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Full Length Research Paper

Polygalacturonase production from *Rhizomucor pusillus* isolated from fruit markets of Uttar Pradesh

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Polygalacturonase (PGases) production from *Rhizomucor pusillus* was enhanced when various production parameters were optimized. Maximum PGases activity was obtained after 120 h at 45°C and pH 5.0 with pectin (pure) (1.5% w/v) as carbon source, urea (0.3% w/v) as nitrogen source and $MnSO_4$ (0.2% w/v) as mineral supplement. PGases from *R. pusillus* was partially purified and subjected to characterization studies. Maximum PGases specificity was observed when polygalacturonic acid was used as the substrate. The PGases was stable at 50°C up to 4 h of incubation and pH condition of between 4.0-5.0. The stability of PGase decrease rapidly above 60°C and above pH 5.0. To our knowledge, it was the first time that a polygalacturonase enzyme was purified in this species. It would be worthwhile to exploit this strain for polygalacturonase production. Polygalacturonase from this strain can be recommended for commercial production because of its constitutive and less catabolically repressive nature, thermo stability and wide range of pH. However, scales up studies are needed for better output for commercial production.

Key words: Thermophilic fungi, *Rhizomucor pusillus*, pectinase, polygalacturonase.

INTRODUCTION

Due of large variety of pectins in plant material, they posses many pectinolytic enzyme systems which can degrade them (Bene et al., 2002). The hydrolysis of pectin backbone is obtained by the synergistic action of several enzymes, including pectin methylesterase (EC. 3.1.11.1), endo-polygalacturonase (EC. 3.2.1.15), exopolygalacturonase (EC. 3.2.1.67), pectate lyase (EC. 4.2.2.2) exo-pectate lyase (EC. 4.2.2.9) and endopectin lyase (4.2.2.10) (Soares et al., 2001; Gummadi and Panda, 2003).

Pectinolytic enzymes are of prime importance for plants as they help in cell-wall extension and softening of some plant tissues during maturation and storage (Aguilar and Huirton, 1990; Sakai, 1992). Plant pathogenicity and spoilage of fruits and vegetables by rotting are some other major manifestations of pectinolytic enzymes (Fraissinet-Tachet and Fevre, 1996; Singh et al, 1999).

They have been used in many industrial and biotechnological processes (Hoondal et al., 2002). Commercial enzyme preparations used in processing of food, traditionally, comprising of the mixtures of polygalacturonase, pectate lyase, and pectin esterase, are almost exclusively derived from fungal species, especially *Mucor* and *Aspergillus* (Lang and Dornenburg, 2000). Preparations containing pectin-degrading enzymes used in the food industry are of fungal origin because fungi are potent producers of pectic enzymes and the optimal pH of fungal enzymes is very close to the pH of many fruit juices, which range from pH 3.0 to 5.5 (Ueda et al., 1982). Due to the potential and wide applications of pectinases, there is a need to highlight recent developments on several aspects related to their production.

Higher cost of the production is perhaps the major constraint in commercialization of new sources of enzymes. In addition, technical constraint includes supply of cheap and pure raw materials and difficulties in

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achieving high operational stabilities, particularly to temperature and pH (Phutela et al., 2005). Polygatacturonases are the most extensively studied among the family of pectinolytic enzymes. Literature highlighting the optimization, biochemical characterization, genetics and strain improvement studies of polygatacturonases from mesophilic fungi (Marcus et al., 1986; Thakur et al., 2010) is available. However, there are few studies where stable polygatacturonases have been reported from thermophilic fungi. Therefore, in the present article, we described the isolation of polygalacturonases producing fungi, purification, and characterization of polgalacturonases with appreciable activity and temperature and pH stability.

MATERIALS AND METHODS

Isolation and screening of thermophilic fungal isolates for pectinolytic activity

Pectinase producing fungi were isolated from various soil, decayed fruits and other vegetables were collected from different fruits markets of western Uttar Pradesh by using the modified pectin agar medium (Singh and Sandhu, 1986). The inoculated plates were incubated at 50°C for 5-7 days. The cultures were further screened by sub culturing on Yeast soluble starch agar (YPSS) medium. Pectin utilization was detected by flooding the culture plates with freshly prepared 1% Cetrimide solution and allowed to stand for 20-30 min. A clear zone around the colonies against a white background (of the medium) indicates the ability of an isolate to produce pectinase. Based on screening, the isolated fungi were identified up to genus level by examining the morphological characters following Dubey and Maheshwari (2006). Highest pectinase producer isolate was selected for further studies and the culture was sent to MTCC, Chandigarh for species identification.

Medium for solid-state fermentation (SSF) and enzyme production

The solid state cultivation was carried out in 250 ml Erlenmeyer flasks containing 15 g of basal medium (g): Pectin-1.0, Urea-0.3, Sucrose-3.14, $(NH_4)_2SO_4$-1.26, KH_2PO_4-0.65, $FeSO_4$-0.29, Sugarcane bagasse-23.1. The flasks were inoculated with 2 ml spore suspension containing (10^6 spores ml^{-1}), which was obtained from a five day agar slant. The pH of the media was adjusted to 5.5-6.0 by using 1.0 N HCl/1.0 N NaOH (Acuna-Argulles et al., 1995). Final moisture will be adjusted to 70%. The cultivation was carried out at 50°C for 4-5 days. The fermented media was extracted with minimum volume of distilled water. The flasks were shaken vigorously and kept for one hour and filtered through cheese cloth. The crude enzyme was extracted by adding 100 ml of citrate buffer (0.1 M, pH 5.0) to each flask. The extract was centrifuged at 11,200 g for 15 min at 4°C and the supernatant was filtered through Whatman No.1 filter paper to remove spores completely. The filtrate was used for enzymatic assays. One unit of enzyme activity is defined as the enzyme that releases 1 µmol ml^{-1} min^{-1} galacturonic acid under standard assay conditions.

Optimization studies for enzyme production

The effect of various physical and biochemical parameters were analyzed for the production of polygalacturonases. The different parameters analyzed were effect of natural substrates like different complex carbon source (wheat bran, rice bran, orange bagasse and pectin from pomegranate, lemon and orange) and purified commercial carbon sources (glucose, sucrose, galactose and starch), nitrogen sources Urea, yeast extract, Casein, Tryptone, Peptone, $NaNO_3$ and $(NH_4)_2SO_4$, incubation temperature between 30 and 80°C, effect of initial pH (adjusted with 1.0 N HCl/1.0 N NaOH in a range of 3-9 with one unit increment) and effect of salt concentration.

Enzyme assay

The PGases activity was assayed by estimating the amount of reducing sugar released under assay conditions. Polygalacturonase activity was measured by determining the amount of reducing groups released according to the method described by Nelson (1944) and modified by Somogyi (1952). The substrate used for assay was 1% PGA (polygalacturonic acid), that is, 1.0 g of PGA in 100 ml of 0.1 M citrate buffer, pH 5.0. The assay mixture was prepared with the following components: 0.2 ml enzyme, 0.1 ml of 0.1 M $CaCl_2$, 0.5 ml of 1% solution of polygalacturonic acid (PGA). Blank was prepared for each sample by boiling the reaction mixture before the addition of substrate. Tubes were incubated at 37°C for one hour. The reaction was stopped by heating at 100°C for 3 min. 0.5 ml of the solution mixture was taken and analyzed for reducing sugars by Nelson-Somogyi method. Final volume was made up to 2 ml in both sample and standard tubes with distilled water. 1.0 ml of alkaline copper tartarate was added and kept for 10 min. The tubes were cooled and 1.0 ml of arsenomolybdate reagent was added to each of the tubes. Final volume was made up to 10 ml in each tube with distilled water. The absorbance of blue color was recorded at 620 nm after 10 min.

The amount of galacturonic acid released per ml per minute was calculated from standard curve of galacturonic acid. One unit of enzyme activity is defined as the enzyme that releases 1 µmol ml^{-1} min^{-1} galacturonic acid under standard assay conditions.

Partial purification of enzyme

The filtrate recovered after SSF was centrifuged at 11,200 g for 20 min at refrigerated condition. Solid ammonium sulfate was slowly added to the supernatant of crude enzyme preparation so as to reach 20% saturation. Addition of ammonium sulfate was carried out with continuous stirring in an ice bath, and then it was kept at 4°C overnight. The precipitated protein was removed by centrifugation at 11,200 g for 30 min at 4°C. Ammonium sulfate was added to the supernatant to 80% saturation. The precipitated protein was again separated by centrifugation at 11,200 g for 30 min at 4°C. The precipitated protein was dissolved in sodium acetate buffer (0.1 M; pH 5.0).

Characterization of PGases

The substrate specificity of the purified enzyme was determined by using various substrates in the reaction mixture for enzyme assay. The various substrates used were polygalacturonic acid, pectin, xylan, galactose and cellulose at 0.1% (w/v) (Thakur et al., 2010).

The temperature stability of enzyme was estimated by incubating the enzyme for 4 h at temperature from 30 to 80°C in assay buffer and then measuring the remaining activity by standard assay determined the inactivation temperature. The pH stability of enzyme was evaluated by varying the pH of the reaction mixture between 3.0 and 9.0 at increments of 1.0. Activity was then assessed under standard conditions.

Table 1. Polygalacturonase activity of crude enzyme extract of different isolates.

Fungal sp.	Source	PGases activity (U/ml)
Mucor sp. 1	Tomato	12.78
Tricoderma sp. 1	Pineapple	6.13
Mucor sp. 2	Sweet lime	19.27
Aspergillus sp. 1	Soil	1.92
Aspergillus sp. 2	Orange	7.81
Penicillium sp. 1	Brinjal	0.76
Rhizomucor sp. 1	Orange	32.57
Rhizomucor sp. 2	Apple	3.45
Alternaria sp. 1	Pomegranate	9.67
Tricoderma sp. 2	Soil	1.08
Aspergillus sp. 2	Pineapple	14.10
Mucor sp. 3	Guava	23.42

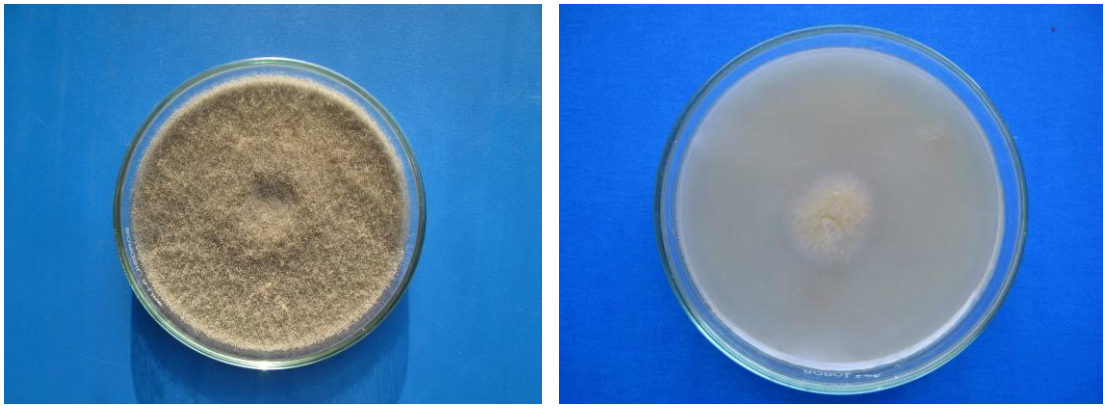

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Figure 1. (a): Five day old culture of isolate 7 on modified pectin agar medium. (b): Clear zone (3.5mm) indicates polygalacturonase activity after 2 days of incubation at 50°C.

RESULTS

Screening and selection of fungal isolate

The phytopathogenic character of pectic enzymes led to the selection of spoiled fruits and vegetables as the sources for pectinolytic microorganisms. Tomato, orange, apple, pineapple, sweet lime, lemon, carrot, gavava, pomegrate and brinjal were the spoiled fruits and vegetables used in this study. In addition to this, soil was also analyzed for pectinolytic microorganisms. Thermophilic fungal strains isolated from various pre collected samples from different fruits markets of western Uttar Pradesh, were purified and their cultural and morphological characteristics were examined according to the method described by Dubey and Maheshwari (2006). A total of 40 fungal strains were isolated from 15 different soil, decayed fruits and other vegetables samples. Different species of *Mucor*, *Aspergillus*, *Penicillium, Rhizopus* and *Tricoderma* were isolated. High pectinase producing strains were further screened semi quantitatively by plate assay method. Twelve different isolates were further screened by solid state fermentation. The results indicate that isolate 7 shows maximum activity for polygalacturonase (Table 1). The isolate was further identified as *Rhizomucor pusillus* by Microbial Type Culture Collection (MTCC), Chandigarh, India (Figure 1).

Effect of various parameters on enzyme production

A proper combination of various conditions was established in order to achieve maximum PGases production by *R. pusillus* in SSF. Different conditions were altered and the level of enzyme production was

Table 2. Effect of initial temperature on polygalacturonase production from *Rhizomucor pusillus* under SSF.

Temperature (°C)	PGases activity (U/ml)
30	13.29
40	21.73
45	43.17
50	19.72
55	6.42
60	0.00
70	0.00

Table 3. Effect of initial pH on polygalacturonase production from *Rhizomucor pusillus* under SSF.

pH	PGases activity (U/ml)
3	3.16
4	14.42
5	34.14
6	11.81
7	6.35
8	16.87
9	2.14

Table 4. Effect of incubation time on polygalacturonase production from *Rhizomucor pusillus* under SSF.

Time (h)	PGases activity (U/ml)
24	0.00
48	7.93
72	15.84
96	23.17
120	39.78
144	21.67
168	11.91
192	5.73

monitored.

R. pusillus was cultured under various temperature conditions ranging from 30-80°C. There was no detectable growth of the microorganism below 30°C and above 55°C and hence there was no enzyme activity. Maximal activity was observed at 45°C for PGases. Enzyme activity was comparatively less at below 40°C as well as above 50°C. Enzyme shows maximal activity at 45°C (Table 2). The initial pH was varied from 3.0-9.0 for finding out the optimum pH for the production of PGases by *R. pusillus*. PGases produced maximal activity at pH 5.0. Above pH 5.0 the activity of enzyme began to decrease with respect to pH, but at pH 8.0 there was a slight increase in the activity. There was very little or no activity at pH 9.0 for PGases (Table 3).

PGases activity was found maximum at the 120th h that is on the 5th day. PGases activity was absent at the 24th h that is the 1st day. In the first day there was no marked growth of the fungus. 48th h onwards, PGases activity increased, and reached its maximum on 120th h, and then it began to decline (Table 4). The concentration of carbon sources in the medium was 1.5% (w/v). Maximal activity was obtained when pectin (pure) was added to the production medium. With glucose and starch, the enzyme showed lesser activity when compared to pectin (Table 5). The concentration of nitrogen sources in the medium was 0.3% (w/v). PGases had maximum activity when urea was incorporated in the medium. All other nitrogen sources had an inhibitory effect on PGases production (Table 6). The salts were added in a concentration of 0.2% in the fermentation media for the production of PGases. Enzyme activity was higher than the control in the media added with $MnSO_4$ and $FeCl_3$. All other salts showed comparative lesser activity than the control (Table 7).

Table 5. Effect of carbon sources on polygalacturonase production from *Rhizomucor pusillus* under SSF.

Carbon sources (1.5%)	PGases activity (U/ml)
Glucose	26.78
Pectin (pure)	41.13
Sucrose	34.17
Galactose	31.29
Starch	19.54
Orange	30.45
Lemon	37.86
Control	38.07

Table 6. Effect of nitrogen sources on polygalacturonase production from *Rhizomucor pusillus* under SSF.

Nitrogen sources (0.3%)	PGases activity (U/ml)
Urea	29.13
Yeast extract	6.76
Casein	2.49
Tryptone	12.56
Peptone	4.23
$NaNO_3$	0.92
Control	24.74

Characterization of PGases activity

The maximum PGases specificity was observed when polygalacturonic acid was used as the substrate.

Table 7. Effect of salts concentration on polygalacturonase production from *Rhizomucor pusillus* under SSF.

Salts (0.2%)	PGases activity (U/ml)
Control	32.46
$CaCl_2$	21.92
$BaCl_2$	17.84
$FeCl_3$	34.57
$ZnCl_2$	13.59
$MnSO_4$	37.71
$MgSO_4$	9.73

Table 8. Substrate specificity of polygalacturonase from *Rhizomucor pusillus.*

Substrate (0.1%)	Enzyme activity (U/ml)	Relative activity (%)
Polygalacturonic acid	13.23	100
Pectin	7.54	57
Xylan	1.45	11.0
Galactose	0.88	6.7
Cellulose	5.00	37.8

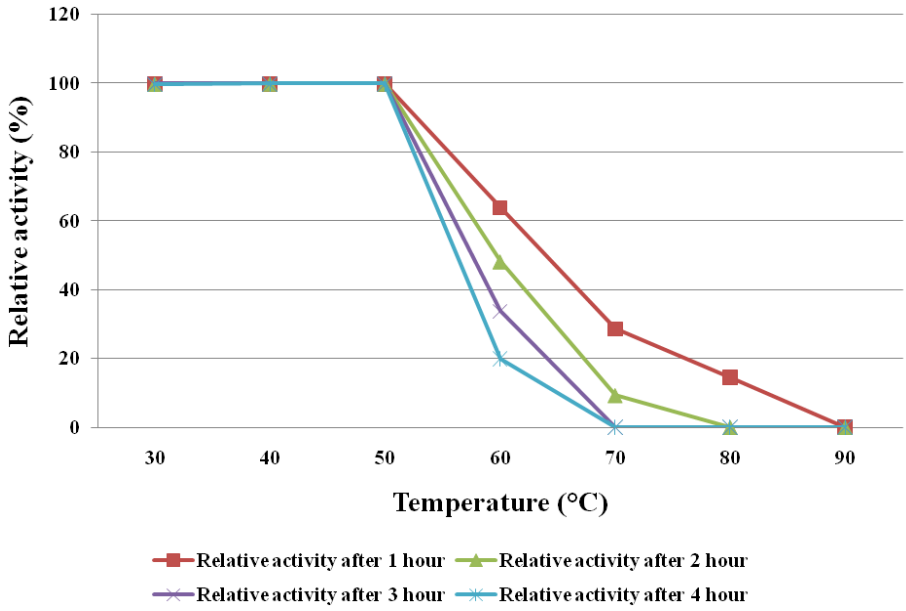

Figure 2. Effect of temperature on polygalacturonase stability from *Rhizomucor pusillus.*

Assuming it as 100%, almost half (57%) activity was expressed with pectin and only 37.8% activity was expressed with cellulose (Table 8).

The enzyme was stable at 50°C up to the 4th h. After two hours the PGases activity was only 48.2 and 9.30% at 60 and 70°C respectively and then the enzyme became suddenly inactive. From 80°C onwards the enzyme activity was lost during the first hour itself (Figure 2).

The enzyme was stable at pH conditions 4.0-5.0. Above pH 5.0, enzyme stability began to decrease. At pH 8.0 after the 4th h the residual activity was 40.21% of that of the control. No activity was determined at pH 9.0 at the 4th hour (Figure 3).

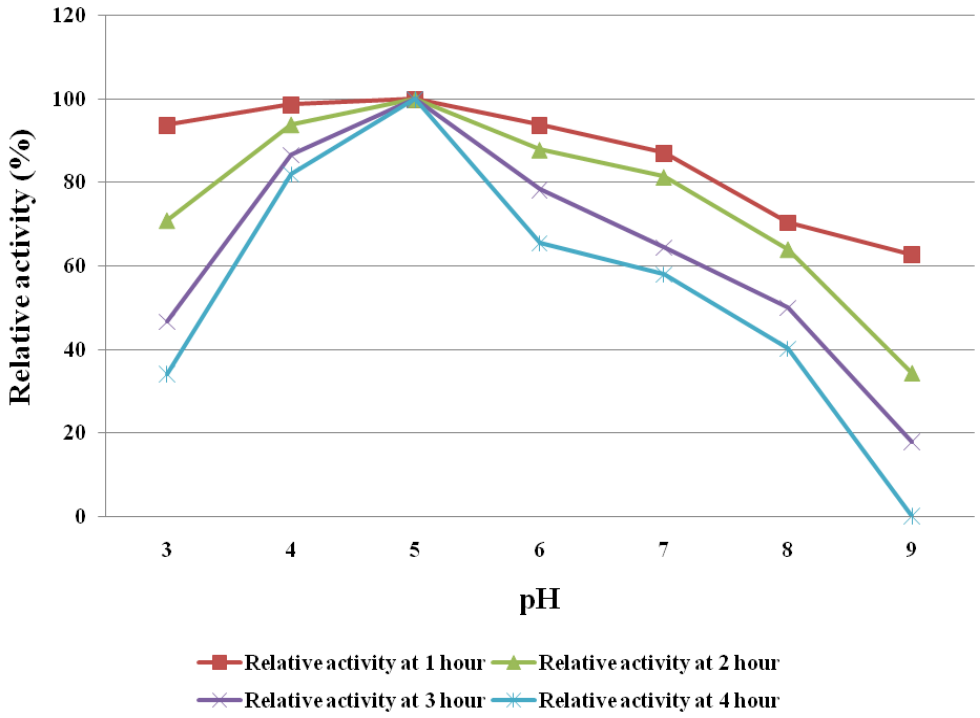

Figure 3. Effect of pH on polygalacturonase stability from *Rhizomucor pusillus.*

DISCUSSION

Different species of *Mucor*, *Aspergillus*, *Penicillium, Rhizopus* and *Tricoderma* were isolated using pre collected soil, decayed fruits and other vegetable samples using enrichment culture techniques. Similar results were also reported by different co-workers: *Rhizopus* (Trescott and Tampion, 1974), *Mucor* (Alves et al., 2002) and *Tricoderma* (Nabi et al., 2003).

The primary screening for pectinase activity was done using disc plate method (Acuna-Argulles et al., 1995). A clear zone around the colonies against a white background (of the medium) indicates the ability of an isolate to produce pectinase. Different chemicals were used by different co-workers to detect pectin utilization such as hexadecyl-trimethyl-ammonium-bromide (Ahmad et al., 2006) and trimethyl ammonium bromide (Asoufi et al., 2007). In the present study, pectin utilization was detected by flooding the culture plates with freshly prepared 1% Cetrimide solution (Tewari et al., 2005). Twelve isolates were selected after primary screening and subjected to SSF. Isolate 7 (identified as *R. pusillus* by MTCC, Chandigarh) was found to be the most potent one for polygalacturonase production. Polygalacturonase activities of *R. pusillus* were found to be higher than other related reports. The maximum reported polygalacturonase activity of *Mucor* sp. 7 is 15.2 U/ml (Tsereteli et al., 2009) and *Mucor circinelloides* is 9.15 U/ml (Thakur et al., 2010). In the present study polygalacturonase activity was 32.57 U/ml. No report on polygalacturonase production from *Rhizomucor pusillus* is available in the literature. However it is evident from this work that this strain is a hyper productive one and is suitable for further studies.

The present study was concentrated on analyzing the physical and chemical parameters (such as initial temperature, pH, various carbon and nitrogen sources, etc.) that influence the production of polygalacturonase in solid state fermentation. Among the various parameters, the foremost one studied was the pH. PGases was maximally produced at an initial pH of 5.0. Kunte and Shastri (1980) obtained similar results where maximum PGases activity was observed at pH 4.4. In *Thermoascus aurantiacus* maximum activity was observed at pH 5.0 by Martins et al. (2002).

Initial temperature for optimum PGases production was observed at 45°C. Phutela et al. (2005) reported that optimum temperature for PGases production of *A. fumigatus* was 50°C. Freitas et al. (2006) studied the effect of temperature on PGases production by *Monascus* sp. N8 and *Aspergillus* sp. N12 and the optimum temperature was found to be 45°C. Thakur et al. (2010) studied the same for *Mucor circinelloides* and the optimum temperature was found to be 42°C.

PGases activity was recorded maximum at the 120th h that is on the 5th day of incubation. Previous studies on this aspect revealed different results for different strains for example maximum PGases activity was reported on the 5th day for *Penicillium* sp. (Gupta et al., 1997) and on the 4th day for *Alternaria alternate* (Kunte and Shastri,

1980). Generally enzyme activity increase with increase in incubation time and reached its maximum after 3rd day. This also supports the present study.

Best carbon source was observed to be pectin (pure). These results are in harmony with previous works such as Maria et al. (2002) for *Mucor* sp., Marques et al. (2006) for *Mucor ramosissimus* and Thakur et al. (2010) for *Mucor circinelloides*.

Similarly best nitrogen source was observed to be urea. Gokhale et al. (1992) also reported the capacity of urea to prevent the drop in pH in an unbuffered fermentation medium. Kuhad et al. (2004) reported optimal production from *Streptomyces* sp. when urea was added to the basal medium devoid of yeast extract and peptone.

Mineral nutrition is very much necessary for the better growth and the metabolism of microorganisms. Couri et al. (2003) observed that addition of Fe^{2+} and/or Zn^{2+} ions was significantly positive to the enzyme production. On the other hand, Cu^{2+} and Mn^{2+} ions had almost no effect on these parameters. Saeed et al. (2007) also observed that metal ions such as Mn^{2+} increased polygalacturonase activity by up to 37%. Thakur et al. (2010) observed that addition of metal ions such as Mn^{+2}, Co^{+2}, Mg^{+2}, Fe^{+3}, Al^{+3}, Hg^{+2}, and Cu^{+2} had inhibitory effect on polygalacturonase production. These findings also supports the present study.

The maximum specificity was observed when polygalacturonic acid was used as the substrate. Previous works supporting the present study are that of Mohamed et al. (2003, 2006) and Saad et al. (2007).

The enzyme was stable at 60°C for 1 h. Thakur et al. (2010) reported that the enzyme was stable up to the 4th h at 42°C. Andrade et al. (2011) reported the enzyme retained about 82 and 63% of its activity at 60 and 70°C respectively, after 2 h of incubation.

In the present study, it was observed that the maximum stability of the enzyme was between pH 4.0-5.0 followed by a fall in stability at higher pH. Previous works supporting the present study are reported for *Aspergillus sojae*-5.0 (Tari et al., 2007), Mucor *circinelloides*-5.5 (Thakur et al., 2010) and *Cylindrocarpon* destructans-5.0 (Sathiyaraj et al., 2011).

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