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Optimization of Processing Parameters for Production of Pectinolytic Enzymes from Fermented Pineapple Residue of Mixed *Aspergillus* species

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Abstract

The present study aims at exploring the production of pectinolytic enzymes from an easily available and cheaper agro-residue (dried pineapple residue) fermented by newly isolated mixed culture of *Aspergillus fumigatus* and *Aspergillus sydowii* through solid-state fermentation. The optimum conditions that influence the extraction of pectinases from the fermented substrate were temperature, pH and incubation period, 35°C, 5.0 and 48h respectively. Among the carbon sources tested for maximum production of enzymes, 2% starch yielded maximum polygalacturonase (PG), whereas 3% sucrose yielded pectin lyase (PL) and 3% glucose enhanced production of pectate lyase (PAL).

Keywords: Pectinases, pineapple fruit residue, *Aspergillus fumigatus, Aspergillus sydowii, m*ixed culture**.**

1. Introduction

Pectinases are the group of enzymes involved in de-polymerization of pectic polymers. This group of enzymes consists of pectin esterase, PE (E.C 3.1.1.11), polygalacturonase, PG (E.C 3.2.1.15), pectate lyase, PAL (E.C 4.2.2.2) and pectin lyase, PL (E.C 4.2.2.10) (Yadav *et al.,* 2008). 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. The side chains of pectin molecule consist of rhamnose, galactose, arabinose and xylose. The carboxyl groups of galacturonate are esterified with methanol and based on the degree of esterification, the pectic substances were differentiated into protopectin, pectin, polygalacturonic acid and pectinic acid (Gummandi and Kumar, 2006).

Pectinolytic enzymes are of significant importance in the current biotechnological era with their all embracing applications in fruit juice extraction and its clarification. In addition, are involved in degumming of plant fibers, vegetable oil extraction, tea and coffee processing, and in alcoholic beverages etc. They have a share of 25% in the global sales of food enzymes (Yugandrah *et al.,* 2008). Emerging new applications underline the importance of large-scale production of these enzymes (Gummandi and Kumar, 2005).

Carbon sources especially of agrarian source are more suitable because they are cost effective, renewable and available in large quantities (Yugandhar *et al.,* 2008). Hence we made an attempt to examine the utility value of inexpensive, renewable and readily available raw material like pineapple residue powder (pectin-rich agrowaste) which is a byproduct of fruit juice industry and was not reported in literature so far, for the production of pectinases. To meet the demand of pectinases for different industrial applications, its production was carried out by co-culture of newly isolated mixed culture of *Aspergillus fumigatus* and *Aspergillus sydowii* 7373.09 ITCC grown on pineapple fruit residue.

The yield of pectic transeliminases (PL and PAL) in amount is much lower, compared to other pectinases. For example, the amount of PL and PAL produced are less than 1U (or 1mU/ml) (Alana *et al.*,1989, 1990; Nakagawa *et al.,* 2000; Panda and Naidu, 2000). Very few strains such as *Candida boidinii* and *Paenibacillus sp.* are known for producing two enzymes together, i.e. PL and PAL (Gummandi and Kumar, 2007). The use of mixed cultures or microbial associations for production of multi enzyme complexes is not new as Stoilova (Stoilova *et al.,* 2008) experimented with microbial association of *Aspergillus niger* and *Fusarium moniliforme* for overproduction of

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laccase and pectinase. Hence an attempt was made to co-culture two fungal strains for obtaining PG, PL and PAL in an appreciable amount, in single solid state fermentation process which can reduce the cost of enzymes production. In solid-state fermentation, growth of microbes occurs on the insoluble substrates in the absence of any free liquid phase and with optimum moisture content necessary for proper growth of microbes as well as production of enzymes.

2. Materials and Methods

2.1. Chemicals

D-Galacturonic acid was procured from Sigma Chemical Co., St. Louis, USA. All other chemicals used were of analytical grade, supplied by Hi Media Lab., Bombay, India.

2.2. Isolation and screening of micro-organisms

Soil samples containing microbes were collected from soils (local fruit market) containing citrus fruit residues. 1g soil was mixed in 20 mL distilled water and after settling 1.0 mL of supernatant was taken in a 250-mL Erlenmeyer flask and mixed with 10-mL medium containing (g/L) citrus pectin, 10; NaH_2PO_4, 7.8 ; KH_2PO_4, 13.6 ; $CoCl_2$, 0.014 ; $MgSO_4.7H_2O$, 0.246 and $(NH_4)_2SO_4$, 2.0, at pH 5.0 by shaking. A loopful of the homogenate was then streaked onto the nutrient medium containing agar (3%) and incubated at 30°C for 24 h to 72 h. All morphologicaly different colonies were purified by repeated streaking. Culture exhibited maximum growth was identified as mixed culture of *Aspergillus fumigatus* and *Aspergillus sydowii* 7373.09 ITCC by Indian Type Culture Collection (ITCC), division of I.A.R.I, New Delhi, and was employed for further experiments. These two strains were separated by repeated streaking. An experiment was carried out using these strains separately and in mixed form in different flasks (Figure 1) under the similar conditions.

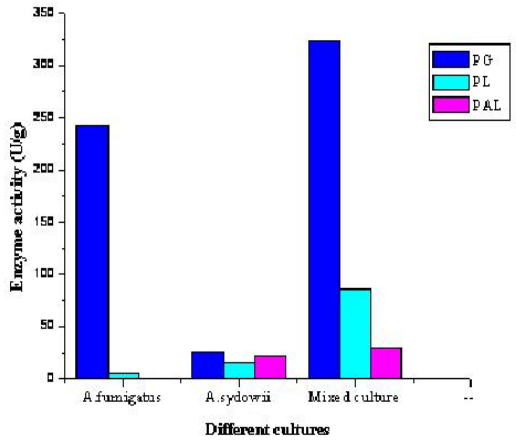


Figure 1. Production of pectinases under solid state fermentation by single cultures and mixed culture.

On comparing them (Figure 1) it becomes clear that *A. sydowii* can produce PG, PL and PAL in very small amount whereas *A. fumigatus* can produce PG and PL only. But when they were co-cultured they can produce three enzymes together in an appreciable amount in a single fermentation process. From industrial point of view it is very beneficial to obtain them together as it reduces the cost of process and consumption of energy too.

The isolated culture was sub-cultured after every 2 weeks onto the agar medium and maintained at 4°C.

2.3. Inoculum

For inoculum preparation, the isolated mixed culture was grown at 35°C for 7 days, and the spores (1×10^7) were scraped into 25-mL of sterile Tween 80 solution, 10 mL of which was used to inoculate 5g of substrate.

2.4. Preparation of substrate

Pineapple residue was obtained after extraction of juice from the fruits which was previously peeled and decored. Pineapple residue was spread on trays and dried in an oven at 60°C for 24 h. The dried residue (chemical composition (g/100g); Protein, 0.5; Fat, 0.12; Carbohydrate, 13.12; Fiber, 1.4; Sucrose, 9.85; Glucose, 5.99; Fructose, 1.73; Ash, 0.27 and Vitamin C, 47.8 (mg)) was grinded and sieved with a screen of ISS mesh no. 40 to obtain an average particle size of 425 µm and stored in polyethylene bags at temperature (30±5°C).

2.5. Enzyme production

Solid-state fermentation (SSF) was carried out in a sterile 250-mL Erlenmeyer flask, mouth plugged with non-absorbing cotton, which contains weighed amount of pectin-rich agro-residue (dried pineapple residue) moistened to desired moisture level with mineral salt solution containing (g/L): $(NH_4)_2SO_4$, 0.1; $MgSO_4.7H_2O$, 0.5; KH_2PO_4, 0.5 and $FeSO_4.7H_2O$, 0.05 at pH 5.0 and then sterilized at 15 psig (121°C) for 15 min followed by cooling to the desired temperature for inoculation with required volume of inoculum. The flask was then incubated under static condition for appropriate time. In order to estimate enzyme activity, measured volume of sterile water was added to the flask containing fermented substrate, mixed properly, filtered to obtain 40-mL extract and then centrifuged at 10,000g for 10 min. The obtained supernatant was used as crude enzyme solution for assay.

2.6. Enzyme assays

Polygalacturonase (PG) was assayed by measuring the reducing sugars released from the action of pectinase on citrus pectin using 3,5-dinitrosalisylic acid (DNS) reagent (Miller, 1959) from the reaction mixture containing 0.8 mL of crude enzyme solution and 0.2 mL (1%) citrus pectin in 0.2M acetate buffer of pH 5.0, incubated for 10 min at 40°C. One unit of PG is defined as the amount of enzyme that liberates 1 µmol of galacturonic acid per min under the assay conditions.

Pectin lyase (PL) activity was determined spectrophotometrically by monitoring the increase in absorbance (Albershiem, 1959). The reaction mixture contains the following: 1.25-mL of 0.15M citrate-phosphate buffer (pH 5.5), 0.25 mL of culture filtrate and 1.0 mL of 1% (w/v) citrus pectin. Pre-incubation were carried out at 40°C for 15 min. The reaction was started by adding pectin, control tubes contained the enzyme previously inactivated by incubation for 10 min at 100°C. One unit of PL activity was the amount of enzyme which produced an increase of one unit of A_{235} per minute.

Pectate lyase (PAL) activity was measured by the increase of A_{235} of 4,5 unsaturated reaction product. First, 0.3 mL of 1% (w/v) polygalacturonic acid neutralized by NaOH was mixed with 1.5-mL of $CaCl_2$ solution (0.0005 M in 0.1 M Tris-HCl [pH 9.0]) and 1.1 mL of distilled water. Then, 0.1 mL of enzyme sample was quickly added and the increase in A_{235} was measured (Collmer *et al.*, 1988). One unit of PAL was defined as the amount of enzyme which produces 1 µmol of unsaturated product per min. The molar extinction coefficient for the unsaturated product at 235 nm (ε_{235}) is 4,600 M^{-1} cm^{-1}.

2.7. Statistical analysis

All the experimental results reported are average of triplicate values, which are represented in the respective graphs with error bars.

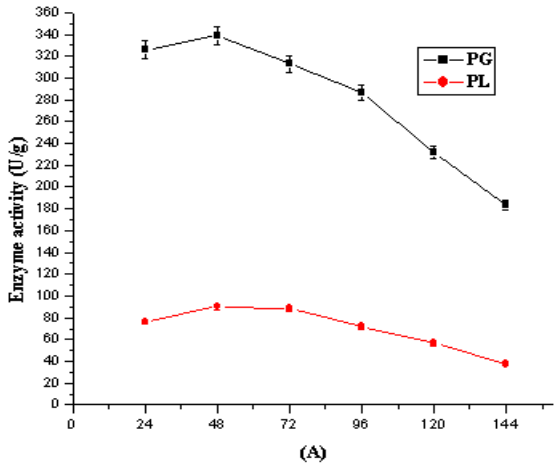
3. Results and Discussion

3.1. Optimization of Process Parameters for Pectinases Production

Several experiments were carried out for pectinase production by isolated mixed culture of *Aspergillus fumigatus* and *Aspergillus sydowii* 7373.09 ITCC under SSF using pineapple residue powder as substrate. To determine the optimal values different process conditions were optimized, the range within which the parameters have been studied were incubation period (24-144h), pH (4-8), moisture content (50-90%), substrate concentration (2-8g) and temperature (25-50°C).

3.2. Effect of Incubation Period.

The experiments were done at different time duration (from 24 to 144 hours) at an interval of 24h for pectinases production under SSF at 30°C at pH 5.0. Experimental data from Figure 2 indicate that the production of PG, PL and PAL increased with fermentation duration upto 48h beyond that, the production of the enzymes decreased gradually may be due to the depletion of nutrients. Whereas Natalia Martin (Martin et al., 2004) reported maximum polygalacturonase yield using wheat bran: sugarcane bagasse: orange bagasse (1:1:1) as substrate after 96h of incubation, under SSF conditions by Penicilliun sp.EGC5. The time of incubation depends on the growth rate of the microorganism and its enzyme production pattern.



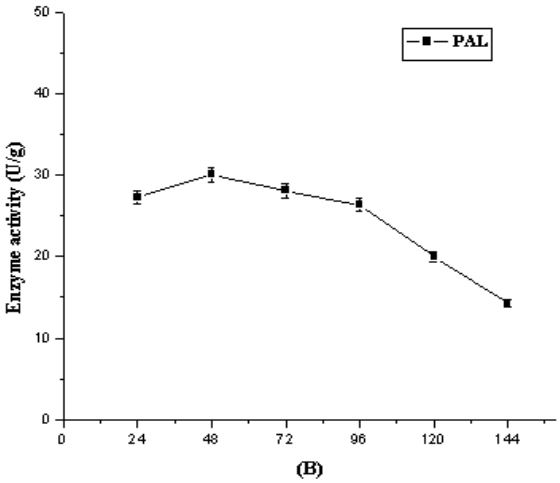
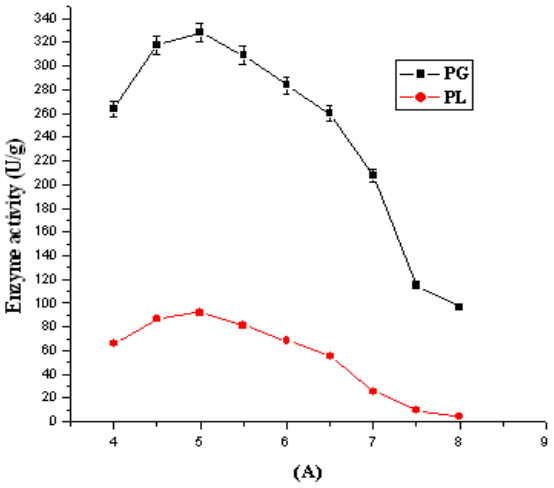


Figure 2. Effect of incubation time (h) on pectinases production under SSF **(A)** PG and PL **(B)** PAL.

3.3. Effect of pH

The impact of pH of the medium on enzyme production by isolated mixed culture under SSF was studied by adjusting the pH in a range of 4.0 to 8.0 at a difference of 0.5. The results from Figure 3 indicate that the production of polygalacturonase (328.1 U/g), pectin lyase (92.3 U/g) and pectate lyase (28.7 U/g) in terms of activity increased with medium pH upto 5.0 and further increase in pH causes decrease in activity of enzyme, which could be due to the fact that as the acidity of fermentation medium increases the activity or production of enzymes decreases. Whereas Friedrich (Friedrich et al., 1994) and Hayashi (Hayashi et al., 1997) reported an optimum pH of 4.5 and 7.0 for maximum PL and PAL production by A. niger A138 and Pseudomonas marginalis MAFF03-01173, respectively.



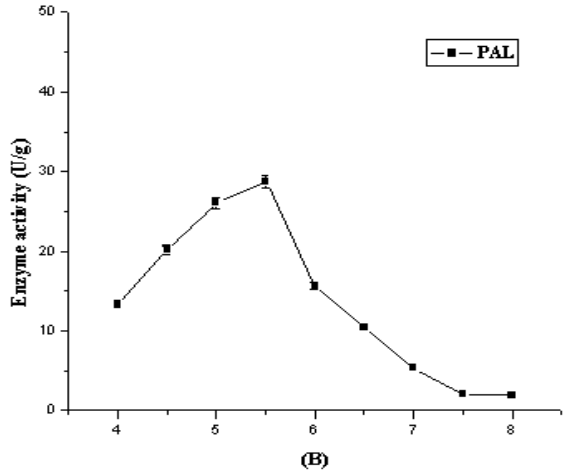


Figure 3. Effect of pH on production of pectinases under SSF at 30°C for 48h (A) PG and PL (B) PAL.

3.4. Effect of moisture content

The moisture content of substrates affects their physiochemical properties, microbial growth as well as enzyme production and thus an overall productivity of process. Experiments were carried out with different moisture levels ranging from 50 to 90% at a difference of 10 and the results of enzyme production are shown in Figure 4.

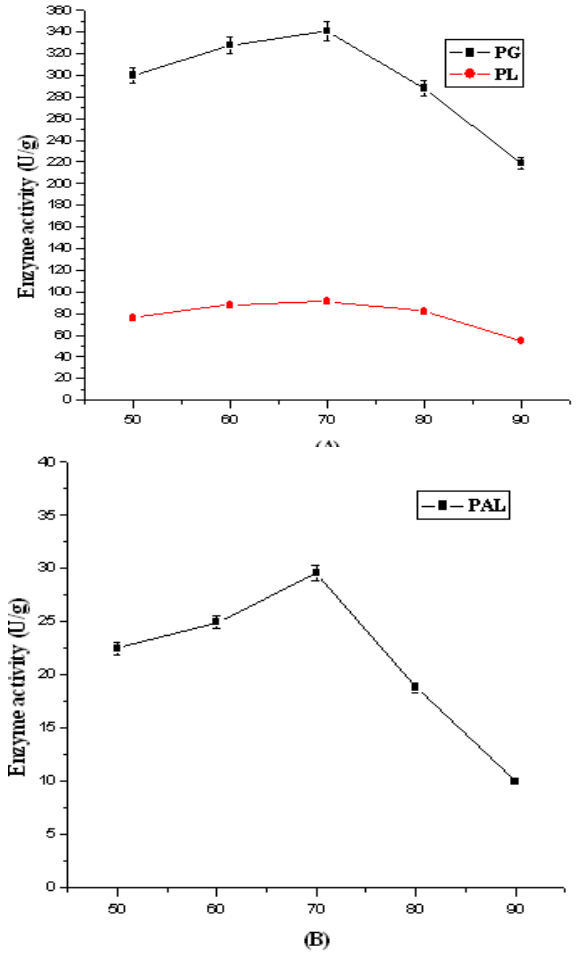


Figure 4. Effect of moisture content (%) on production of pectinases under SSF at 30°C and pH 5.0 for 48h fermentation (A) PG and PL (B) PAL.

It was found that on every 10% increase in moisture from 50% moisture level, enzyme activity increases to 70% moisture content in substrate and a further increase in moisture content decreases pectinase activity. Microbes generally grow near the outer surface of the substrate particle, at the same time evaporation of water takes place due to generation of heat during microbial growth. But water uptake in new biomass and evaporation are thus localized at the surface of the substrate particles. Hence the optimum humidity allows the entry of nutrients easily through the cell membrane, which favours maximum enzyme production. Any deviation from the optimum humidity results in the decrease in enzyme activity, which may be due to osmotic imbalance inside the cell causing cell lysis. Here the maximum production of PG (340.2 U/g), PL (90.3 U/g) and PAL (29.6 U/g) were obtained at 70% moisture content.

3.5. Effect of Substrate Concentration

The effect of substrate concentration on dried pineapple pulp powder was studied by incubating 1 to 8 g amounts at 30°C for 48 h at 70% moisture (Figure 5). Increasing the substrate concentration from 2 to 5 g gradually increased enzyme production, but above 5 g yield decreased significantly. The data from Figure 5 show that on increasing substrate concentration from 2g to 5g, the production of enzymes gradually increased but above which it decreased significantly. When the substrate amount was increased above the optimum level, the increased bed height might have caused inadequate diffusion of the liquid medium across the bed as well as in contact of microbes, hindering the process of biotransformation. In this experiment, the maximum enzyme activities, PG (340.8U/g), PL (91.6U/g) and PAL (30.4U/g) were observed in case of 5g powdered pineapple pulp.

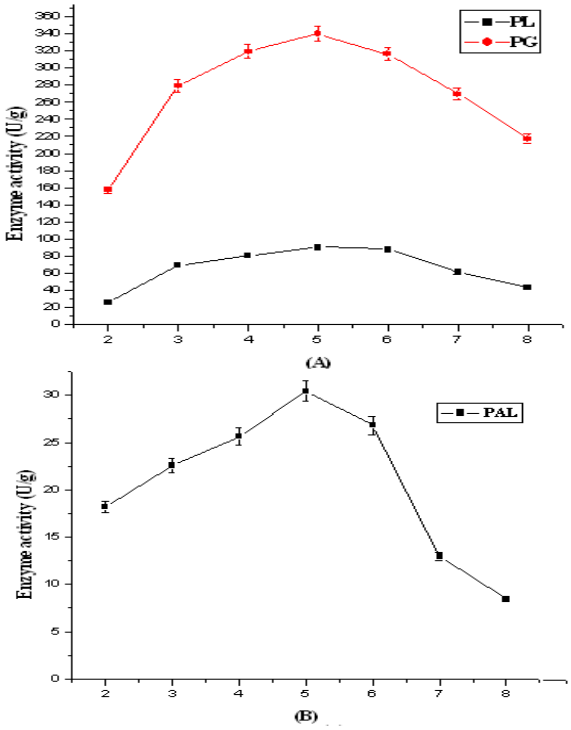


Figure 5. Effect of substrate concentration (g) on pectinases production under SSF using pineapple pulp as a substrate at 30°C and pH 5.0 with 70% moisture for 48h (A) PG and PL (B) PAL.

3.6. Effect of Temperature

Temperature is a very important factor for microbial growth as well as microbial product formation. That is why several experiments were carried out at different temperatures (25-50°C) to see the effect of temperature on pectinase production and the results are shown in Figure 6 indicates that maximal production of enzymes depend on fermentation temperature and the maximum yield in terms of activity of PG (343.2U/g), PL (96.6U/g) and PAL (31.6U/g) was obtained at 35°C. However, the enzyme activities predominantly decreased due to inactivation of enzymes at temperatures higher than that of 35°C. de Frietas (de Frietas et al., 2006) reported an optimum temperature 50°C for PG production by SSF using Aspergillus sp. N12 strain, whereas Gummandi (Gummandi et al., 2007) and Nakajima (Nakajima et al., 1999) reported an optimum temperature 30°C and 37°C for PL and PAL production with A. niger NCIM548 and Clostridium butyricum-beijerineki, respectively.

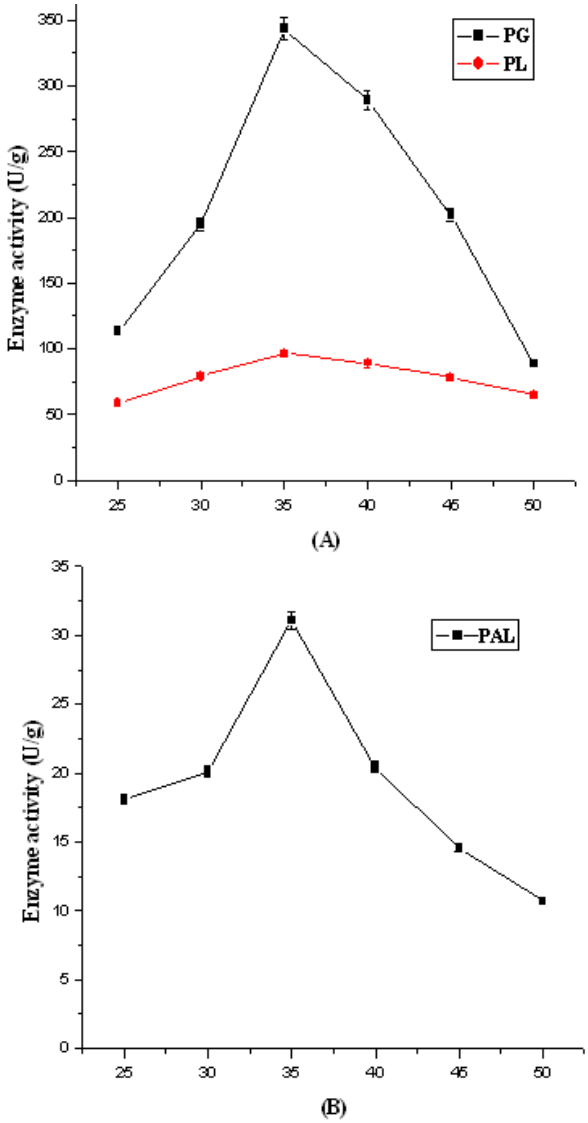


Figure 6. Effect of temperature (°C) on pectinases production under SSF using pineapple pulp as substrate with 70% moisture at pH 5.0 for 48 h (A) PG and PL (B) PAL.

3.7. Effect of Carbon Sources

The influence of variety of carbon sources on production of pectinases was studied using different concentration (1-5%) of carbon sources for each of glucose, pectin and sucrose separately along with 5g of powdered pineapple pulp and the results are indicated in Figure 7. The maximum yield of PG was obtained with 2% starch (433.6 U/g) and 2% glucose (408.2 U/g) whereas of PL with 3% pectin (253.6 U/g) and 3% sucrose (296.7 U/g) and PAL with 1% pectin (48.3 U/g) and 3% glucose (57.6 U/g) under the optimized conditions. The data shown in Figure 7 signify that on increasing concentration of carbon sources, enzyme activity started decreasing due to substrate inhibition as well as catabolic repression. Probably, this is due to the presence of a high galacturonic acid concentration from pectin degradation. These findings are similar to that reported by Aguilar and Huitron (Aguilar and Huitron, 1987) and of Maldonaldo (Maldonaldo et al., 1989) which showed that the production of these enzymes is directly correlated with substrate concentration as shown in Aspergillus sp.

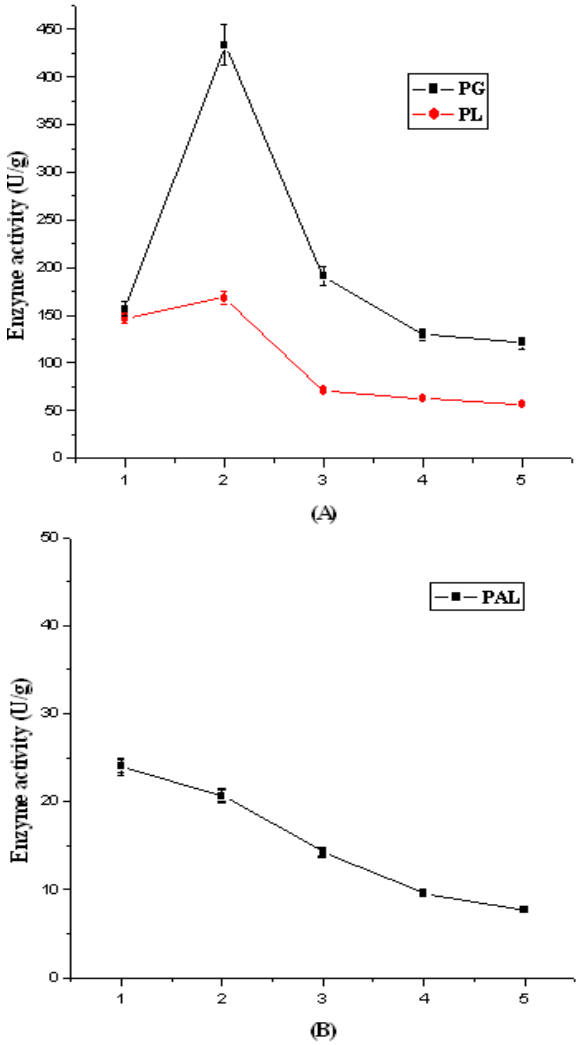


Figure 7. Effect of different concentrations of starch (%) on pectinases production under optimized conditions (A) PG and PL (B) PAL.

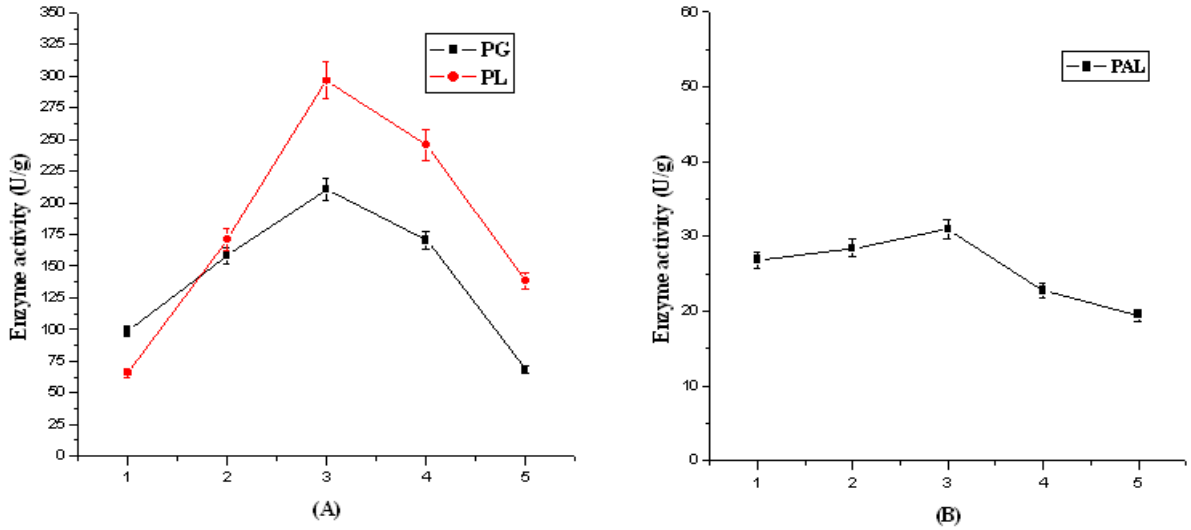


Figure 8. Effect of different concentrations of sucrose (%) on pectinases production under optimized conditions **(A)** PG and PL **(B)** PAL.

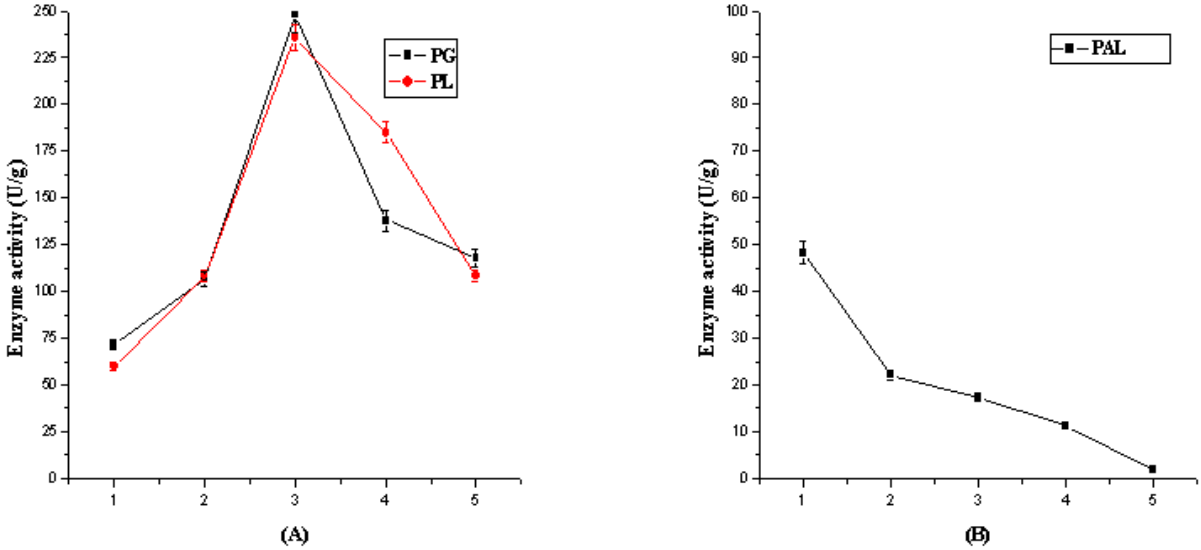


Figure 9. Effect of different concentrations of pectin (%) on pectinases production under optimized conditions **(A)** PG and PL **(B)** PAL.

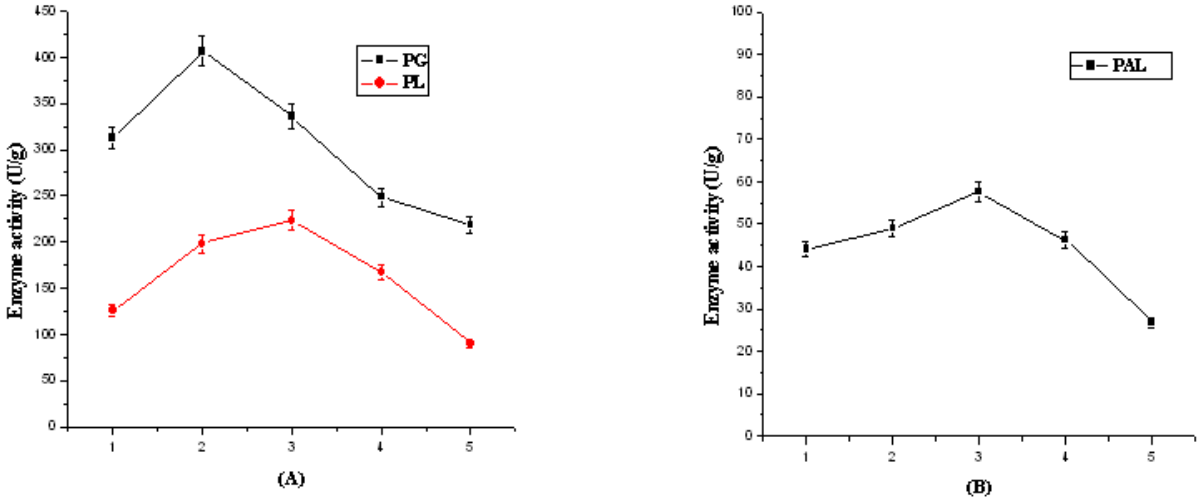


Figure 10. Effect of different concentrations of glucose (%) on pectinases production under optimized conditions **(A)** PG and PL **(B)** PAL.

4. Conclusion

Pineapple residue an easily available and cheaper agro-residue was successfully used as a solid state support by mixed culture of *Aspergillus fumigatus* and *Aspergillus sydowii* at 35°C for production of pectinolytic enzymes and it has not been reported so far. Optimization of processing parameters resulted in higher production of pectinases. The effect of carbon sources such as glucose, sucrose, starch and pectin were also investigated on the production of pectinases and starch was indentified to induce maximum PG at 2% (w/v).

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