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Screening and Isolation of Pectinase from Fruit and Vegetable Wastes and the Use of Orange Waste as a Substrate for Pectinase Production

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Abstract

Pectinase producing fungal strains were isolated from spoiled fruits, vegetables and soil. Of the thirteen strains isolated, Penicillium citrinum, obtained from rotten tomatoes, was found to be the most potent producer of pectinase. A comparative study on the production of pectinase under solid state and submerged fermentation systems as well as the effect of initial pH, temperature, various carbon and nitrogen sources and salts were also analyzed. Higher production of pectinolytic enzymes was observed under solid state fermentation at 30^{0}C and these enzymes were seen to have a constitutive nature.

Keywords: *Penicillium citrinum*, pectinase, polygalacturonase, pectin lyase, solid state fermentation, submerged fermentation.

Introduction

Pectic substances are the complex polysaccharides present in the middle lamella of plants and are degraded by a group enzymes, pectinases. Pectinases are both plant and microbial in origin. Pectin degrading property of pectinases using in these enzymes in fruit juice industry. Many plant pathogenic bacteria and fungi have long been known to produce pectinolytic enzymes and it is widely accepted that the production of these enzymes is a major means by which these microorganisms invade the host tissue[1]. Even though, occurrence of pectinolytic enzymes have been reported in a large number of bacteria and fungi, most commercial preparations of pectic enzymes are obtained from fungal sources[2]. This is because the pH optima of enzymes produced by fungal strains are in a range naturally found in materials to be processed. Hence fungal strains were selected in this study. Microorganisms require carbon, nitrogen, minerals, sometimes growth factors, water and oxygen if aerobic, as elements for cell biomass, energy, biosynthesis and cell maintenance. The maximum production of some metabolites requires the incorporation of specific inhibitors in the medium either to minimize formation of metabolic intermediaries or to prevent further metabolism of the desired product[3]. The prime ingredients of the media are water, energy sources, sources for carbon, nitrogen and minerals, chelators, growth factors, buffers, precursors, inhibitors etc. The literature on the various cultural conditions for the PG and PL production from *Penicillium citrinum* is scanty. This work is a preliminary study on the impact of various physiological as well as biochemical conditions for the better output of pectinase from *Penicillium citrinum*.

Material and Methods

The chemicals used were purchased from Himedia, Merck and Sigma and the spoiled fruits, vegetables and soil used in the study were collected locally (Kottayam, Kerala, India).

Isolation of pectinolytic fungi: In order to get a cheap source for the pectinase production, fungi were isolated from locally available spoiled fruits, vegetables and soil by using the modified pectin agar medium proposed by Jayashankar and Graham[4]. In brief, two fold dilutions of the spoiled samples were plated in pectin agar medium and incubated at room temperature for 4-6 days. The strains grown in the plate were subcultured into pectin agar medium separately and flooded with 1% cetrimide for primary screening. The pectinolytic fungi produced a clearing zone when exposed to cetrimide.

Composition of the media used for isolation (g/l, wt/vol.): Pectin 5.00, K_2HPO_4 0.50, $MgSO_4.7H_2O$ 0.10, NaCl 0.20, $CaCl_2.2H_2O$ 0.20, $FeCl_3.6H_2O$ 0.01, Yeast extract1.00, Agar 20.00. pH-5.0.

Secondary screening: The fungal strains which showed clearing zones in pectin agar medium were selected for secondary screening by estimating the polygalacturonase (PG) and pectin lyase (PL) production under solid state fermentation by using the modified medium of Maldonaldo and Strasser de Saad[5].

The composition of the salt solution used for solid state fermentation media g/100ml (weight/volume): Urea 0.30, K_2HPO_4 0.65, $(NH_4)_2SO_4$ 1.26, $MgSO_4$ 0.02, $FeSO_4$ 0.029, Pectin 1.50, Glucose 0.50. pH-4.5

Enzyme assays: Polygalacturonase activity was measured by determining the amount of reducing groups released according to the method described by Nelson[6] and modified by Somogyi[7]. One unit of enzyme activity has been defined as the amount of enzyme that releases one µ mol of galacturonic acid /min under the assay conditions.

Pectin lyase activity was measured by the reaction between unsaturated end products of pectin degradation and

thiobarbituric acid[8]**.** One unit of activity is the amount of enzyme causing a change in absorbance of 0.01 under the conditions of the assay.

All experiments were conducted in triplicate and the mean values of all the sets of observations were taken for evaluation of results.

Identification: All strains were identified up to genus level by examining the morphological characters according to the method described by Dubey and Maheswari[9]. The strain produced pectinase maximally in the secondary screening was identified at microbiology laboratory of IMTECH, Chandigarh and deposited in their culture collection (MTCC 6590). This particular strain was isolated from rotten tomato samples.

Impact of various factors: The effect of various physical and biochemical parameters analyzed were: - comparison of solid and submerged fermentation methods, initial pH on enzyme production, incubation temperature, carbon and nitrogen sources and the various salts. Preparation of solid substrates and inoculums, inoculation and incubation, recovery and assay of enzymes were carried out as described in previous sections.

Results and Discussion

As the main objective of this study was to isolate pectinolytic enzymes from the cheap sources, spoiled fruits, vegetables and soil were selected. The strain which produced maximally was cultured by changing the various parameters for the better output.

The fungal strains isolated from spoiled fruits and vegetables underwent cetrimide treatment for pectinolytic identification. Of them, thirteen strains showed pectinolytic activity (table1) and they were further cultured under solid state fermentation for determining pectinase activity (table 2).The strain isolated from rotten tomato samples which showed maximum activity was send to IMTECH, Chandigarh for further identification and it is identified as *Penicillium citrinum* Thom. (figure 3) and deposited in their culture collection (MTCC 6590).

Table-1
Primary screening of Pectinolytic fungi isolated from different sources

SlNo	*Source*	Pectinolytic fungi
1	*Soil*	*Penicillium* sp., *Rhizopus* sp.
2	Tomato	*Rhizopus* sp., *Penicillium* sp., *Aspergillus* sp.
3	Orange	*Penicillium* sp., *Rhizopus* sp.
4	Apple	*Neurospora* sp.
5	Pineapple	*Mucor* sp.
6	Lemon	*Rhizopus* sp., *Mucor* sp.
7	Carrot	*Penicillium* sp.
8	Brinjal	*Aspergillus* sp.

sp.: species

Table-2
Secondary screening for pectinolytic fungi under solid state fermentation

Sl. No	Fungi	Polygalacturonase units/gds*	Pectin lyase units/gds*
1	*Rhizopus* SBSS1	1.510	1.756
2	*Rhizopus* SBST2	0.935	1.544
3	*Rhizopus* SBS03	0.777	1.386
4	*Rhizopus* SBSL4	1.081	1.575
5	*Mucor* SBSP1	0.625	1.455
6	*Mucor* SBSL2	1.110	1.171
7	*Aspergillus* SBST1	3.265	1.298
8	*Aspergillus* SBSB2	11.323	1.400
9	*Penicillium* SBSS1	6.638	1.389
10	*Penicillium* SBST2	33.547	2.070
11	*Penicillium* SBSO3	9.610	1.70
12	*Penicillium* SBSL4	7.839	1.622
13	*Neurospora* SBA1	6.138	1.427

*gds: grams/dry weight of substrate

In the present study, a significant activity of activity could detect for both polygalacturonase and pectin lyase (33.0545 units/gds and 2.070 units/gds respectively). Also, because of the availability of this strain from the cheap sources and its comparatively better production it can be recommended for further studies and commercial use.

Penicillium citrinum was cultured under solid state and submerged fermentation methods for comparing the efficacy of the two systems in the enzyme production. Solid state fermentation was done by using wheat bran as a solid substrate. Cultural conditions were identical for both types of fermentations. But a higher pectinase production was observed in solid state fermentation than submerged fermentation. (table 3) and it has been reported that exo-, endo polygalacturonase as well as pectin lyase production is in SSF than in SmF[10,11]. The higher production of solid state fermentation is due to the fact that catabolic repression is less affected by solid state fermentation than by submerged fermentation[10,12]. The quantity of the enzyme per litre of the extract from solid state fermentation is 10 times more than with submerged fermentation technique.

The initial pH in the medium was varied from 2.0-9.0, and a 0.5 unit difference was tested between pH 4.0-6.0.Even though maximum polygalacturonase (PG) and pectin lyase (PL) production were observed at pH 5.0 and 8.0 respectively.

Studies done in *A.niger*[13] and *Penicillium* sp.[10] (pH5.0 for PG) support this. Small peaks were also observed at pH 8.0 and 4.5 (figure 1). The two pH optima may be due to the presence of two isoenzymes[14]. Studies conducted in P.sp.[15] say that pectin lyase production is better in alkaline pH.

Table-3
Comparison of submerged and solid state fermentation in the production of polygalacturonase and pectin lyase from *Penicillium citrinum*

Type of fermentation	Submerged fermentation* (µmol/ml/min)	Solid state fermentation* (Units/gds)
Polygalacturonase	0.067	34.5
Pectin lyase	0.052	2.07

gds: grams/dry weight of substrate

Penicillium citrinum was cultured under various temperature conditions (4.0-50^{0}C). Maximal activity was observed at 30±2^{0}C (room temperature) for both polygalacturonase and pectin lyase (figure2). Temperature is directly related to the metabolic activities of the microorganism and it affects the proper growth and product formation by the organism[16]. Similar observations were reported in *Aspergillus niger* (30^{0}C)[17,18] and *Penicillium frequentans* (30-35^{0}C) and it is also reported that lower temperature slows down the hydrolysis of pectin.

Effect of various carbon sources on the production of polygalacturonase and pectin lyase by *Penicillium citrinum* was studied. In this study glucose is used as a sole carbon source. In addition to this various other carbon sources like pectin, polygalacturonate, galactose, fructose, xylan, sucrose and CM cellulose have been tried. Xylan and fructose were found to be inducers of Polygalaturonase and Pectin lyase respectively (table 4). Previous studies on the effect of carbon sources on different strains are *Penicillium frequentans*[18], *Aspergillus japonicas 586*[19], and in *Aspergillus niger*[20] in submerged fermentation the pectinase activity is inhibited by the presence of glucose and other sugars in the medium. In solid state fermentation presence of glucose does not inhibit pectinase production, since solid state fermentation is less affected by catabolic repression. That may be the reason for better production of polygalacturonase and pectin lyase in presence of glucose than its absence.

However, when orange peel alone was used as the carbon source, a better production of ploygalacturonse was observed (287.22 units/gds). This is a major finding and can be used for the cheap production of pectinase in large scale by using the orange waste as the sole carbon source.

Carbon sources other than pectin induced pectinase production like, galacturonic acid**,** fructose and mannose induced polygalacturonase production in *Geotrichum candidum*[21], and galactose and starch induced pectinase production in *Bacteroides ovatus*[17]**.** But it varies in different strains. The observation indicates the constitutive nature of the enzymes and also the stimulating capacity of carbon source for the production of polygalacturonase and pectin lyase also varies from strain to strain.

Table4
Effect carbon sources on polygalacturonase production from* Penicillium citrinum *under SSF

Sl. No	*Carbon source (1.5%)*	Polygalacturonase* (Units/gds)	Pectin lyase (Units/gds)
1	Wheat bran+ Glucose	14.511 ± 0.016	1.231
2	Wheat bran+ Glucose+ pectin	34.4 ± 0.012	1.9
3	Wheat bran + Pectin	6.060 ± 0.023	0.241
4	Wheat bran+ Glucose+ PGA	17.173 ± 0.030	1. 465
5	Wheat bran+ Glucose+ Xylan	46.597 ± 0.021	2.165
6	Wheat bran+ Glucose+ CM Cellulose	14.577 ± 0.025	1.298
7	Wheat bran+ Glucose+ Galactose	5.768 ± 0.032	0.422
8	Wheat bran+ Glucose+ Fructose	1.182 ± 0.030	2.638
9	Wheat bran+ Glucose+ Sucrose	2.193 ± 0.109	1.340

gds: grams / dry weight of substance, PGA: polygalacturonic acid

Microbes use nitrogenous compounds for the synthesis of proteins, enzymes and nucleotides[22]. The nitrogen sources used were urea, yeast extract, casein, tryptone and peptone, while urea was taken as control. Here polygalacturonase and pectin lyase had maximum activity when urea was incorporated in the medium. All others had inhibitory effect in the production of both polygalacturonase and pectin lyase (table 5). The result is in agreement with the previous reports[23,24] where only urea activated the production of polygalacturonase. The inhibitory effect of other nitrogen sources may be due to disturbance in the balance between carbon and nitrogen source in the medium. As a result, the pH control of the system may be lost.

In the case of polygalacturonase, activity was enhanced by $CaCl_2$ only. While for pectin lyase the addition of various salts had no impact on the enzyme activity (table 6). Mg^{2+} and Mn^{2+} were the activators of polygalacturonase, and $NaCl_2$, $BaCl_2$, $FeCl_3$ and $ZnCl_2$ were the potent inhibitors of polygalacturonase[25].

Table5
Effect of nitrogen sources on polygalacturonase production from* Penicillium citrinum *under SSF

Sl.No	Nitrogen sources (0.3%)	Relative activity (%) PG	Relative activity (%) PL
1	Urea (Control)	100	100
2	Yeast extract	8.010	69.850
3	Casein	3.494	28.050
4	Tryptone	1.327	71.710
5	Peptone	0.800	82.530

PG: polygalacturonic acid, PL-pectin lyase

Table-6
Effect of salts on polygalacturonase production from *Penicillium citrinum* under SSF

Sl. No	Salts (0.02%)	Relative activity (%) PG	Relative activity (%) PL
1	Control	100	100
2	$CaCl_2$	121.800	42.340
3	$BaCl_2$	37.500	52.540
4	$FeCl_3$	5.810	-
5	$ZnCl_2$	7.500	41.792
6	$NiCl_2$	37.550	31.010
7	$MnSO_4$	37.390	29.800

PG: polygalacturonic acid, PL-pectin lyase

Conclusion

Pectinases are novel enzymes which are integral in fruit processing industry. In this study a group of pectinolytic fungi were isolated from cheap sources like spoiled fruits and vegetables and *Penicillium citrinum* was found to be the potent source for pectinase. Polygalacturonase and pectin lyase production was higher in solid state fermentation by using wheat bran as the solid substrate than submerged fermentation at room temperature.

Pectinase produced had a better constitutive and less catabolically repressive nature. Previous reports on the production of pectinase by *Penicillium citrinum* under solid state fermentation are less. Since the SSF has of special economic interest for countries with large amount of agroindustrial residues and orange peel can be used as a carbon source for pectinase production in large scale. Larger, scale-up studies are needed for the better output for commercial production.

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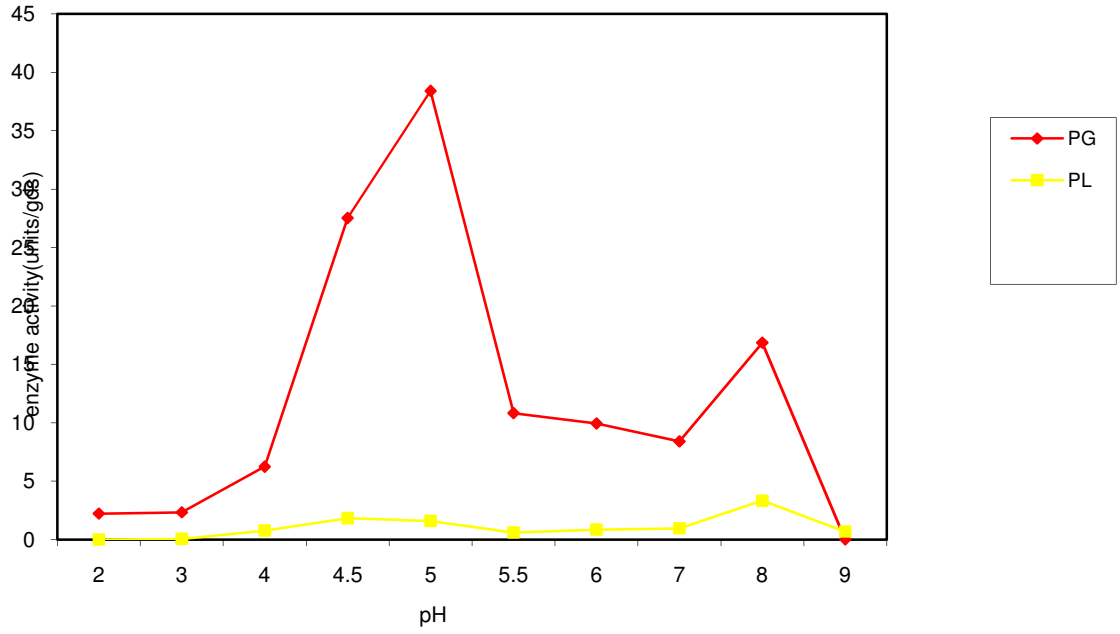

Figure-1
Effect of pH on the production of PG and PL from P.citrinum under SSF

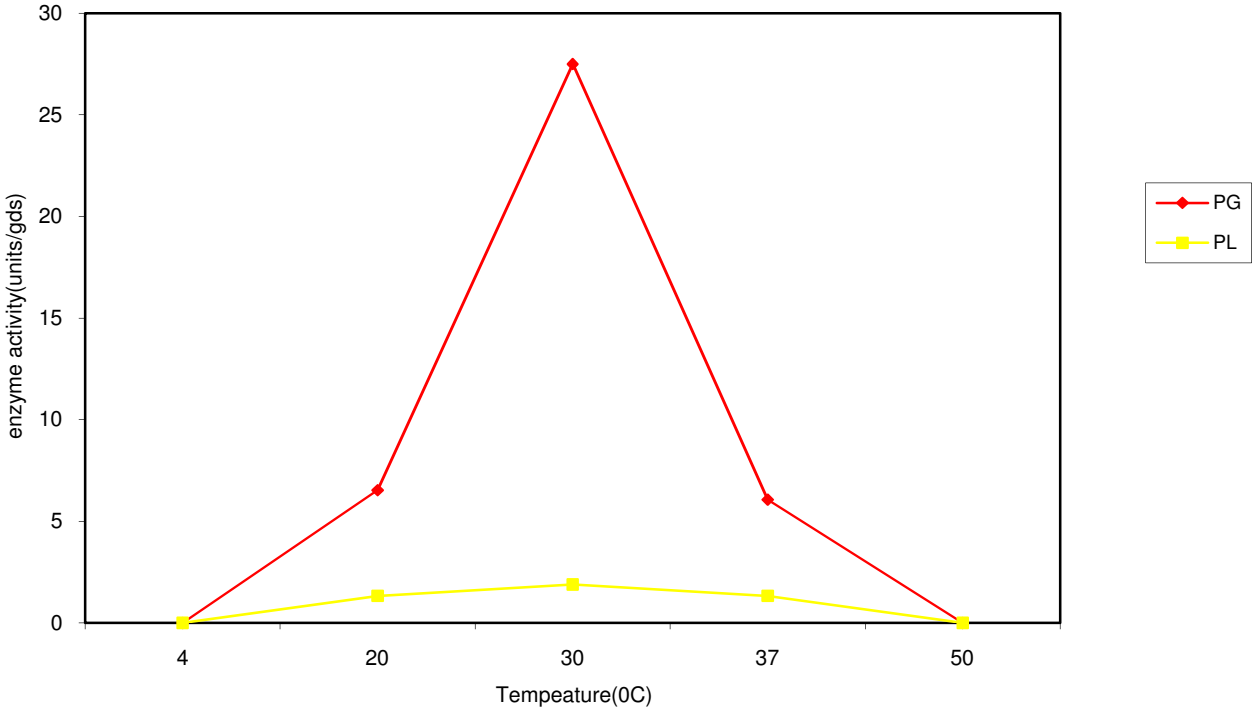

Figure-2
Effect of temperature in the production of PG and PL under SSF from P.Citrinum

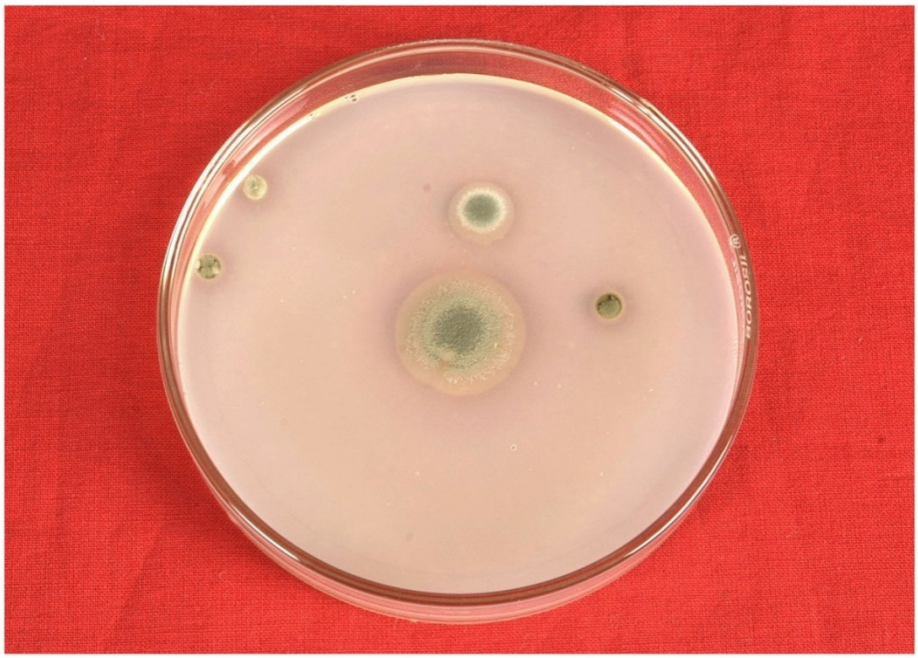

Figure-3
Photograph of Penicillium citrinum showing the clearing zone, when flooded with cetyl trimethyl ammonium bromide.

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