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Research Article

PRODUCTION OF POLYGALACTURONASE FROM *ASPERGILLUS FUMIGATUS* ITCC 6915 USING FACTORIAL DESIGN

***R. Gupta and A. Lakhanpal**
Department of Biotechnology, Himachal Pradesh University, Summerhill, Shimla- 171005, India
**Author for Correspondence*

ABSTRACT

Twenty strains which were able to grow on medium containing pectin as the sole carbon source were isolated from the soil samples rich in pectic and fruit waste collected from fruit and vegetable market of Shimla, Himachal Pradesh, India. The strain PG-2 which gave maximum activity was selected and identified at ITCC, IARI, New Delhi as *Aspergillus fumigatus* (ITCC 6915). The maximum polygalacturonase production was obtained when organism was grown for 72 h at 30°C and pH 6.5 on Rotary Shaker (150 rpm) with apple pectin (2.5% w/v) and ammonium oxalate (1.5% w/v) as carbon and nitrogen source respectively. Polygalacturonase showed maximum activity in sodium citrate buffer (50 mM) with pH 4.8 at 60°C reaction temperature for 15 minutes incubation. Best substrate for enzyme reaction was found to be polygalacturonic acid with concentration 0.9% w/v. The K_m and V_{max} values of the enzyme were found to be 0.11 mg/ml and 5.71 µmol/ml/min respectively. Various phenolics, thiols, protein inhibitors and metal ions had inhibitory effect on the polygalacturonase activity. Utilizing a 2^6 factorial experimental design, concentration of apple pectin, ammonium oxalate and magnesium sulphate was reduced with higher polygalacturonase production over the medium selected. After optimizing all the parameters there was 5.57- fold increase in polygalacturonase activity.

Keywords: *Polygalacturonase, Polygalacturonic Acid, Isolation, Production, Optimization, Aspergillus Fumigatus*

INTRODUCTION

Pectinase is a general term for enzymes, such as pectolyase, pectozyme and polygalacturonase (Anisa *et al.,* 2013) which are involved in the breakdown of pectin from a variety of plants (Jayani *et al.,* 2005). Pectin is one of the most widely available polysaccharide in nature after cellulose, starch and chitin. The basic unit of pectin is α, D-galacturonate which is linked through α-1,4-glucosidic linkages (Singh and Mandal 2012). Pectinases are classified on the basis of their preferred substrate (pectin, pectic acid or oligo-D-galacturonate), the degradation mechanism (transelimination or hydrolysis) and the type of cleavage, random [endo-] or terminal [exo-] (Kashap *et al.,* 2001). The ability to synthesize pectinolytic enzymes is very common in groups of microorganisms (Juwon and Emmanuel 2012). Polygalacturonases are produced by numerous fungi and bacteria and also by higher plants (Lei *et al.,* 1985) (Kester and Visser, 1990) (Riou *et al.,* 1992) (Sathish and Palanivelu, 1998) (Jayani *et al.,* 2012). Most of the commercial polygalacturonases are produced by *Aspergillus* species (Mathew *et al.,* 2008). Among various Aspergilli, *Aspergillus fumigatus* is the most common in nature with a wide range of temperature tolerance and ability to produce large array of enzymes. Microbial pectinases have tremendous potential in food, beverage and textile industries as to hydrolyze the pectic substances. These are used in degumming of plant fibers, paper making, tea leaves and coffee fermentation, and in the treatment of waste waters (Hoondal *et al.,* 2002) (Ricard and Reid 2003). Pectinases also help the maintenance of ecological balance by decomposition and recycling of waste plant materials (Hagerman and Austin 1986). Pectinases from food and food Bio products processed waste alone account to a total of one-third quarter of world's food enzyme production (Prathyusha and Suneetha 2011). Pectinolytic enzymes are essential in the decay of dead plant material by nonpathogenic microorganisms and thus assist in recycling carbon compounds in the biosphere (Alana *et al.,* 1989; Zeni *et al.,* 2010). These enzymes not only provide an economically viable alternative, but are also environmental friend.

Due to the potential and wide applications of pectinases, there is a need to highlight recent developments on several aspects related to their production. In view of the above points the present study was aimed at isolation of polygalacturonase producing strain and further optimization using factorial design of production and reaction conditions for polygalacturonase.

MATERIALS AND METHODS

Chemicals, Microorganism, Production Medium

The media components namely magnesium sulphate ($MgSO_4$), dipotassium hydrogen phosphate (K_2HPO_4) and ammonium oxalate [$(COONH_4)_2H_2O$] were obtained from Himedia Laboratories, India. Apple pectin, D-galacturonic acid monohydrate and polygalacturonic acid were obtained from Sigma Chemicals Co. (USA). All other chemicals were of analytical grade. Twenty strains which were able to grow on medium containing pectin as the sole carbon source were isolated from the soil samples rich in pectic and fruit waste collected from fruit and vegetable market of Shimla, Himachal Pradesh, India. Strain PG-2 was selected on the basis of highest enzyme activity and identified at ITCC, IARI, New Delhi as *Aspergillus fumigatus* (ITCC 6915).

The initial liquid medium used in submerged fermentation for the production of polygalacturonase from strain PG-2 was composed of 2% (w/v) citrus pectin,0.5% (w/v) yeast extract, 0.1% (w/v) K_2HPO_4,0.01% (w/v) $MgSO_4$with pH 4.0 (Kaur *et al.*, 2004). Further, optimization of components of production medium was done to maximize enzyme production. Spore inocula were prepared by growing the culture on PDA slant at 30°C for 5 days. The spores were dispersed in 10 ml of sterile saline containing 0.1% Tween 20 and 1 ml was taken as the inoculum.

Enzyme Assay

Polygalacturonase activity was determined by standard colorimetric method using polygalacturonic acid as substrate. The reducing sugars released were measured by arsenomolybdate method of Nelson (1944) and Somogyi (1952). One unit of enzyme activity was defined as the amount of enzyme required to release one micromole of galacturonic acid per ml per minute under standard assay conditions.

Optimization Studies for Polygalacturonase Production

Various carbon sources (citrus pectin, apple pectin, xylose, mannitol, dextrose, glucose, fructose, galactose, maltose, lactose and sucrose, 1% w/v each) were individually added to the medium. Different concentrations of optimized carbon source (0.25%-3.25% w/v) were used and the culture supernatant was assayed for enzyme activity. Different nitrogen sources (yeast extract, casein hydrolysate, sodium nitrate, potassium nitrate, ammonium sulphate, ammonium nitrate, ammonium chloride, ammonium oxalate, ammonium dihydrogen orthophosphate, calcium nitrate, peptone and urea 1% w/v each) were also added individually to the production medium. Optimized nitrogen source was used with different concentrations (0.25%-2.0% w/v) in the production medium and the culture supernatant was assayed for enzyme activity. Various metal ions (Mg^{+2}, Zn^{+2}, Ca^{+2}, Mn^{+2}, Na^{+1}, Fe^{+2}) and inorganic salts (K_2HPO_4, KH_2PO_4, $NaHCO_3$, Na_2HPO_4, $Na_2H_2PO_4$, $C_2H_3NaO_2$) were tested for the effect on enzyme activity. Effect of incubation time was studied by incubating the enzyme in production medium for different time periods (24 h-144 h) at the interval of 24 h and measuring the polygalacturonase activity. The production medium was incubated at different temperatures viz. 25°C-60°C. The production medium of varying pH viz. 3.0-8.0 was inoculated with the culture and incubated in the rotary shaker at optimized time and temperature.After inoculation of the different media with the inocula, the flasks were placed on a Rotary shaker with different agitation rate (100 rpm-200 rpm).

Optimization of Reaction Conditions for Polygalacturonase

Sodium citrate buffer with varying pH (4.5-6.5) and different molarity (25mM-200 mM) was used to perform enzyme reaction. Enzyme reaction was carried out at different temperatures (30ºC to 70ºC) and time periods ranging from 1 to 30 minutes at interval of 5 minutes to work out the optimum reaction temperature and incubation time respectively. Different substrates viz. polygalacturonic acid, citrus pectin (DE-89%), apple pectin (DE-78%), potato dextrose agar and amylopectin (0.1% w/v each) were used to check substrate affinity of enzyme. Selected substrate was used with varying concentrations (0.1%-1.5%

w/v). Lineweaver-Burke curve was plotted to calculate K_m and V_{max} of enzyme. Varied concentrations of enzyme (5 µl-40 µl) were used to find optimum enzyme concentration. The effect of various additives (metal ions, phenolics, thiols and protein inhibitors in 1 mM concentration each) on enzyme activity was studied. The stability of enzyme was studied at different temperatures viz. 55ºC, 60ºC, 65ºC and 70ºC.

Designing 2^6technological Factorial Experiments on Optimized Medium Components to Study Their Effect on Production of Polygalacturonase by Aspergillus Fumigatus

In order to reduce the cost of production medium for the production of *Aspergillus fumigates* with high polygalacturonase activity, technological combinations were applied in designing the factorial experiments. For this purpose, instead of one parameter being varied, different combinations of optimum and next lower level of optimized physicochemical parameters were used. The experiment was carried out with optimized physical and chemical parameters. The physicochemical factors considered were medium pH, incubation temperature, carbon source (apple pectin), nitrogen source (ammonium oxalate), metal ion ($MgSO_4$) and inorganic salt (K_2HPO_4). In each case, polygalacturonase production by *Aspergillus fumigatus* was done after 72 h incubation. Total 64 combinations (2^6 factorial) were obtained on the basis of above parameters. The previously optimized medium was considered as control to compare the polygalacturonase activity with different technological combinations.

Statistical Analysis

The results were statistically analysed by using Student's t-test. 2^6technological factorial experiments were designed to study the effects of optimized medium components on production of polygalacturonase by *Aspergillus fumigatus*.

RESULTS AND DISCUSSION

Favourable production and reaction conditions were determined to enhance the polygalacturonase activity. Apple pectin (2.5% w/v) was found to be the most favourable carbon source for the polygalacturonase production from *Aspergillus fumigatus*. In its absence, very low polygalacturonase activity was detected. Previously, commercial pectin (9.68% w/v) was optimized as best carbon source for the production of pectinase from *Aspergillus niger* (Akhter *et al.*, 2011) and pectin methyl ester (1% w/v) was the best source followed by apple pectin for the polygalacturonase production from *Mucor circinelloides* (Thakur *et al.*, 2010). The source of nitrogen in the growth medium has a very important role in microbial growth and enzyme production (Mrudula and Anitharaj 2011). In the present studies, supplementation of ammonium oxalate (1.25% w/v) as the nitrogen source to the production medium increased the polygalacturonase production. However the production was reduced in the presence of calcium nitrate. Previously, calcium nitrate (0.1% w/v) was observed to give maximum polygalacturonase production from *Byssochlamys fulva* (Gupta and Kalpana 2011) and $(NH_4)_2SO_4$ (1.69% w/v) for the production of pectinase from *Aspergillus niger* (Akhter *et al.*, 2011). Magnesium sulphate (0.02% w/v) and K_2HPO_4 (0.1% w/v) were found to increase the polygalacturonase production from *Aspergillus fumigatus*. Incubation time for maximal polygalacturonase production was found to be 72 h; similar result was reported for maximal polygalacturonase production by a diploid construct from two *Aspergillus niger* overproducing mutants (Loera *et al.*, 1999). The decrease in the activity after 72 h could be due to the depletion of nutrients in the medium. A temperature of 30°C (Table 1) was reported to be the optimum growth temperature which was similar to the findings of Mathew *et al.*, (2008); Lima *et al.*, (2008); Gupta and Kalpana (2011).

The maximum enzyme activity occurred with an initial pH of 6.5. Earlier a pH range of 5.5-6.5 has been reported for maximum polygalacturonase production from *Aspergillus niger* (Arguelles *et al.*, 1995), 3.5-5.5 for polygalacturonase production by *Mucor flavus* (Gadre *et al.*, 2003) and 4.5-6.0 for polygalacturonase production by *Aspergillus awamori* (Abbasi and Mortazavipur 2011). It was noticed that optimum level of rotation needed for the maximum production of enzyme was 150 rpm and on increasing the rpm level, there was a decrease in the production; this could be due to the fact that the increase in the rpm level had resulted in the coagulation of the organism to form lumps and decrease in the rate of mass transfer (Palaniyappan *et al.*, 2009).

Table 1: Effect of temperature on the production of polygalacturonase from *Aspergillus fumigatus*

Temperature (°C)	Activity (U/ml)	Relative activity (%)
25	4.12 ±0.06	70.06
30	**5.88 ±0.10**	**100**
35	4.77 ±0.28	81.12
40	4.65 ±0.04	79.08
45	3.81 ±0.08	64.79
50	2.97 ±0.35	50.51
55	1.60 ±0.13	27.21
60	1.14 ±0.03	19.38

Values are mean ± S.D. of 3 replicates

In the present study, maximum polygalacturonase activity was found with citrate buffer (0.05 M, pH 4.8). Previously, citrate buffer with pH 5.5 has been reported for maximum polygalacturonase activity from *Penicillium* SPC-F20 (Mathew *et al*. 2008) and from *Byssochlamys fulva* (Gupta and Kalpana 2011). In another study, pH of 7.0 was reported to be optimum for maximum activity of polygalacturonase from *Penicillium ostreatus* (Freixo *et al.*, 2010). Incubation temperature of 60°C was best for enzyme activity from *Aspergillus fumigatus*. In previous studies, it was reported at 55°C for polygalacturonase from *Streptomyces* sp. (Beg *et al.*, 2000), 50°C from *Byssochlamys fulva* (Gupta and Kalpana 2011) and 30°C from *Penicillium* sp. (Rashad *et al.*, 2010). Maximum activity of polygalacturonase was observed after 15 minutes of incubation. Similar result was reported for polygalacturonase from *Byssochlamys fulva* (Gupta and Kalpana, 2011), while maximum activity of polygalacturonase from *Mucor circinelloides* was achieved after 20 minutes of incubation (Thakur *et al.*, 2010). While studying effect of substrate on enzyme activity it was highest with polygalacturonic acid (0.9% w/v) and lowest with amylopectin. Earlier, polygalacturonase from *Penicillium capsulatum* (Gillespie *et al.*, 1990) and from *Byssochlamys fulva* (Gupta and Kalpana 2011) was also found to have high affinity for polygalacturonic acid. In the previous study, 0.5% w/v substrate concentration was optimized for maximum polygalacturonase activity from *Aspergillus niger* (Thibault and Mercier 1979) and *Byssochlamys fulva* (Gupta and Kalpana 2011). While Dinu (2001) reported 0.75% w/v as optimal concentration of substrate for maximum polygalacturonase activity from *Aspergillus niger*. V_{max} value was 5.71 µmol/ml. The K_m (0.11 mg/ml) value was found to be very low which shows high affinity of the enzyme towards substrate (Figure 1).

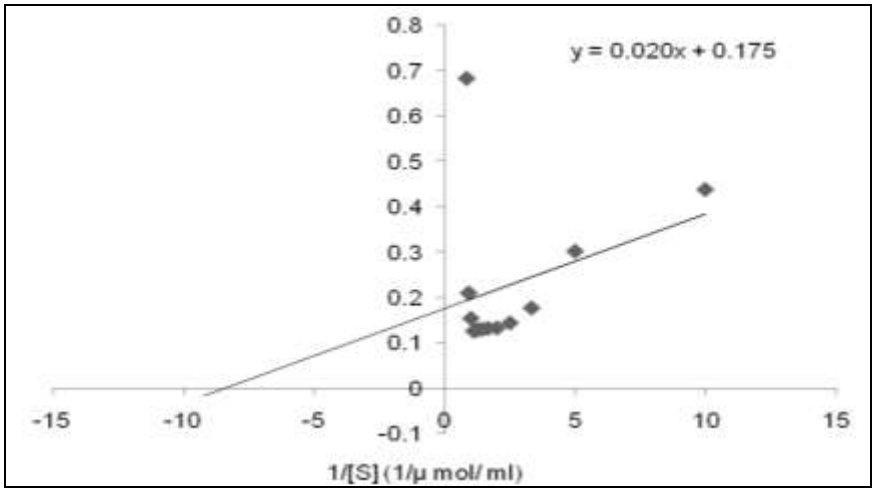

Figure: 1 Lineweaver-Burke plot for polygalacturonase from *Aspergillus fumigatus*

In a previous study, K_m for polygalacturonase from *Aspergillus niger* was found to be 1.42 mg/ml (Mohsen *et al.*, 2009) and 0.54 mg/ml (Rombouts and Pilnic 1980). The K_m and V_{max} values reported for

Fusarium oxysporum polygalacturonase were 4.1 mg/ml and 5.5 µmol/ml respectively (Al-Najada *et al.*, 2012). The K_m and V_{max} values of pectin methylesterase from *Lycopersicon esculentum* were found to be 0.115 mg/ml and 1.03 µmol/ml/min respectively (Kant and Gupta 2012). In a recent study, K_m and V_{max} values for polygalacturonase from *Aspergillus niger* have been reported to be 0.083 mg/ml and 18.21 µmol/ml/min respectively (Kant *et al.*, 2012). Enzyme inhibitors are substances which alter the catalytic action of the enzyme and consequently, slow down or stop catalysis in some cases. All the phenolics, thiols and protein inhibitors strongly inhibited the polygalacturonase activity from *Aspergillus fumigatus*. The effect of metal cations as inhibitors for polygalacturonase was in the order of Al^{3+}< Zn^{2+}< Li^{2+}< Ca^{2+}<Fe^{3+}< Mn^{2+}. Thus the enzyme did not require any metal ion to express its activity. The effect of phenolics as inhibitor for polygalacturonase was in the order of salicylic acid>cinnamic acid> p-coumaric acid>ferulic acid. All the thiols tested had significant ($p< 0.001$) inhibitory effect on polygalacturonase activity (Table 2). Their inhibitory effect was in the order ascorbic acid> $HgCl_2$> ß-mercaptoethanol> SDS.

Table 2: Effect of phenolics, thiols and protein inhibitors on the activity of polygalacturonase from *Aspergillus fumigatus*

Additives	Activity (U/ml)	Relative activity (%)
Phenolics		
Ferulic acid	2.81 ±0.04*	36.25
Cinnamic acid	1.10 ±0.01*	14.19
Salicylic acid	0.97 ±0.01*	12.51
p-Coumaric acid	2.28 ±0.02*	29.41
Thiols		
$HgCl_2$	2.03 ±0.05*	26.19
β -mercaptoethanol	2.76 ±0.03*	35.61
SDS	3.04 ±0.05*	39.22
Ascorbic acid	1.50 ±0.02*	19.35
Protein inhibitors		
EDTA	0.83 ±0.03*	10.78
Sodium arsenate	1.05 ±0.006*	13.54
Sodium azide	1.00 ±0.04*	12.90
Potassium permanganate	0.90 ±0.02*	11.61
Control	**7.75 ±0.04***	**100**

*Values are mean ± S.D. of 3 replicates. *p < 0.001 as compared to control*

The enzyme was strongly inhibited by EDTA and potassium permanganate. Previously, comparable results have been obtained for the effect of protein inhibitors on the polygalacturonase from *Byssochlamys fulva* (Gupta and Kalpana 2011). Thermostability profile of enzyme was determined and it was found that at 55°C, the half life was 3 h 15 minutes approximately. At 60°C the half life was observed to be 2 h 25 minutes approximately. At 65°C and 70°C half life of enzyme was found to be approximately 1 h 40 minutes and 1 h 10 minutes respectively (Figure 2). In a previous study, the half life of polygalacturonase at 60°C was observed to be 2 h from *Penicillium* sp. (Mathew *et al.*, 2008). At 30°C the enzyme showed a considerable stability up to 8 h, at 40°C the half life of the enzyme was 6 h for polyagalacturonase from *Byssochlamys fulva* (Gupta and Kalpana 2011). For all 64 combinations of factorial experiment designed on physicochemical parameters, maximum polygalacturonase production was obtained with 15th combination (pH 6.5, temperature 30^{o}C, apple pectin 2.25%, ammonium oxalate 1.0%, $MgSO_4$ 0.015%, K_2HPO_4 0.1%, w/v). This combination showed more activity (7.52 U/ml) than the previously optimized control medium (7.18 U/ml). Polygalacturonase from *Aspergillus fumigatus* also showed good activity in 1st, 21st, 46th and 58th combinations. After optimization, activity of polygalacturonase was found to be 7520 U/litre.

Table 3: Technological combinations on optimized physico-chemical parameters for polygalacturonase production by *Aspergillus fumigatus*

S.No.	pH	IT (^{o}C)	Apple Pectin (% w/v)	Ammonium oxalate (% w/v)	$MgSO_4$ anhydrous (% w/v)	K_2HPO_4 (% w/v)	EA (U/ml)
1	**6.5**	**30**	**2.5**	**1.25**	**0.02**	**0.1**	**7.18**
2	6.5	30	2.5	1.25	0.02	0.15	6.01
3	6.5	30	2.5	1.25	0.015	0.1	6.30
4	6.5	30	2.5	1.25	0.015	0.15	5.02
5	6.5	30	2.5	1.0	0.02	0.1	5.31
6	6.5	30	2.5	1.0	0.02	0.15	6.09
7	6.5	30	2.5	1.0	0.015	0.1	6.37
8	6.5	30	2.5	1.0	0.015	0.15	5.67
9	6.5	30	2.0	1.25	0.02	0.1	4.21
10	6.5	30	2.0	1.25	0.02	0.15	4.05
11	6.5	30	2.0	1.25	0.015	0.1	5.88
12	6.5	30	2.0	1.25	0.015	0.15	4.55
13	6.5	30	2.0	1.0	0.02	0.1	3.81
14	6.5	30	2.0	1.0	0.02	0.15	6.21
15	**6.5**	**30**	**2.0**	**1.0**	**0.015**	**0.1**	**7.52**
16	6.5	30	2.0	1.0	0.015	0.15	6.49
17	6.5	35	2.5	1.25	0.02	0.1	6.12
18	6.5	35	2.5	1.25	0.02	0.15	7.02
19	6.5	35	2.5	1.25	0.015	0.1	3.25
20	6.5	35	2.5	1.25	0.015	0.15	5.92
21	**6.5**	**35**	**2.5**	**1.0**	**0.02**	**0.1**	**7.09**
22	6.5	35	2.5	1.0	0.02	0.15	4.89
23	6.5	35	2.5	1.0	0.015	0.1	4.12
24	6.5	35	2.5	1.0	0.015	0.15	6.31
25	6.5	35	2.0	1.25	0.02	0.1	7
26	6.5	35	2.0	1.25	0.02	0.15	5.07
27	6.5	35	2.0	1.25	0.015	0.1	6.97
28	6.5	35	2.0	1.25	0.015	0.15	4.13
29	6.5	35	2.0	1.0	0.02	0.1	5.74
30	6.5	35	2.0	1.0	0.02	0.15	6.36
31	6.5	35	2.0	1.0	0.015	0.1	7.08
32	6.5	35	2.0	1.0	0.015	0.15	4.32
33	7.0	30	2.5	1.25	0.02	0.1	5.75
34	7.0	30	2.5	1.25	0.02	0.15	3.28
35	7.0	30	2.5	1.25	0.015	0.1	2.25
36	7.0	30	2.5	1.25	0.015	0.15	2.75
37	7.0	30	2.5	1.0	0.02	0.1	3.91
38	7.0	30	2.5	1.0	0.02	0.15	5.00
39	7.0	30	2.5	1.0	0.015	0.1	6.31
40	7.0	30	2.5	1.0	0.015	0.15	6.97
41	7.0	30	2.0	1.25	0.02	0.1	4.62
42	7.0	30	2.0	1.25	0.02	0.15	2.65
43	7.0	30	2.0	1.25	0.015	0.1	2.09
44	7.0	30	2.0	1.25	0.015	0.15	4.77
45	7.0	30	2.0	1.0	0.02	0.1	5.99
46	**7.0**	**30**	**2.0**	**1.0**	**0.02**	**0.15**	**7.02**

47	7.0	30	2.0	1.0	0.015	0.1	5.80
48	7.0	30	2.0	1.0	0.015	0.15	4.45
49	7.0	35	2.5	1.25	0.02	0.1	2.56
50	7.0	35	2.5	1.25	0.02	0.15	3.14
51	7.0	35	2.5	1.25	0.015	0.1	4.45
52	7.0	35	2.5	1.25	0.015	0.15	6.88
53	7.0	35	2.5	1.0	0.02	0.1	1.94
54	7.0	35	2.5	1.0	0.02	0.15	3.39
55	7.0	35	2.5	1.0	0.015	0.1	5.94
56	7.0	35	2.5	1.0	0.015	0.15	4.72
57	7.0	35	2.0	1.25	0.02	0.1	6.11
58	**7.0**	**35**	**2.0**	**1.25**	**0.02**	**0.15**	**7.31**
59	7.0	35	2.0	1.25	0.015	0.1	5.69
60	7.0	35	2.0	1.25	0.015	0.15	5.34
61	7.0	35	2.0	1.0	0.02	0.1	6.01
62	7.0	35	2.0	1.0	0.02	0.15	6.28
63	7.0	35	2.0	1.0	0.015	0.1	3.08
64	7.0	35	2.0	1.0	0.015	0.15	4

IT-Incubation temperature; EA-Enzyme Activity

The results of factorial combinations revealed that the physicochemical parameters were dependent on each other as they worked better in combination and enhanced the polygalacturonase activity as compared to the individually optimized parameters. Factorial combination also proved to be very satisfactory on economical point of view because it reduced concentration of apple pectin, ammonium oxalate and magnesium sulphate with higher polygalacturonase activity over the medium selected after study of the individual parameters. Previously, response surface methodology (RSM) was applied to optimize the fermentation conditions of *Bacillus licheniformis* 3x05 for polygalacturonase production (Hou *et al.*, 2011).

The result of present study revealed that *Aspergillus fumigatus* is a potential polygalacturonase producer. Rather, this is the first report of production of polygalacturonase from mesophilic *Aspergillus fumigatus.* The optimized production and reaction conditions did enhance the enzyme production in submerged fermentation. Further study will be carried out on the purification of polygalacturonase from *Aspergillus fumigatus* for the industrial use of this highly demanding enzyme all over the world. The data presented in this research would provide reference for future work with *Aspergillus fumigatus* ITCC 6915.

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