Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2013, 5(1):336-347

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Optimization and characterization of polygalacturonase enzyme produced by gamma irradiated *Penicillium citrinum*

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ABSTRACT

The activity of polygalacturonase(PGase) produced by Penicillium citrinum was studied in solid state fermentation (SSF) using sugar beet pulp as substrate. A full factorial design was used to study the effect of 5 variables (yeast extract , pH , inoculum size, incubation period, and incubation temperature) on polygalacturonase production. Maximum polygalacturonase (PGase) production (132 U/g dry fermented substrate(U/gdfs) was obtained in 8 days at 30°C and pH 5.5 with yeast extract as the best nitrogen source (1.2%) inoculated with 1 ml of spore suspension (1.8×10^5 spores/ml). Using the optimized conditions of factorial design, maximum PGase production has been obtained by using 0.7 kGy of gamma radiation with an activity 152.2U/gdfs as compared to the parent strain (un-irradiated). The enzyme was partially purified using 75% ammonium sulphate precipitation, dialysis, and gel filteration chromatography on sephadex G-100. The optimum pH and temperature of the enzyme were found to be 6.0 and 40°C respectively. The enzyme was found to be stable in the pH range 4-8 and showed high stability at temperature range 20-60°C. The effect of metal ions on enzyme activity was investigated. It was found that Mg^{+2}, and Zn^{+2} stimulated PGase activity.

Keywords: *Penicillium citrinum ;* Polygalacturonase(PGase) ; Factorial design ; gamma radiation.

INTRODUCTION

Currently, the fundamental exploitation of agricultural and food wastes, which participate in pollution, is the controlled biological degradation of the wastes by microorganisms for the production of valuable compounds such as proteins, polysaccharides, oligosaccharides, vitamins, hormones, enzymes and other raw materials for medicinal and industrial uses [1].

Pectinases are widely used in the disintegration of plant tissues in the fruit and vegetable processing industries, for increasing juice extraction, for decreasing viscosity of the concentrates and for making pectic complexes soluble to complete sedimentation and clarification of juices [2, 3] and wines [4].

Pectinolytic enzymes are classified according to their cleavage of the galacturonan part of the pectin molecule.They can be distinguished into pectinesterases (PE, E.C 3.1.1.11), which modify pectin esters into low methoxyl pectins or pectic acid and pectin deploymerases, which split the glycosidic linkages between galacturonosyl (methyl ester) residues. Polygalacturonases (PGs) split glycosidic linkage next to free carboxyl groups by hydrolysis, while pectate lyases (PALs) split glycosidic linkages next to free carboxyl groups by β- elimination. Both endo types of PGs and PALs (E.C3.2.1.15 and E.C 4.2.2.2, respectively) are known to randomly split the pectin chain. Exo-polygalacturonases (exo-PGs, E.C 3.2.1.67) release monomers or dimmers from the non-reducing end of the chain, whereas exopectate lyases (exo-PALs, E.C 4.2.2.9) release unsaturated dimers from the reducing end. Highly

methylated pectins are degraded by endo-pectin lyases (endo-PL,E.C 4.2.2.10) and also by a combination of PE with PG or PAL [5, 6].

The ability to synthesize pectinolytic enzymes is very common in groups of microorganisms, but fungi are preferred on the industrial scale. This is because about 90% of the enzymes produced may be secreted in the culture medium [7]. PGs, the most abundant and extensively studied of the pectinolytic enzymes, typically exist in multi-gene families and may have both endo [8] and exo activities [9].

The synthesis of pectolytic enzymes by microorganisms has been reported to be highly influenced by the componenets of the growth medium. Most extracellularly induced enzymes are known to be synthesized in higher quantities when inducers are present in the cultivation medium [10].

The production of pectolytic enzymes using different substrates and the effect of physical parameters such as temperature, aeration rate and type of fermentation were investigated [11] and [12].Pectolytic enzymes have been reported to be induced by several substances. In many cases pectin itself has been used.Other investigators had used complex media such as beet sugar, wheat bran, ground nut meal, citrus fruit peels, etc. [13, 14].

Polygalacturonases appeared to be the most frequently encountered pectic enzymes. They are formed in the majority of plant tissues particularly in ripening fruits. Also many plant pathogenic and saprophytic microorganisms produced polygalacturonases [15] ,[16].Most fungi that exhibited pectolytic activity produce polygalacturonases either as sole pectic enzymes as observed in *Sacchromyces fragilis*, or in association with either or both pectinmethylesterases and pectinlyase [17]. The critical role of these enzymes in the degradation of the host middle lamella and cell walls, leading to plant tissues merceration and cellular death had been documented [18, 16 ,19]. The infection of cotton seedlings by *Rhizoctonia solani*, soft rot yam and sweet potato, brown rot of apple by *Monilinia* species and deterioration of tomato by *Botryoplodia theobromae* Pat have been reported to produce polygalacturonases and other cell wall degrading enzymes [20, 21,22,23, 17 ,16]. The culture filterates of *Collectotrichum lagnarum* exhibited polgalactuonase activity [24].

Pectinase production from microorganisms has been reported under both submerged and solid state fermentations. Solid state fermentation (SSF) is defined as the cultivation of microorganisms on moist solid substrate, preferably on agricultural residues like wheat bran, rice husk, etc. that can in addition, be used as carbon and energy source. SSF takes place in the absence and near absence of free water thus being close to the natural environment to which microorganisms are adapted [25].

The optimization process condition under SSF is generally done by varying one condition at a time approach [26] However, these strategies are laborious and time consuming especially for a large number of variables. Full factorial design (FFD) is an experimental strategy for seeking the optimum conditions in a multivariable study [27, 28]. The optimization process searches for a combination of parameter levels that simultaneously satisfy the requirements placed (i.e. optimization criteria) on each one of process parameters and response.

The most important prerequisite for microbial production is the isolation of enzyme hyper-productive mutants, which are obtained by subjecting parental strains to successive mutagenic treatments. Therefore, *P.citrinum* isolate was exposed to gamma radiation treatment to determine the effect of irradiation exposure on pectinase synthesis.

This study was undertaken to evaluate the production of polygalacturonase (PG) (E.C., 3.2.1.15.) by *P.citrinum* under SSF using sugar beet pulp as the substrate, optimization of the fermentation parameters [incubation period, medium pH, yeast extract, inoculums size and temperature] were also taken into consideration by applying full factorial design(FFD). This work also aimed to irradiate the wild strain to certain doses certain doses of gamma radiation to select a high producer isolate. Also, characterization of the partially purified enzyme has been investigated.

EXPERIMENTAL SECTION

Microorganisms

Fungal strains (from 1-14 as mentioned in table1) were provided from Pharmaceutical Microbiology Lab, Drug Radiation Research Department, National Center for Radiation Research &Technology (NCRRT)Nasr City-Cairo-Egypt. Fungal colonies were maintained on potato-dextrose agar medium, stored at 4ºC and freshly subcultured every four weeks.

Screening of Microorganisms for pectinolytic activity

Fourteen fungal strains were assayed for pectinase activity using sugar beet pulp containing medium. Ten grams of sugar beet pulp, which act as carbon source as well as pectinases inducer [29], were placed in 250ml Erlenmeyer flask and moistened with 20 ml distilled water in which the following nutrients were dissolved (0.4g Na_2HPO_4 + 0.08g KH_2PO_4+ 0.5% yeast extract(Oxoid)) and autoclaved at 120°C for 30 min. Each flask was inoculated with 1 ml of spore suspension (about 1.8×10^5 spores/ml) prepared in Tween 80, 0.1% v/v and incubated at 25ºC for 7 days at static state. Visual observations regarding growth were made on each day and samples were drawn periodically to assess the enzyme production. Enzyme was extracted with 50ml of 0.2M sodium acetate buffer (pH 5.5) and filtered. The filtrate was centrifuged at 5000g for 15 min at 4 ºC and used as source of the crude enzyme. Pectinase activity in the filtrate was assayed according to the method reported by [30].

Polygalacturonase assay

Enzyme activity was determined using pectin as substrate. The reaction mixture, containing equal amounts of 1% pectin prepared in sodium acetate buffer(o.2 M; pH5.5) and suitably diluted crude enzyme, were incubated at 50ºC in water bath for 10 min. The reaction was stopped with 0.5ml dinitrosalycyclic acid solution (DNS) [30] after which the mixture was boiled for 5 min and cooled. The colour density has been recorded at 540nm using (JASCO V-560 UV-visible spectrophotometer). The amount of reducing sugar released was quantified using glucose as standard. The enzyme activity (U/gdfs) was calculated as the amount of enzyme required to release one micromole (1μmol) equivalent of galactouronic acid per minute.

Protein assay

The protein content of the crude enzyme was determined by the Folin method of Lowery et al [31] using Bovine Serum Albumin (BSA) as standard.

Application of factorial design for optimization of polygalacturonase production by P.citrinum under solid state fermentation.

A full factorial two-level design(2^5) was performed to confirm the optimization of independent factors level by taking incubation period (7 and 8 days), pH (5.0 and 5.5), inoculum size ($1.8\times10^5$and 3.6×10^5 spores/ml) ,temperature (25 and 30ºC) and nitrogen content(0.5% and 1.2%) in this study.The level of independent factors were optimized by studying each factor in the design at two different levels(-1, and +1),Table 2).The minimum[coded as(-1)] and maximum [coded as(+1)] range of experimental values of each factor used. A set of 32 experiments was performed. The quality of fitting the first-order model was expressed by the coefficient of determination R^2 and its statistical significance was determined by F-test. The sugar beet pulp had been used as the sole carbon source.

Effect of gamma radiation on polygalacturonase production

All irradiation processes were carried out at the National Center for Radiation Research and Technology (NCRRT) Nasr City-Cairo-Egypt. Irradiation facility was Co-60 Gamma chamber 4000-A india. The source gave average dose rate 3.696 kGy/hr at the time of experiments. The fungal strain was grown on PDA for 8days and subjected to gamma radiation at doses (0.1, 0.2, 0.5, 0.7, 1, 1.5 and 2 kGy). The tested cultures have been investigated for its enzyme activity.

Partial purification of crude enzyme

i-Ammonium sulphate precipitation

The cell free filtrate was brought to 75% saturation by mixing with ammonium sulphate slowly with gentle agitation and allowed to stand for 24 hrs at 4ºC. After the equilibration , the precipitate was removed by centrifugation (5000 rpm at 4°C for 15 min).The obtained precipitate has been dissolved in 50ml of 0.2M sodium acetate buffer, pH (5.5) to be dialyzed.

ii- Desalting by dialysis

According to Karthik et al [32], the precipitate was desalted by dialysis by the following protocol: 10cm dialysis bag was taken and activated by rinsing in distilled water. One end of the dialysis bag is tightly tied and the obtained precipitate is placed into the bag. Then the other end of the dialysis bag is tightly tied to prevent any leakage. After that, dialysis bag has been suspended in a beaker containing 0.2M sodium- acetate buffer (pH 5.5) to remove low molecular weight substances and other ions that interfere with the enzyme activity.

iii -Gel filtration chromatography

The dialyzed enzyme fraction was further purified by gel filteration chromatography [33]. It was loaded on sephadex G-100 column and eluted with 0.2M sodium- acetate buffer (pH 5.5) with the flow rate of 20 ml/hr. Total 40 fractions(each 5 ml) were subsequently collected and its protein content was measured by using spectrophotometer

at λ280. The fractions that have high absorbance at λ280 were collected and evaluated for its PGase activity. The fractions showing higher enzyme activity were collected together for further characterization.

Enzyme characterization:

Effect of different pH values:

a-On enzyme activity

The enzyme PGase activity was determined in different buffers using sodium acetate (pH 4.0, 5.0),sodium citrate (pH 6.0, 7.0), and sodium phosphate (pH 8.0).The relative activities were based on the ratio of the activity obtained at certain pH to the maximum activity obtained at that range and expressed as percentage.

b- On enzyme stability

The pH stability of the enzyme was determined by exposing the purified enzyme first at various pH values (4 to 8) using different pH buffer solutions as mentioned previously for 2 hours. Afterwards, aliquots of the mixtures were taken to measure the residual polygalacturonase activity (%) with respect to control, under standard assay conditions.

Effect of different temperatures:

a-On enzyme activity

The optimum temperature was determined by incubating each reaction mixture at variable temperatures (20-70ºC). The relative activities (as percentages) were expressed as the ratio of the purified polygalacturonase obtained at certain temperature to the maximum activity obtained at the given temperature range.

b-On enzyme stability

Thermal stability of the enzyme was investigated by measuring the residual activity after incubating the enzyme at various temperatures ranging from 20 to 70°C for 10 min.

Effect of different metal ions on the activity of polygalacturonase enzyme produced by P.citrinum.

For determination the influence of Ca^{+2}, EDTA, Cu^{+2}, Zn^{+2}, Mg^{+2}, Ba^{+2} and Co^{+2} on PGase activity. The listed ions were added to the reaction mixture at concentration (1mM). Activity without added metal ions was taken as 100% activity.

RESULTS AND DISCUSSION

Screening of Polygalacturonase Producing Microorganisms:

Table (1) showed the values of polygalacturonse activity produced by the tested fourteen fungal strains. The results clearly showed that *P.citrinum* was the best producer of polygalacturonase enzyme with the maximum value of 129.2U/gdfs, followed by *P.brevi-compactum* that yield 123.2U/gdfs of enzyme activity. These results confirmed those of Fontana et al [34]. They concluded that the high value of the enzyme could be due to presence of substrate rich pectin that increases enzyme activity. Also Favela-Torres et al [35] evidenced the production of PGase by *Aspergillus*, *Fuasrium,Penicillium*, *Thermoascus*, *Lentinus* species on various substrates during solid substrate fermentation and submerged fermentation ,owing to hydrolysis of pectin and pectin containing materials for fungal growth. It was reported an increase in the level of pectinase production at pH 5.0, temperature 34°C, and inoculums size of 1×10^5 spores/ml [36].

Table (1).Screening of fungal strains for production of polygalacturonase enzyme

F ungal strains	Enzyme activity (Units /gdfs*)
Aspergillus niger 1	86.2 ± 2.2
Aspergillus niger 2	115.3± 1.9
Aspergillus niger 3	92.3± 1.1
Aspergillus niger 4	96.3± 1.05
Penicillium chrysogenum	121.4± 1.14
Sclortium rolfsii	115.7± 1.9
Gliocladium virens	95.7± 2.1
Pleurotos ostreatus	102.4± 2.1
Rhizoctonia solani	83.1± 2
Alternaria alternata	86.2± 2
Penicillium citrinum	129.2±2
Trichoderma veridi	62.1± 2.1
Aspergillus oryzea	96.8± 1.9
Penicillium brevi-compactum	123.2± 2.2

**- gdfs: Units of pectinase per gram dry fermented substrate*

Optimization of cultural conditions using full factorial design(Experimental statistical analysis design)

The experimental conditions and the results of enzyme activity are shown in Table2. In the present study, the maximum value of PGase activity obtained was 132U/gdfs which was observed in the 14th run with the specific conditions of initial pH 5.5, incubation period of 8 days, inoculums size (1.8×10^5 spores/ml),N content (1.2%) and temperature (30°C). To construct a first model that can predict the PGase activity (dependent variable) as a function of physical parameter (independent variables included N content, temperature, pH, inoculums size and incubation period). ANOVA analysis comprises classifying and cross classifying statistical results and testing whether the means of specified classification differ significantly. This was carried by Fisher's statistical test for the analysis of variance. The fisher's F-test showed a value of 2.84 which is much greater than that of the tabulated F (0.02) and that demonstrates that the model terms are significant (Table 3).

The model equation fitted by regression analysis is given by:
predicted= 78.55+8.19 N content+2.35 incubation period- 12.25 inoculum size-2.11 pH+ 14.95 temperature -0.38(N content *incubation period) -7.42(N content *inoculum size) -0.55 (incubation period*inoculum size) +5.85(N content *pH) +1.21(incubation period*pH) -3.61(inoculum size*pH)+1.74(N content*temperature)+0.67(incubation period*temperature)+6.37(inoculum size*temperature)-2.65(pH*temperature).

The model determination coefficient (R^2=0.74) suggested that the fitted model could explain 74% of the total variation.This implies a satisfactory representation of the process by the model. The coeffecient of determination R^2 value always lies between 0 and 1.The closer the value of R^2 is to 1.0,the stronger the model and the better it predicts the response (Fig 1).

Both the T-value and P-value statistical parameters were used to confirm the significance of factors studied. The t-value that measured how large the coefficient is in relationship to its standard error. The P-value is the chance of getting a larger –t-value (in absolute value) by chance alone. The larger the magnitude of the-t-value and smaller the P-value, more significant is the corresponding coefficient [37].

Table (4) showed that inoculum size, N content and temperature had significant positive effect on PGase production; however, the interaction between them was not significant. Conversely, pH and incubation period displayed negative effect. The results of the present study showed that the maximum production of PGase have been obtained at the inoculum size (1.8×10^5 spores/ml)for SSF, but decrease with the increase in the inoculum size. This view of result is supported by Ghanem et al [38] who concluded that optimum inocula density is an important consideration for SSF process since over crowding of spores can inhibit growth and development. It is worth mentioned that higher inocula levels besides increasing spores density, increase water content of the medium as well. Temperature is one of the critical parameter that might be controlled to get the optimum production of the enzyme. Our results revealed that the optimum temperature that gave high polygalacturonase activity was 30°C .This is in agreement with Kitpreechavanich [39] who evidenced that temperature considered to be a significant controlling factor for enzyme production. Patil and Dayanand [36] explained this by pointing out that temperature is known to influence the metabolic rate of the organism involved in the process, and this determines the amount of end product. Temperature in SSF is maintained at 30°C, as it cannot be precisely controlled. This could be due to due that solid state fermentation has solid substances have limited heat transfer capacity.

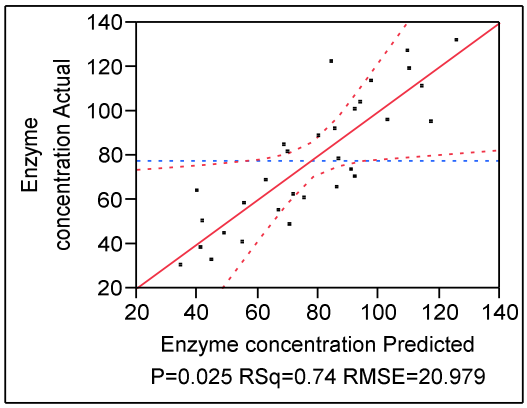

Fig 1.Plot of predicted versus actual polygalacturonase production

Our study clarify that (N content as yeast extract) exert a significant positive effect on polygalacturonase production at 1.2% concentration. This could be attributed to that yeast extract served as an inducer of exopectinase by *P.citrinum* [40].

Table (2): Effect of the variables and their interactions in the 2^5 factorial design on polygalacturonase production by *P.citrinum* under solid state fermentation.

Trials	Response(Enzyme production) (U/gdfs*)	Factors				
		N content(%)	Incubation period(days)	Inoculum size (spores/ml)	pH	Temperature (ºC)
1	86.6	-	+	+	-	+
2	103.7	+	+	+	-	+
3	113.6	-	-	+	-	+
4	70.3	+	-	+	-	+
5	100.8	-	+	-	-	+
6	111.5	+	+	-	-	+
7	65.9	-	-	-	-	+
8	119.4	+	-	-	-	+
9	60.9	-	+	+	+	+
10	73.5	+	+	+	+	+
11	55.6	-	-	+	+	+
12	122.4	+	-	+	+	+
13	88.9	-	+	-	+	+
14	132.0	+	+	-	+	+
15	81.9	-	-	-	+	+
16	94.8	+	-	-	+	+
17	58.2	-	+	+	-	-
18	44.7	+	+	+	+	-
19	40.5	-	-	+	-	-
20	50.1	+	-	+	-	-
21	62.1	-	+	-	-	-
22	78.4	+	+	-	-	-
23	84.5	-	-	-	-	-
24	91.9	+	-	-	-	-
25	64.0	-	+	+	+	-
26	38.7	+	+	+	+	-
27	30.4	-	-	+	+	-
28	33.1	+	-	+	+	-
29	48.8	-	+	-	+	-
30	127.2	+	+	-	+	-
31	68.6	-	-	-	+	-
32	97.8	+	-	-	-	-

**U/gdfs: unit/gram dry fermented substrate*

Table3: ANOVA for PG production in solid state fermentation

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	15	18897.609	1259.84	2.8624
Error	15	6601.935	440.13	**Prob > F**
C. Total	30	25499.544		0.0250*

Table (4): Regression coefficient for polygalacturonase activity under different physical parameters

Predictor	Coef	Std Error Coeff	t Ratio	Prob>|t|
Intercept	78.552734	3.822781	20.55	<.0001*
Yeast extract(0.4,1)	8.1972656	3.822781	2.14	0.0488*
Incubation period(7,8)	2.3464844	3.822781	0.61	0.5485
Inoculm size(1.8,3.6)	-12.25977	3.822781	-3.21	0.0059*
pH(5,5.5)	-2.108984	3.822781	-0.55	0.5893
Temp(25,30)	14.958984	3.822781	3.91	0.0014*
Yeast extract*Incubation period	-0.383984	3.822781	-0.10	0.9213
Yeast extract*Inoculm size	-7.427734	3.822781	-1.94	0.0710
Incubation period*Inoculm size	-0.553516	3.822781	-0.14	0.8868
Yeast extract*pH	5.8589844	3.822781	1.53	0.1462
Incubation period*pH	1.2097656	3.822781	0.32	0.7560
Inoculm size*pH	-3.608984	3.822781	-0.94	0.3601
Yeast extract*Temp	1.7410156	3.822781	0.46	0.6553
Incubation period*Temp	0.6777344	3.822781	0.18	0.8617
Inoculm size*Temp	6.3714844	3.822781	1.67	0.1163
pH*Temp	-2.652734	3.822781	-0.69	0.4983

Effect of radiation

Data in Table (5) clearly indicated that gamma irradiation potentiate the productivity of the enzyme to its maximum value (152.2U/gdfs) post exposure to 0.7kGy. This enhancement of enzyme production might have been due to either, an increase in the gene copy number or the improvement in gene expression, or both [41, 42, 43, 44]. A gradual decrease in the enzyme activity after exposure to the different doses of 1, 1.5 and 2 kGy was observed. The complete inhibition of the enzyme activity obtained at dose 2kGy. This could be explained by damage or deterioration in the vitals of the microorganism as radiation causes rupturing in the cell membrane .This major injury to the cell allows the extracellular fluids to enter in to the cell.Inversely, it also allows leakage out of ions and nutrients which the cell brought inside.Membrane rupture may result in the death of a cell and decrease in the enzyme synthetic activity due to radiation exposure [45]. El-Batal [46] evidenced that production of pectinases increased by gamma irradiated interspecific hybrids of *Aspergillus.sp* using agroindustrial wastes.

Table (5): Effect of different Radiation doses on polygalacturonase production by Penicillium citrinum

Radiation dose (kGy)	Enzyme activity (U/gdfs)
Unirradiated (control)	132±1.9[a]
0.1	137.8±2.1[b]
0.2	142.2±1.3[c]
0.5	145.5±2.1[d]
0.7	152.2±2.2[e]
1	100.2±2.3[f]
1.5	95.5±2[g]
2.0	N.D[h]

**-gdfs: Units of pectinase per gram dry fermented substrate.*
** Groups with different letters have significant between each other.*
**Groups with same letters have non significant with each other.*
**N.D: not detected.*

Enzyme purification

Polygalacturonase produced by *P.citrinum* was purified using ammonium sulfate precipitation, then underwent dialysis and gel filtration. Results observed in Table (6), indicate a decrease in total protein and total activity, whereas specific activity increased. Ammonium sulphate precipitation (salting out) is useful for concentrating dilute solutions of proteins. It is also useful for fractionating a mixture of proteins, since large proteins tend to precipitate at first while smaller ones will stay in solution. After protein precipitation and taken back in buffer, the solution contained a lot of residual ammonium sulfate which was bound to the protein. One way to remove this excess salt, that is the dialyses of protein. The pectinase enzyme was purified from crude sample by ammonium sulfate fractionation and further dialysis was carried out. The ammonium-dialysate fractionated sample, 75% showed purification fold of 1.2 and the yield of 13% as ammonium sulfate precipitation serves as an intermediate step prior to other chromatographic step used in the purification [47].

In contrast, elution profile of the crude enzyme subjected to gel filtration on sephadex G-100 column chromatography showed purification fold of 1.6 and yield of 0.78 % (Table 6).The obtained poor yield of enzyme in the present study may be explained on the findings of Sharma et al [48]who reported that working with high concentrated protein solutions often leads to loss of yields. Both enzyme activity at 540 nm and protein content at 280 nm were determined for each fraction fig(2). The enzyme activity has been detected between the fraction No.16 to the fraction No.20, while fraction No.10 to the fraction No.13 had no enzyme activity suggesting a number of isoforms of PGase. According to Viniegra-Gonzalez [49] variation in the isoforms of extracellular enzymes obtained by SSF can be attributed to alteration of the water potential(a_w) that results in changes in the permeability of fungal membranes, limitation of sugar transport and presence or absence of inducer. It is even reported that pectinases produced by the same microorganism have exhibited different molecular weights, degrees of glycosylation and specificities. These variations may be due to the post transitional modification of a protein from a single gene or may be the products of different genes[50, 51].

Table(6):Purification of PGase secreted by *P.citrinum*

Purification step	Protein(mg)	Total activity(U)	Specific activity(U/mg)	Purification	Yield(%)
Crude exract	1300	2500	1.9	1	100
$(NH_4)SO_4$	1000	2275	2.3	1.2	13
G-100	720	2192	3.0	1.6	0.78

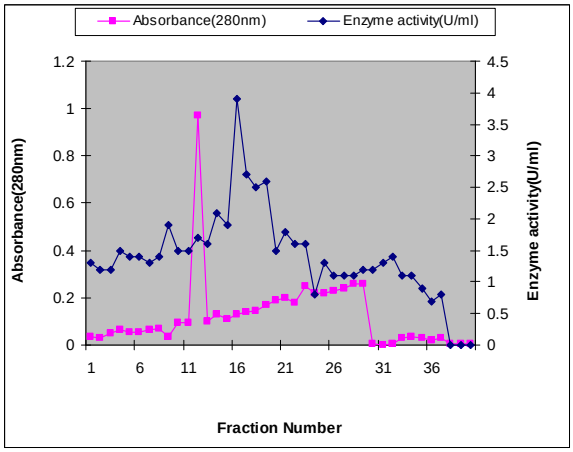

Fig.2.Gel filteration profile of polygalacturonase.

Enzyme characterization

Effect of pH on polygalacturonase activity and stability

The effect of pH on the polygalacturonase activity is presented in Fig 3. As it can be observed, the enzyme was active over a broad pH range, displaying over 60% of its activity in the pH range of 4.0 up to7.0 with an optimum pH of 6.0. Concerning to the PGase at pH 8, the relative activity decreased down up to 57%. This could be attributed to Histidine residues that have ionizable side-chains, increasing the net negative charge on the molecule in the alkaline pH range and leading to repulsion between the strands, resulting in a destabilization of the hydrogen-bond structure of the enzyme[52].The optimum pH for PGase was higher than the majority of fungal PGase described, and they are acidic enzymes as reported by Favela-Torres et al[49], meanwhile, *P.viridicatum* showed an optimum pH 6.0 as mentioned by Silva et al [52], *Moniliella sp* showed its maximum activity at pH 4.5 and at pH 4.5-5.0 for *Penicillium sp* [53]. The maximum activity of *Monascus sp.* and *Aspergillus sp* for exo-PGase was obtained at pH 5.5 [54]. The effect of pH on the structure and activity of polygalacturonase from *Aspergillus niger* was described by Jyothi et al [55]. They evidenced that the active conformation of PGase was favored at pH between 3.5 and 4.5, alterations in the secondary and tertiary structures resulted in at pH (from 5.0 to 7.0). Comparing the present results for PGase from *P.citrinum*, an important difference between these enzymes is clearly shown.

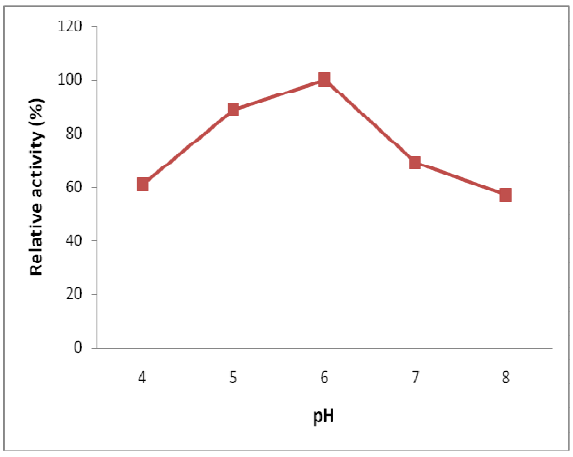

Fig (3): Effect of different pH values on the activity of the purified polygalacturonase enzyme produced by *P.citrinum.*

Stability of pH on enzyme incubated in suitable buffer systems for 2h at 30°C was also investigated during this work. The results presented in table (4) revealed that the polygalacturonase enzyme was stable at the broad pH range of pH 4up to7, retaining more than 66% of its activity. PGase activity was more stable at pH 6.0. However, the stability was significantly reduced to 58% at pH 8. It is reported that the inactivation process is found to be faster at high alkaline pHvalues due to disulfide exchange, which usually occur at alkaline condition [56].

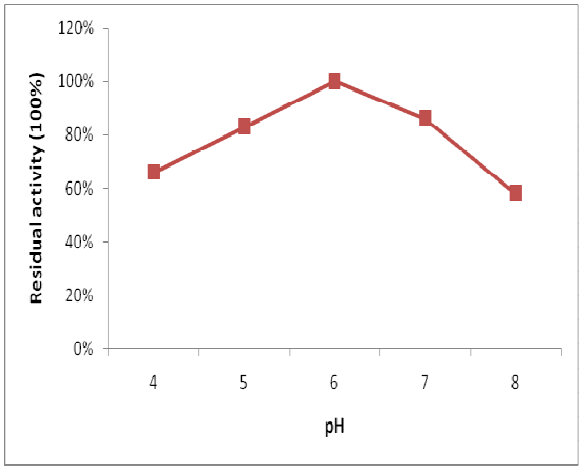

Fig (4): Effect of different pH values on the stability of the purified polygalacturonase enzyme produced by *P.citrinum*.

Effect of temperature on polygalacturonase activity and stability

The results illustrated in (Fig.5) showed that the activity of *P.citrinum* polygalacturonase increased gradually at temperature ranged from 20°C up to 60°C. Moreover the optimum temperature was achieved at 40°C, meanwhile the relative activity was attained 49% at 70°C. This view is supported by Arotupin et al [57], who reported a decline in the enzyme activity with a temperature more than 40°C. This clearly evidenced that the higher temperature resulted in a decrease in the enzyme activity. Similar observation had been reported by Palaniyappan et al [58]. Also,PGase produced by *Aspergillus Flavus, Aspergillus fumigatus* and *Aspergillus repens* exhibited maximum activity at 35°C, 40°C and 45°C respectively [59].These variations in the optimum temperature of fungi of PGase producers suggest a broad range of temperature tolerable by the enzyme. In addition, the nature, sources and differences in the physiological activities of the fungi may be responsible for these observations [60].

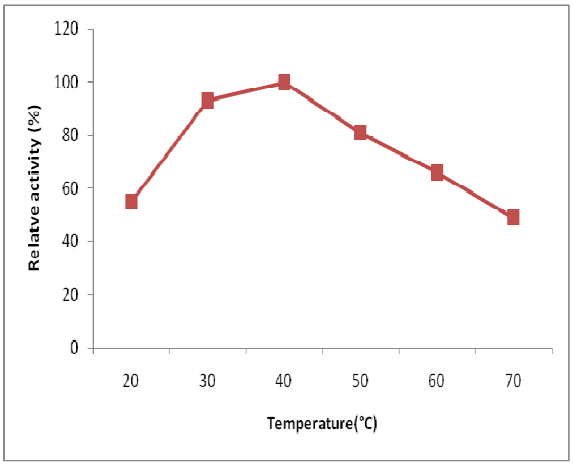

Fig (5) :Effect of temperature on the activity of the purified polygalacturonase enzyme produced by *P.citrinum*.

Thermostability is the ability of enzyme to tolerate against thermal unfolding in the absence of substrates [61]. The thermostability of the purified polygalacturonase was determined by measuring the residual activity of the enzyme after incubation at different ranges of temperatures (20°C - 70°C)after 30 minutes. Fig 6 showed that, the increase in temperature caused an overall increase in the stability up to 60°C; rising temprature above 60°C caused a decline in thermostability. It is worth mentioned that the maximum stability of 100% was observed at 50°C. Similarly, the optimum temperatures for PGase of *Aspergillus niger* and *Penicillium dierckii* were shown to be 50°C and 60°C, respectively [62]. However, the residual activity declined up to 58% at 70°C respectively.

A loss in PGase activity percentage obtained at 70°C from *Aspergillus niger,Botryodiplodia theobromae and Penicillium variabile* was reported by Oyede [63] and Ajayi et al[16]. Daniel et al [64] also reported the thermal inactivation of enzymes at high temperature. It was reported that extremely high temperature lead to deamination, hydrolysis of the peptide bonds, interchange, and destruction of disulphide bonds and oxidation of the amino acids side chains of the enzyme protein molecules [65,64].

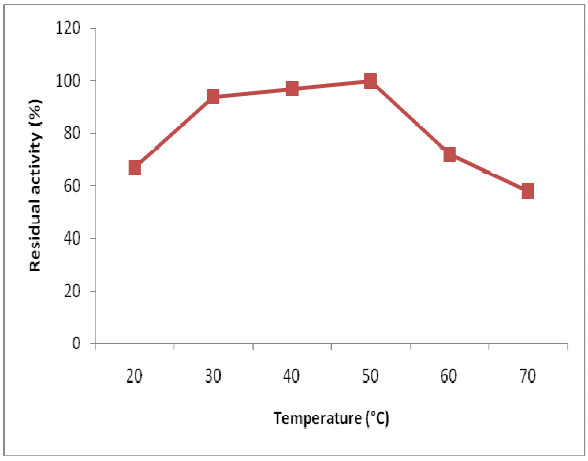

Fig.6: Effect of temperature on the stability of the purified polygalacturonase enzyme produced by *P.citrinum*.

Effect of metal ions on polygalacturonase activity

The effect of metal ions were examined by adding chlorides of Ca^{+2}, Co^{+2}, and Mg^{+2} ; sulphates of Cu^{+2}, Zn^{+2},Cd^{+2} ; EDTA and nitrate of Ba^{+2} at concentration of 1mM to the buffer solution. Results in Fig.7 revealed that the enzyme activity was enhanced in the presence of Mg^{+2} and Zn^{+2} to 12% and 5% respectively, whereas Ca^{+2} resulted in a reduction in the enzyme activity by 12%. Salts such as Ba (NO_3), $CoCl_2.6H_2O$, $CuSO_4.5H_2O$ and EDTA inhibited enzyme activity up to 50%. Jurick et al[66] reported that there was increase in PG enzyme activity by adding magnesium and iron whereas a decrease in activity occurred when calcium and manganese were included in the PGase assay. Also, Rasheedha et al [67] reported that $HgCl_2$, $CoCl_2$ and $CuSO_4$ caused inhibition of pectinase activity by *P.chrysogenum* up to 60%. Similarly $BaCl_2$ and EDTA resulted in the maximum inhibition of pectinases activity up to 40% (Rasheedha et al [67]). Also, Oyede [63] reported the stimulatory role of K^{+2}, Na^{+2} and Mg^{+2} on PGase activity from *Penicillium* variabile, while concentrations of Ca^{+2} beyond 15mM inhibited the enzyme activity. This variation in degrees of stimulation and inhibition could be a function of the sources of enzyme from different mould genera. Also, Murray et al [68] showed that the formation of a chelate complex between the substrate and the metal ions could form a more stable metal-enzyme-substrate complex and stabilizing the catalytically active protein conformation. Added to this, that the metal ions which could not be activating directly, they could act indirectly by ensuring that the true activating metal ions combine only with the active centre. Brown and Kelly [69] affirmed the ability of metal ions often acting as salt or ion bridges between two adjacent amino acids. Famurewa et al. [70] and Sakamoto et al. [71] confirmed the inhibitory activity of EDTA on enzyme. The metal building reagent like EDTA can inactivate enzyme either by removing the metal ions from the enzyme forming coordination complex or by building inside enzyme as a ligand [72]

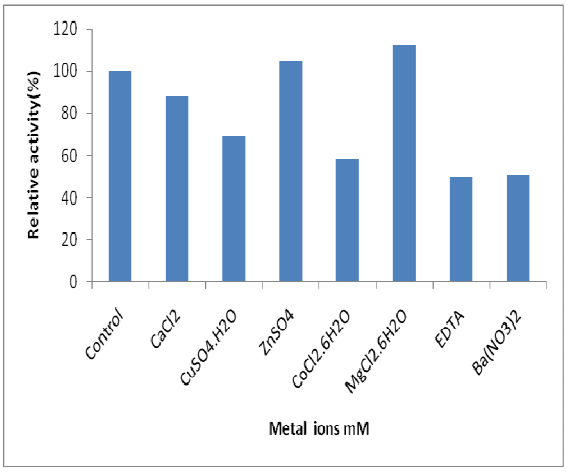

Fig.7.Effect of metal ions on the activity of the purified polygalacturonase enzyme produced by *P.citrinum*.

CONCLUSION

From the above mentioned results, we can conclude that the maximum polygalacturonase activity (152.2U/gdfs) obtained in solid state fermentation using the full factorial optimized conditions comprise: incubation period (8

days), initial medium pH(5.5), temperature (30°C), inocula size (1.8×10^5spores/ml) , yeast extract N content (1.2%) and gamma irradiation dose 0.7kGy .The highest PG activity was achieved at optimum conditions of temperature (40°C) and pH (6.0). Also, we can conclude that the PGase activity was stable at a broad range of pH from 4 upto 8 and temperature (20°C -50°C).The metal ions as Zn^{+2} and Mg^{+2} stimulated the activity of polygalacturonase by *P.citrinum* at 1mM, while EDTA had an inhibitory effect on the enzyme activity.

An important aspect of this study is the possibility of application of gamma radiation in a manner to increase the enzyme production to its maximum value at a certain dose of gamma irradiation above which the enzyme activity gradually decrease.

The overall conclusion, we can evident that the full factorial design can be a practical useful tool for optimizing the reactive parameters for enhancing the activity of pectinases.

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