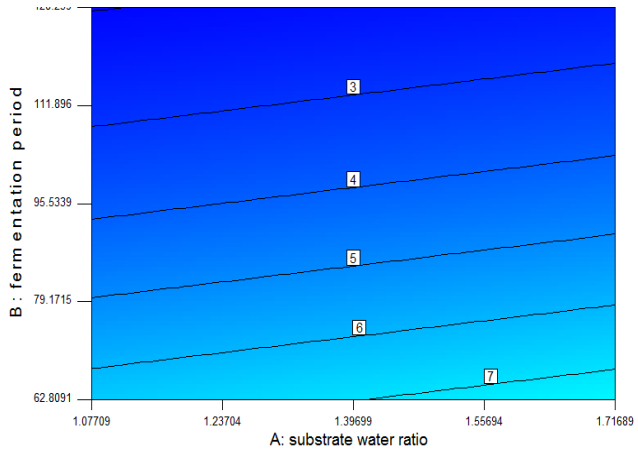

Figure 3: Effect of fermentation period and substrate water ratio on EPG production.

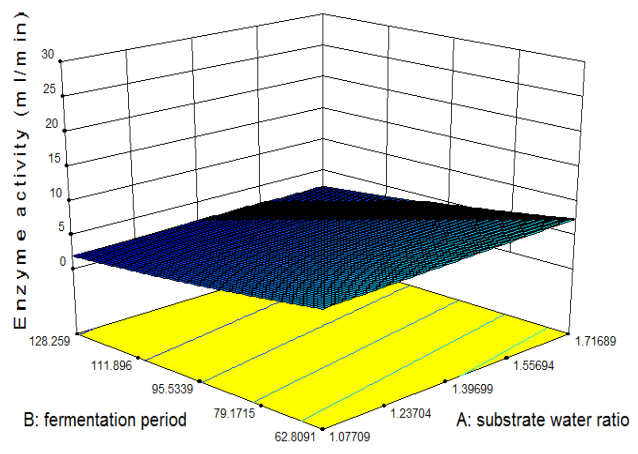

Figure 4: Effect of fermentation period and substrate water ratio on EPG production.

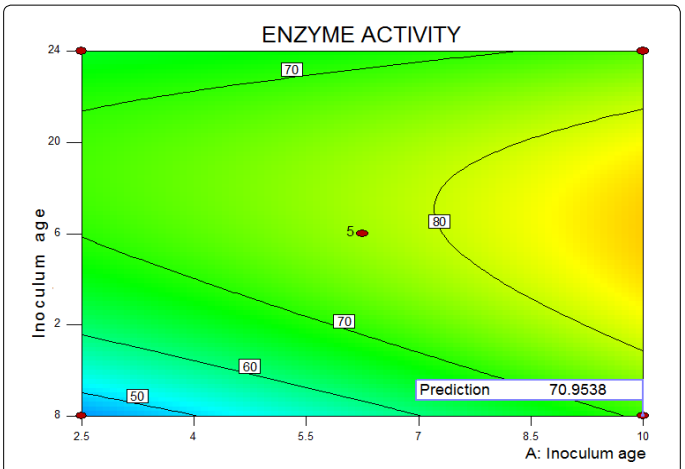

Figure 5: Effect of inoculum size and inoculum age on EPG production.

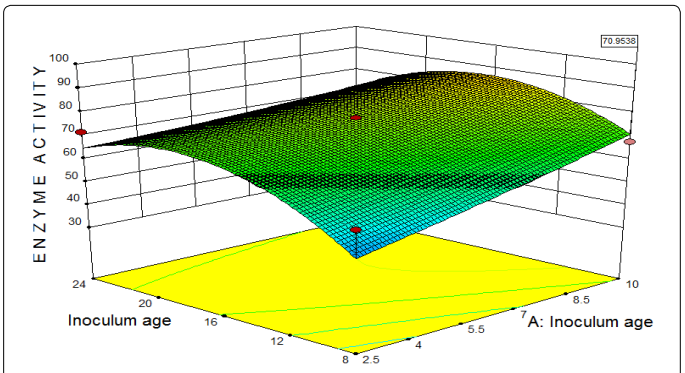

Figure 6: Effect of inoculum size and inoculum age on EPG production.

Carbon sources and nitrogen sources

Different carbon sources (fructose, sucrose, lactose, galactose) and nitrogen sources (Ammonium sulfate, Ammonium chloride, Ammonium nitrate, calcium nitrate) was added in reaction mixture and after 24 hour of fermentation period at 60°C their OD was measured. When ammonium sulfate and fructose was added maximum enzyme activity was observed when concentration of 2.5 mg ammonium sulfate and 2.5 mg fructose. Figures 7 and 8 showing combine effect of Ammonium sulfate and fructose.

Similarly combine effect of lactose and ammonium sulfate was study and maximum enzyme activity was observed when lactose concentration was 2.5 mg and ammonium nitrate concentration was 2.5 mg (Figures 9 and 10).

Combine effect of galactose and ammonium sulfate was observed maximum enzyme activity was observed when ammonium sulfate and galactose concentration was 0.085 mg and galactose concentration was 1.5 mg (Figures 11 and 12). Calcium nitrate and sucrose both have positive effect on enzyme activity and maximum enzyme production was observed when concentration of sucrose and calcium nitrate was 0.5 mg and 2.5 mg (Figures 13 and 14).

Nitrogen and carbon sources repress the enzyme activity and there was no pronounced effect of different carbon and nitrogen sources except calcium nitrate and sucrose in presence of these sources and amplification in enzyme production occur in presence of calcium

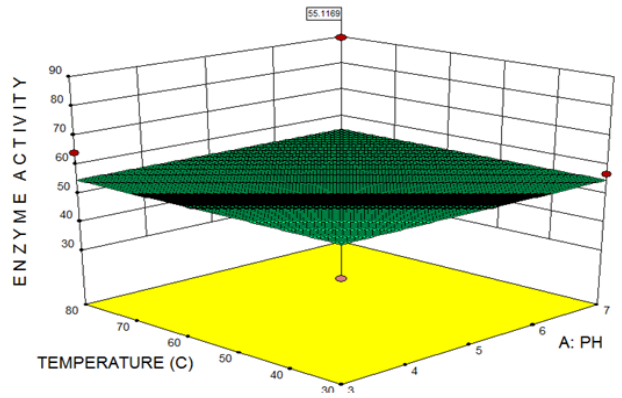

Figure 7: Effect of Temperature and pH on EPG production.

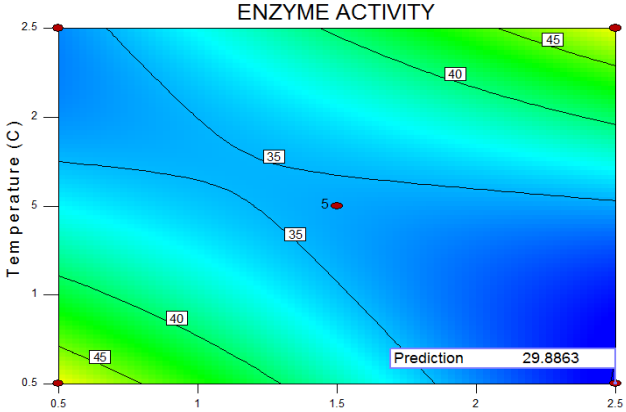

Figure 8: Effect of Ammonium sulfate and fructose on EPG production.

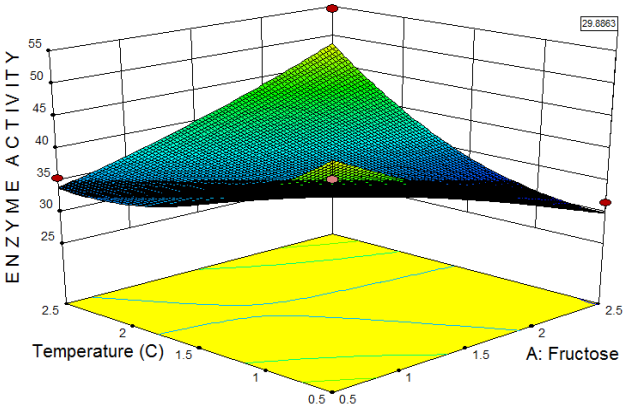

Figure 9: Effect of Ammonium sulfate and fructose on EPG production.

nitrate. When we compare effect of all of the above mention sources among all only in presence of calcium nitrate and sucrose enzyme activity was good. While all other sources decreases the enzyme activity.

Surfactant and mediator

By adding different surfactant (SDS, Tween 80, Tween 20) and mediator(cane molasses changes in enzyme activity was measure mostly these surfactants decrease the enzyme activity except yeast extract and maximum enzyme activity was observe whenever there was increase in concentration of yeast extract and peptone. May be decrease in enzyme activity was observed due to reason that these surfactants was added in combination and they cancel effect of each other and as a result leave negative impact on enzyme production (Figures 15-21).

Result of addition of metal ions

After adding different metal ions in crude extract their effect on enzyme production was measured. Maximum enzyme activity was observed whenever there was high concentration of calcium chloride. Figures 22 and 23 shows combine effect of $Mgcl_2$ and $Cacl_2$.

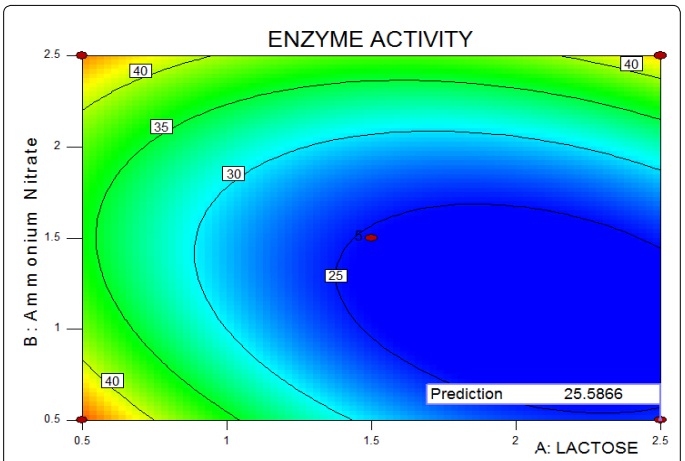

Figure 10: Effect of lactose and ammonium nitrate on EPG production.

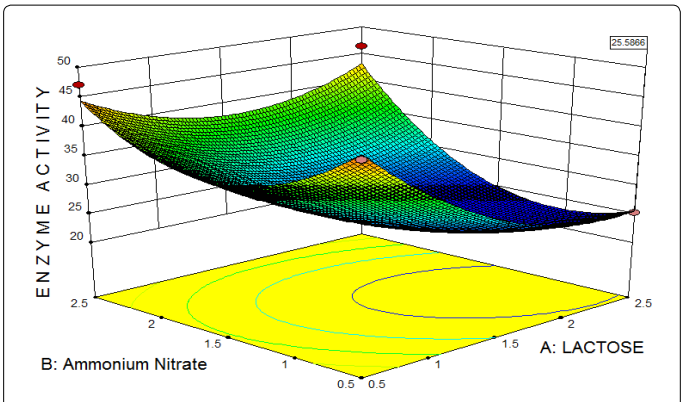

Figure 11: Effect of lactose and ammonium nitarate on EPG production.

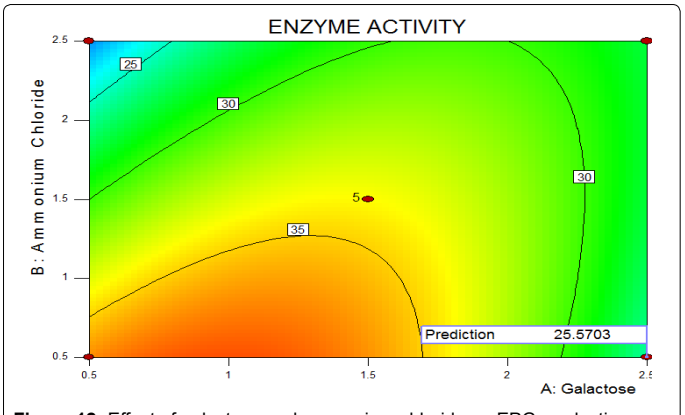

Figure 12: Effect of galactose and ammonium chloride on EPG production.

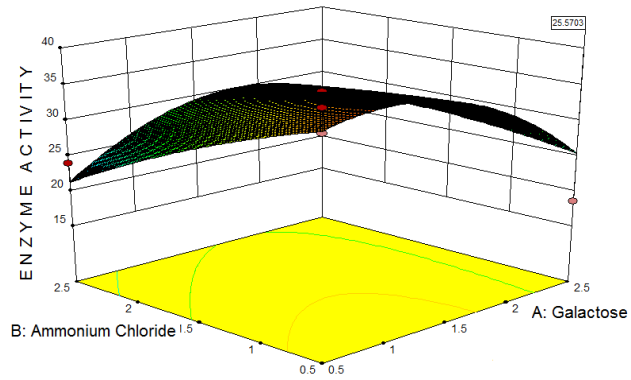

Figure 13: Effect of galactose and ammonium chloride on EPG production.

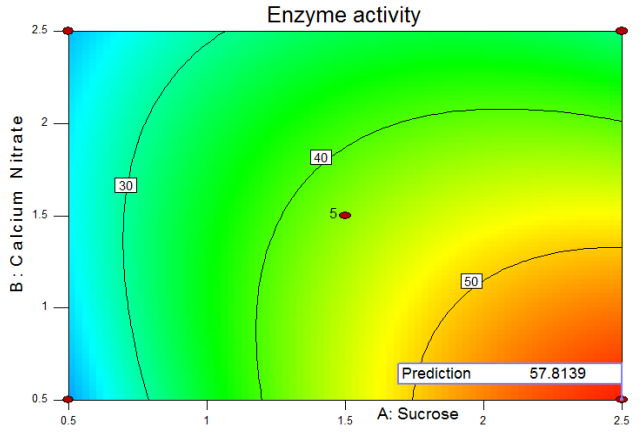

Figure 14: Effect of Sucrose and Calcium Nitrate on EPG production.

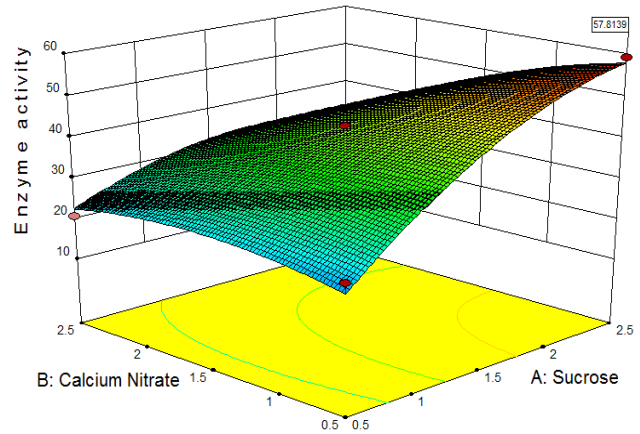

Figure 15: Effect of SDS and Peptone on EPG production.

Enzyme Purification

Enzyme partial purification was done by using Ammonium sulfate, Ethanol, Methanol, and isopropanol. Maximum enzyme activity was observed with 60% Ammonium sulfate and 40% crude extract maximum enzyme activity was 77.4 U/ml/min and protein content was 1.168 (Table 13 and Figure 24).

Partial Purification with Organic Solvents

Partial purification was done by using three organic solvent (methanol, ethanol and isopropanol). Maximum precipitates were observed at 40% ethanol and 60% crude extract maximum enzyme was 94.62 and total protein content was 1.01 (Table 14 and Figure 25).

Methanol gives maximum enzyme activity at 30% methanol and 70% crude extract. Maximum enzyme activity was 1.11 with protein content of 0.89 (Table 15 and Figure 26). Maximum enzyme activity was observed by using isopropanol. Enzyme activity was 116.7 with protein content of 0.31 (Table 16 and Figure 27).

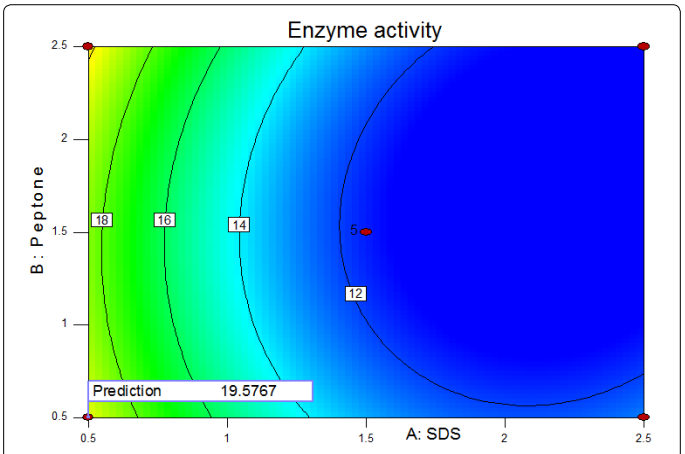

Figure 16: Effect of SDS and petone on EPG production.

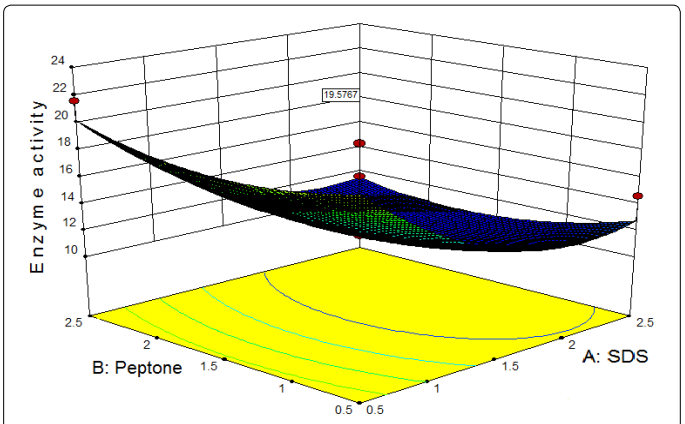

Figure 17: Effect of Cane molasses and Tween 80 on EPG production.

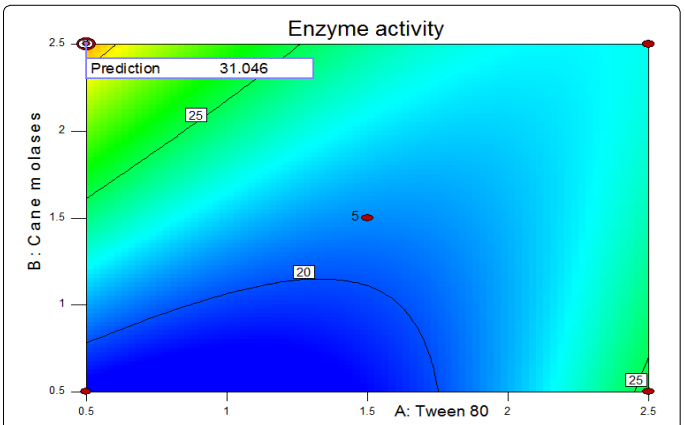

Figure 18: Effect of Cane molasses and Tween 80 on EPG production.

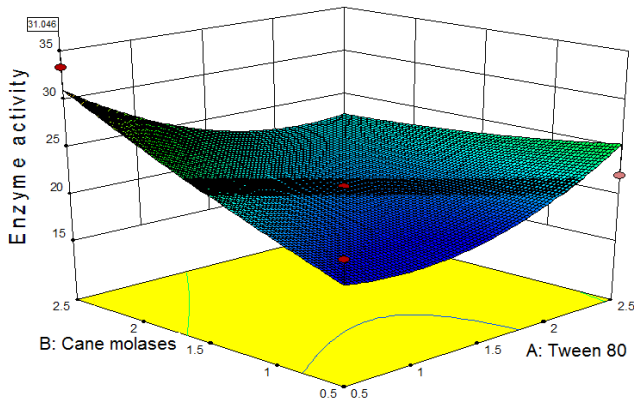

Figure 19: Effect of Tween 20 and yeast extract on EPG production.

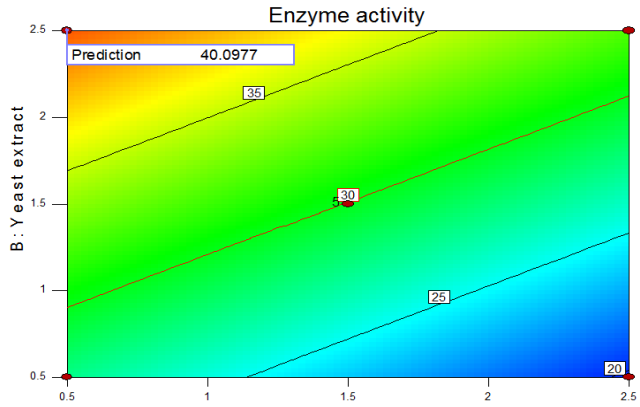

Figure 20: Effect of Tween20 and yeast extract on EPG production.

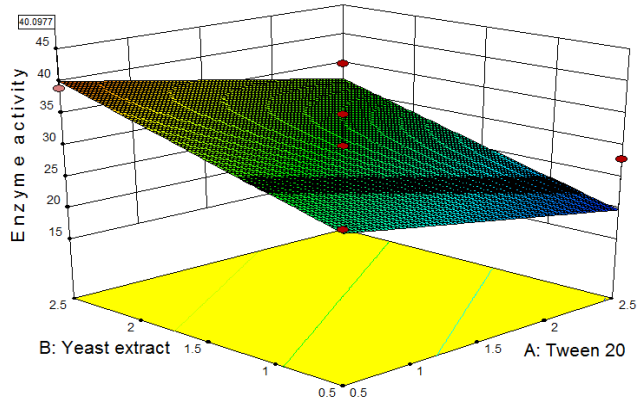

Figure 21: Effect of Tween20 and yeast extract on EPG production.

Gel Filtration

When partially purified enzyme was run through gel filtration and different friction were collected. Friction no 11, 12 and 13 show highest enzyme activity. Friction 13 shows maximum enzyme activity of 139.91 with protein content of 0.33 (Tables 17, 18, Figures 28 and 29).

Characterization of Endopolygalacturonase

Effect of pH on endopolygalacturonase activity

Maximum enzyme activity was observed by using buffer of PH 5. PH 4.8 and 5 show enzyme activity in almost same range so optimum pH for endopolygalacturonase is 5. Enzyme was stable up to pH 9 (Table 19 and Figure 30). During enzyme assay crude enzyme was placed at different temperatures for 30 minutes and maximum enzyme activity was shown at temperature of 60°C.

Study of Enzyme Kinatics

Effect of substrate concentration on EPG : There was increase

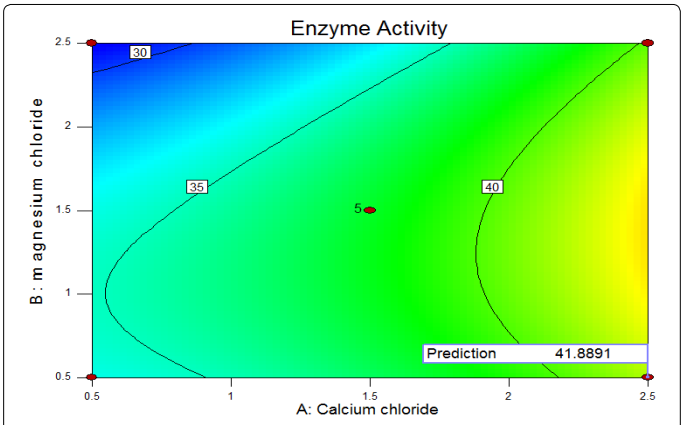

Figure 22: Effect of Magnesium chloride and calcium chloride on EPG production.

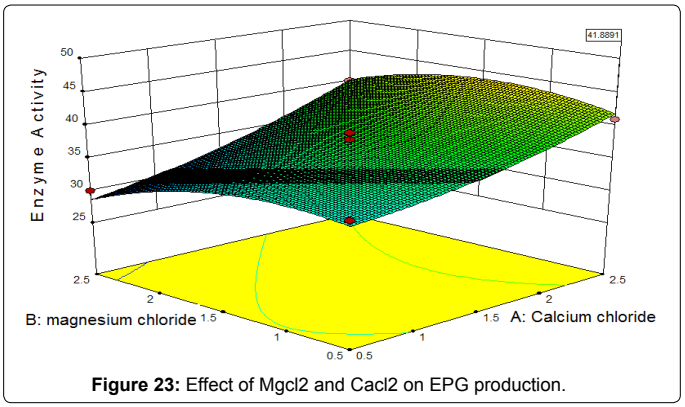

Figure 23: Effect of Mgcl2 and Cacl2 on EPG production.

Source	DF	SS	MS	F-Value	P-Value
Concentration of substrate	2	12956	6478.2	22.73	0.00
OD	21	5984	285.0		
enzyme activity	23	18941			
S=16.8807 R2=65.40%					

Table 13: ANOVA for Enzyme purification with Ammonium sulfate.

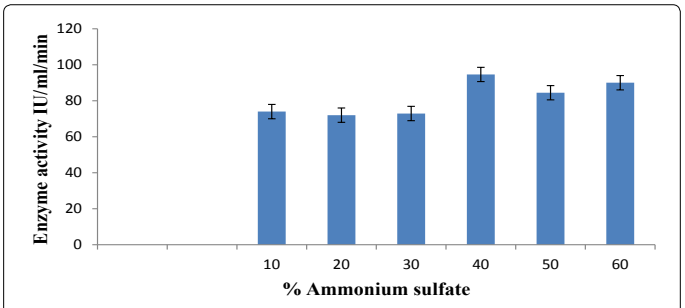

Figure 24: Partial purification of EPG by Ammonium sulfate precipitation.

Source	DF	SS	MS	F-Value	P-Value
Concentration of substrate	3	22412	7470.6	37.60	0.00
OD	20	3974	198.7		
enzyme activity	23	26386			
S=14.0957 R^2=84.94%					

Table 14: ANOVA for Enzyme purification with Ethanol.

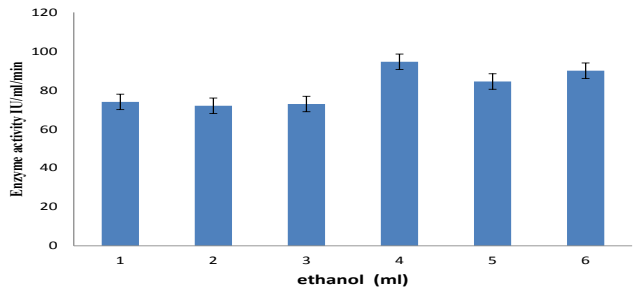

Figure 25: Partial purification of EPG by using ethanol.

Source	DF	SS	MS	F-Value	P-Value
Concentration of substrate	3	32631	10877.1	58.89	0.00
OD	20	3694	184.7		
enzyme activity	23	36325			
S=13.5903 R^2=89.83%					

Table 15: ANOVA for purification of enzyme with methanol.

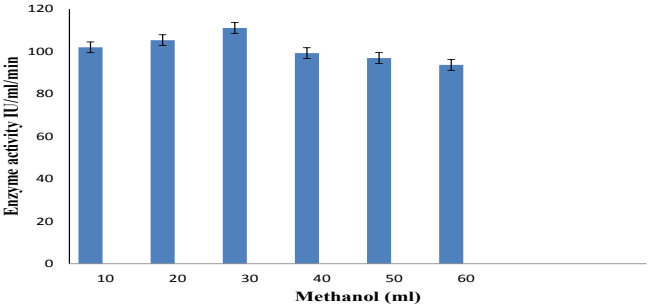

Figure 26: Partial purification of EPG with methanol.

Source	DF	SS	MS	F-Value	P-Value
Concentration of substrate	3	24984	8328.0	30.74	0.00
OD	20	5419	271.0		
enzyme activity	23	30403			
S=16.4607 R^2=82.18%					

Table 16: ANOVA for Enzyme purification with Isopropanol.

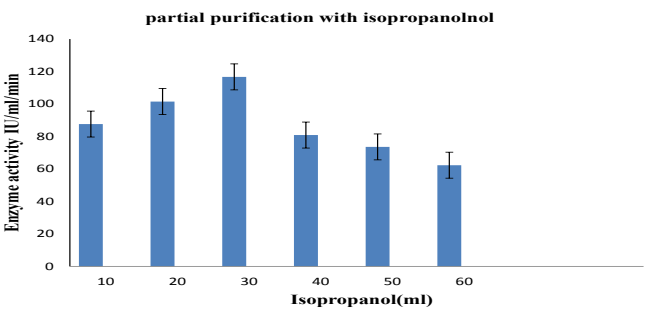

Figure 27: Partial purification of EPG with isopropanol.

Source	DF	SS	MS	F-Value	P-Value
Concentration of substrate	1	20637	20637	16.96	0.00
OD	34	41373	1217		
enzyme activity	35	62009			
S=34.883 R^2=31.32%					

Table 17: ANOVA for Gel Filtration.

S.NO	Volume in ml	OD	E.A	Total protein
Crude extract	1 ml	0.991	65.7	2.98
NH_4SO_4	1 ml	1.427	94.62	1.01
Methanol	1 ml	1.676	111.1	0.89
Isopropanol	1 ml	1.76	116.7	0.31
Gel filtration	1 ml	2.11	139.91	0.33
Ethanol	1 ml	1.168	77.44	0.95

Table 18: Comparison of all purification steps.

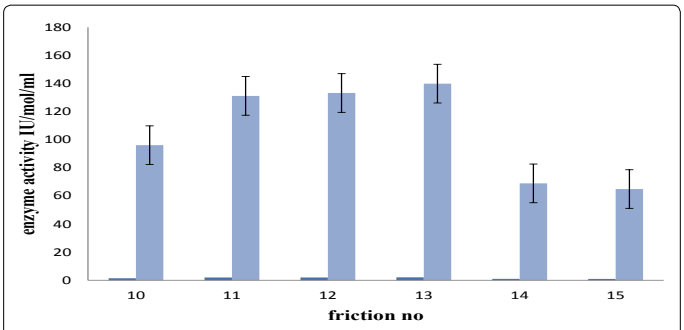

Figure 28: Purification of EPG by using Gel filtration.

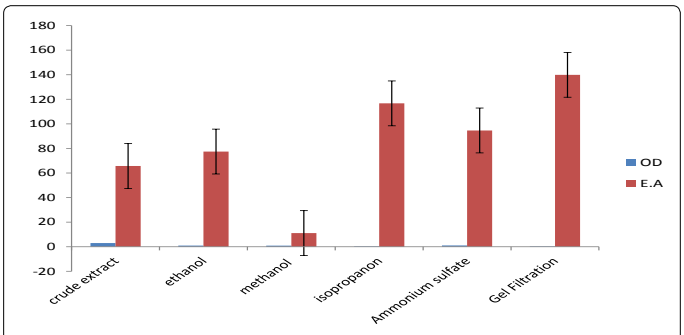

Figure 29: Comparison of different purification steps for EPG production.

Source	DF	SS	MS	F-Value	P-Value
Concentration of substrate	2	17447	8723.37	93.28	0.00
OD	18	1683	93.52		
enzyme activity	20	19130			
S=9.67073 R^2=91.20%					

Table 19: ANOVA for Effect of pH on Enzyme production.

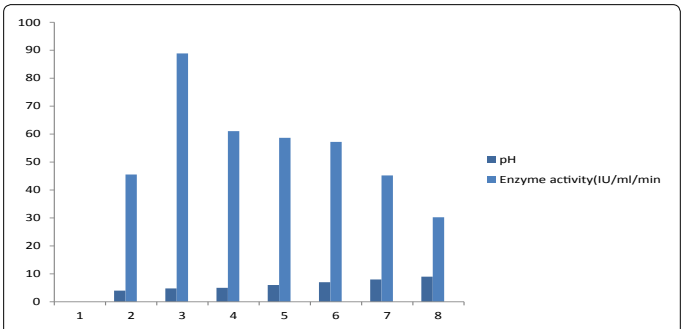

Figure 30: Effect of pH on Enzyme production.

Source	DF	SS	MS	F-Value	P-Value
Concentration of substrate	2	3	3.74	67	0
OD	15	6	0.1318	10	
enzyme activity	17	9	8.73		
S=5.48673 R^2=47.45%					

Table 20: ANOVA for effect of different substrate concentrations on Enzyme production.

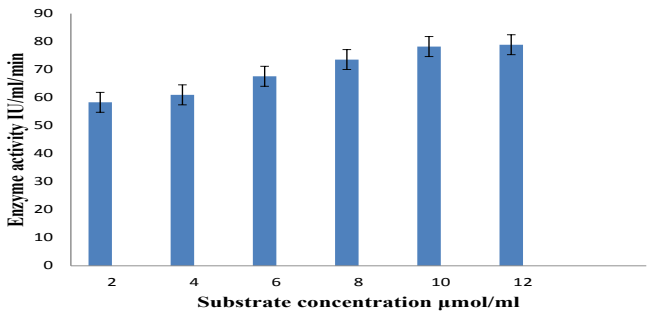

Figure 31: Effect of substrate concentration on Enzyme production.

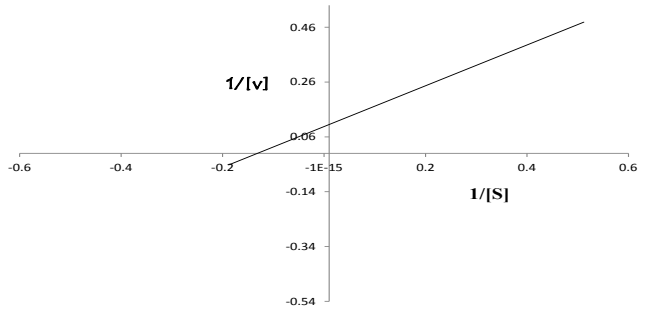

Figure 32: Lineweaver-Burk graph of EPG.

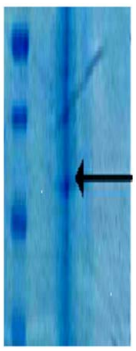

Figure 33: SDS PAGE

in enzyme activity along with increase of substrate concentration. Endopolygalacturonase activity increases when substrate concentration increases from 2 mmol to 4 mmol there was uniform increase in enzyme activity up to 10 µmol of substrate and then there was less steep increase from 10 mmol to 12 mmol (Table 20 and Figure 31)

V_{max} and Km for endopolygalacturonase

Vmax for endopolygalacturonase was and Km was 1.21 mg/ml was 2423 mol/min/mg respectively.

Determination of molecular mass of EPG

Partially purified enzyme after gel filtration was run on SDS-PAGE gave a single band of protein viewing its mass at 67 kDa. It was recognized as Endopolygalacturonase by enzyme assay (Figure 32,33).

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