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PRODUCTION OF PECTINASE BY *Trichoderma harzianum* IN SOLID STATE FERMENTATION OF CITRUS PEEL

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The growth medium of the citrus peel was fermented by *Trichoderma harzianum* in solid state fermentation (SSF) at pH 5.5 and 28°C for 72 hours. It was observed that addition of yeast extract, and tween-80 into the growth medium enhanced pectin lyase production by *Trichoderma harzianum* whereas peptone showed negative effect on microbial growth. The maximum activity of pectin lyase (16.2 U/mL/min) was recorded in the culture filterates harvested after 72 hours of SSF of basal growth medium of citrus peel with 70% moisture at pH 5.5 in the presence of 0.4% yeast extract, 2.5 ml (25%) Inoculum and 0.2% tween-80. The comparison of solid state fermentation (SSF) and liquid state fermentation (LSF) showed that SSF was better than LSF as it gave higher yield of pectin lyase (15.55 U/mL/min) as compared to LSF (10.57 U/mL/min). The enzyme showed maximum activity, at pH 7 and 40°C temperature, but it showed considerable stability upto 60°C. Pectin lyase produced in SSF was partially purified by ammonium sulfate precipitation and residue showed more enzyme activity (41.745 U/mL/min) than supernatant (31.59 U/mL/min) after precipitation with 20% ammonium sulfate.
Key words: Citrus peel, Solid state fermentation, Pectin lyase, Optimization, *Trichoderma harzianum*

INTRODUCTION

Industrial transformation of citrus gives rise to several wastes besides the main products juices and essential oils. The problem arising from waste orange peels disposal are the best studied because of the large yearly production which has led to different but not exhaustive proposals for disposal by single cell protein (SCP) production (Vaccarino *et al.*, 1989; Lo Curto *et al.*, 1992).

Pectinases are a group of enzymes that break the glycosidic bonds of the long chains of galacturonic acid residues of pectic substances, which are the structural polysaccharides of plant cells. Major pectinases are Polygalacturonase, Pectin lyase, Pectate lyase and Pectin esterase (De-Gregorio *et al.*, 2002). Pectinases are useful industrial enzymes for extraction, clarification and liquefaction of fruit juices. They are also used in fabric industry to ret plant fibres such as flax, hemp and jute and in the paper making industry to solve retention problems in mechanical pulps (Tohru, 2001). The pectinolytic enzymes have been used in other process where elimination of pectin is essential as in wine making, coffee and tea processing plants and maceration of vegetable tissues. Recent emerging applications of pectinases are the treatment and degumming of natural fibres (Blandino *et al.*, 2001).

The ability to synthesize the pectinase is wide spread among all microbial groups but moulds (e.g. *Trichoderma harzianum*; *Aspergillus niger*, *Rhizopus*) are preferred because as much as 90% of the enzyme can be extracted into culture medium (Blandino *et al.*, 2001). *Trichoderma harzianum* is a fungus that can resist high temperature. Therefore, pectinase produced from this fungus can be of great importance.

Among processes used for enzyme production solid state fermentation (SSF) is an attractive one because it presents higher productivity per reactor volume, lower capital and operating costs, lower space requirements, simpler equipment and easier downstream processing compared to that of submerged fermentation (SmF) (Pandey *et al.*, 2000).

In this paper production of pectin lyase in solid state fermentation (SSF) of citrus peel by *T. harzianum* is reported.

MATERIALS AND METHODS

Microorganism

Pure culture of *Trichoderma harzianum* procured from NIBGE, Faisalabad was maintained on nutrient agar medium in petriplates (Latif *et al.*, 1995). The pH of medium was adjusted to 5.5 with MHCl/MNaOH and incubated at 28°C for 6 days to allow sporulation.

Substrate

Peel of *Citrus sinensis* (Mussami) a major citrus variety in Pakistan obtained from a fruit juice corner in students market, Hashmi Hall, University of Agriculture, Faisalabad was used as inducer substrate. The coloured part of the peel was scrapped off in a locally made scrapper device. Inner whitish portion (pectin) was chopped into small pieces and dried in oven at 60°C to 5% (w/w) moisture content. It was ground to powder form (40 mm particle size) prior to its use in SSF.

Preparation of Inoculum

Inoculum medium (100 mL) containing (g/100 mL); glucose, 2.5; yeast extract, 0.5; peptone, 0.1; trisodium citrate, 0.5; KH_2PO_4, 0.4; $(NH_4)_2SO_4$, 0.4 and $MgSO_4.7H_2O$ 0.2; was prepared in 500 mL Erlynmyer flask. Its pH was adjusted to 5.5 and autoclaved (121°C for 15 minutes). Culture was transferred into it in laminar air flow and the flask was then kept on shaker (150 rpm) at 28°C for 72 hours to get 10^7-10^8 spores/mL.

Culture Cultivation

All experimental treatments were performed in duplicate. The growth media were sterilized in autoclave (SANYO) for 15 minutes at 121°C temperature and cm^2 pressure 15 lb. After cooling inoculum (2.5 ml) was added to each flask (unless other wise mentioned) in the laminar air flow (DALTON) with the help of sterilized disposable syringe. The inoculated flasks were kept at 28°C for fermentation under still culture conditions for desired time period in incubator (Gallenkamp).

Enzyme Extraction

Pectin lyase was extracted from the fermented broth by a simple contact method (Krishna and Chandrasekaran, 1996). To each flask 100 ml of Tris HCl buffer of pH 8 (assay buffer) was added 2 times and each time samples were shaken (150 rpm) for 1 hour. Contents were filtered and filtrates were centrifuged at 10,000 rpm for 10 minutes at -10°C to remove undissolved matter and fungal spores. The supernatants of two extraction were mixed and subjected to enzyme assay.

Optimization of Conditions

The strategy adopted for standardization of fermentation parameters was to evaluate the effect of an individual parameter and to incorporate it at optimum level for studying the effect of next parameter. Process parameters thus standardized included moisture level (40-80%) adjusted using various volumes of basal mineral medium, fermentation period (24-120 h), inoculum size (1-3 mL), peptone and yeast extract each with concentrations of (0.1-0.5%) and Tween-80 (0.05-0.25%).

Comparison of SSF and LSF

Liquid State Fermentation (LSF) medium was prepared by adding 5g of citrus peel powder in 100 ml medium for comparison with SSF. After autoclaving and inoculation the LSF flasks (in duplicate) were incubated on shaker (150 rpm) for 72 hours under optimum conditions. The SSF flasks containing optimum growth medium were processed as described earlier.

Optimal pH

Optimal pH for maximal activity of crude pectin lyase was determined by measuring enzyme activity using 50 mM tris HCl buffer of different pH 5, 6, 7, 8 and 9.

Optimal Temperature

The enzyme was assayed at different incubation temperatures viz. 30, 40, 50, 60, 70 and 80°C with optimum pH.

Partial Purification

In order to achieve maximal precipitation of enzyme, several ammonium sulfate concentrations (0-80%) were used and it was found that 20% ammonium sulfate concentration gave better purification. Therefore, the extract was saturated to 20% in the second step by adding 5.7 g ammonium sulfate in 50 ml of the extract under constant stirring. Both the supernatant and filterate were subjected to enzyme assay and protein estimation. Protein was estimated by Biuret method (Bardawill and David, 1949).

Pectin lyase Assay

Assay of pectin lyase was performed by the method described by Press and Ashwell (1963). 0.5 mL of enzyme was incubated for 1h with 0.5 mL of 0.5% pectin and 1 mL of 50 mM tris HCl buffer of pH 8 and 1 mL of 0.2 mM $CaCl_2$. After 1 hour the change in absorbance was measured at 548 nm and increase in absorbance was determined. One unit of enzyme activity was defined as change in absorbance per hour caused by 1 mL of enzyme extract.

RESULTS AND DISCUSSION

Optimum conditions

Trichoderma harzianum was cultured in SSF medium for optimization of fermentation parameters like fermentation period, moisture level, inoculum size and concentrations of peptone, yeast extract and tween 80 for maximum production of pectin lyase. The results of optimization experiments have been discussed under the following respective headings.

Moisture level

It was observed that ground citrus peels with 70% moisture yielded maximum pectin lyase activity (8.24 U/mL/min) after 72 hours (Table 1). Pectin lyase production increased with an increase in moisture level from 40 to 70% and a further increase in moisture content (upto 80%) caused a significant ($P<0.05$) decrease in enzyme yield. Low moisture content is known to decrease the solubility of nutrients, low substrate swelling and higher water tension (Romash and Lonsane, 1990). Moisture levels higher than a certain critical level cause aeration problem and decrease the microbial growth and metabolic activities in SSF (Ghanem *et al.*, 2000). Our results are in line

with those of Krishna and Chandrasekran (1996) who reported 70% as optimum moisture level for enzyme production in solid state fermentation.

Table 1. Activity of Pectin lyase produced by *T. harzianum* at varying moisture levels in the presence of 10g of substrate

Moisture level (%)	Enzyme Activity (U/mL/min)
40	4.72E
50	5.15D
60	6.70C
70	8.24A
80	7.91B

(P<0.05)

Fermentation Period

Growth media (70% moisture) were inoculated, and duplicate flasks were harvested after 24, 48, 72, 96 and 120 hours of SSF at pH 5.5 and 28°C. The maximum activity of pectin lyase was observed in culture filtrate harvested after 72 hours of SSF. It was observed that the production of pectin lyase increased with an increase in fermentation time from 24-72 h and peaked (8.42 U/mL/min) at 72 hours (Fig. 1). The decrease in enzyme yield after 72 hours may be a result of variation in pH of the medium during fermentation as a result of formation of citric acid and acetic acid. Also after a certain period of time the microorganism start hydrolyzing the enzymes for synthesis of biomass protein. According to Hours *et al.* (1998) *A. foedidus* grown in apple pulp produced maximal pectin lyase activities after 96 hours. Roberta *et al.* (2001) obtained maximum pectin lyase activity after 65 hours. The difference in incubation time may be due to the difference of organisms and inducer substrates.

Inoculum Size

Growth media (70% moisture) were inoculated with varying inoculum size and incubated in duplicate at pH 5.5 and 28°C for 72 hours. The maximum activity (10.28 U/mL/min) of pectin lyase was obtained from SSF medium fermented with 2.5 mL (25% v/w) inoculum (Table 2). The production of pectin lyase increased with an increase in inoculum size from 1-2.5 ml and a further increase in inoculum level did not favour significantly (P<0.05) higher enzyme production. Optimum inoculum density is important consideration for SSF process since over crowding of spores can inhibit growth and development (Ghanem *et al.*, 2000). Higher inoculum levels besides increasing spore density increase water content of the medium as well.

Effect of Peptone

The source and concentration of nitrogen in the growth medium has a very important role in microbial growth and enzyme production. In this experiment varying concentrations of peptone were used in preoptimized SSF medium of citrus peel. Addition of peptone showed negative impact on enzyme production by *T. harziaum*. All levels of peptone were found to inhibit microbial growth and pectin lyase production (Table 3). Enzyme formation has been reported to be strongly affected by the nature of nitrogen source (Galhaup *et al.*, 2002). Different microorganisms use and assimilate different nitrogenous compounds as additional nitrogen sources. It was therefore, concluded that peptone should not be used in the subsequent study.

Table 2. Activity of Pectin lyase produced by *T. harzinum* with varying inoculum size*

Inoculum size (mL)	Enzyme activity (U/mL/min)
1.0	5.21E
1.5	6.24D
2.0	8.48B
2.5	10.28A
3.0	8.23C

(P<0.05)

* Moisture, 70%; Fermentation period, 72 hours.

Table 3. Activity of Pectin lyase with varying concentrations of Peptone*

Peptone (%)	Enzyme activit y (U/mL/min)
Control	10.30A
0.1	8.14B
0.2	6.70C
0.3	4.84D
0.4	3.91E
0.5	3.37F

(P<0.05)

*Moisture, 70%; Fermentation period, 72 hours; inoculum size, 2.5 mL.

Effect of Yeast Extract

Yeast extract was used to enhance the fermentation rate and pectin lyase production. Five different concentrations (0.1, 0.2, 0.3%, 0.4 and 0.5%) of yeast extract were added into preoptimized SSF medium in duplicate. The control did not receive any yeast extract. The results (Table 4) clearly indicate that 0.4% yeast extract showed maximum pectin lyase activity (13.05 U/mL/min) that decreased with the increase of yeast extract concentration beyond 0.4%. Pereira (1994) observed that *P. griseoroseum* produce maximum PL when cultured in medium containing 0.06% (w/v) yeast extract, without added pectin.

Table 4. Activity of Pectin lyase produced by *T. harzianum* with varying Concentrations of Yeast Extract*

Yeast Extract (%)	Enzyme activity (U/mL/min)
Control	10.29F
0.1	10.94E
0.2	11.56D
0.3	12.06C
0.4	13.05A
0.5	12.59B

*Moisture, 70%; Fermentation period, 72 hours; inoculum size, 2.5 mL.

Effect of Tween 80

Tween 80 (a surfactant) was used to enhance the SSF rate. Addition of tween-80 into the growth medium of citrus peel enhanced pectin lyase production and maximum enzyme yield was noted in SSF medium receiving 0.2% of this surfactant (Table 5). Growth media containing less and more than 0.2% tween-80 showed lower activities of the enzyme. Higher levels of Tween-80 increased the penetration of water into the solid substrate matrix and increase the surface area more than the requirement of the microbe (Fujian *et al.*, 2001). Tween-80 has also been shown to increase enzyme production in fungal species such as *T-reesei* (Mandel and Weber, 1969).

Table 5. Activity of Pectin lyase with varying concentrations of Tween 80*

Tween-80 (%)	Enzyme activity (U/mL/min)
Control	13.09C
0.05	13.62C
0.10	14.16B
0.15	14.81B
0.20	16.20A
0.25	14.20B

*Moisture, 70%; Fermentation period, 72 hours; inoculum size, 2.5 mL; Yeast extract, 0.4%.

Comparison of SSF with LSF

Duplicate flasks containing the optimum growth medium for pectin lyase production were used. It was observed that SSF is better then LSF. ,SSF showed more enzyme production as compared to LSF (Table 6). It has previously been reported (AcunaArguelles 1994; Rodriguez *et al.* 1985; Solis-Pereira *et al.* 1993; Tao *et al.* 1997) that solid state fermentation (SSF) generally allows more production of highly concentrated crude enzymes as compared to liquid state fermentation.

Table 6. Comparison of SSF and LSF under optimum conditions

Type of Fermentation*	Pectin lyase activity (U/mL/min)		
	A	B	Mean
SSF	15.10	16.00	15.55
LSF	10.90	10.25	10.57

Partial Purification

In the first step 20% $(NH_4)_2SO_4$ precipitation gave better purification as compared to other concentrations of this salt. In the second purification step with 20% ammonium sulfate residue indicated the presence of maximum enzyme activity. The analysis revealed 0.37 & 1.98 mg/mL protein and 85.3 & 21.08 U/mg specific enzyme activity for supernatant and residue respectively (Table 7). The purification procedure provided 0.87 and 3.52 fold purification of pectin lyase respectively for residue and supernatant. Moharib *et al.* (2000) reported that pectin lyase produced by *Pichia pinus* growing on mango wastes was successively purified by precipitation with ammonium sulfate followed by chromatography on sephadex G-120. The purification procedure provided 39 fold purification with 24% recovery of pectinase.

Effect of pH on enzyme activity

Pectin lyase showed maximal activity when assayed at a pH 7 (Fig 2). The activity of the enzyme gradually increased with increasing assay pH from 5-7 and declined, thereafter. This is a well establish fact that each enzyme has a characteristic pH optimum for its activity (Lehninger *et al.* 1992) Wei-Chen (1998) observed maximum pectin layse activity at pH 8, the small difference owing to the microbial source difference.

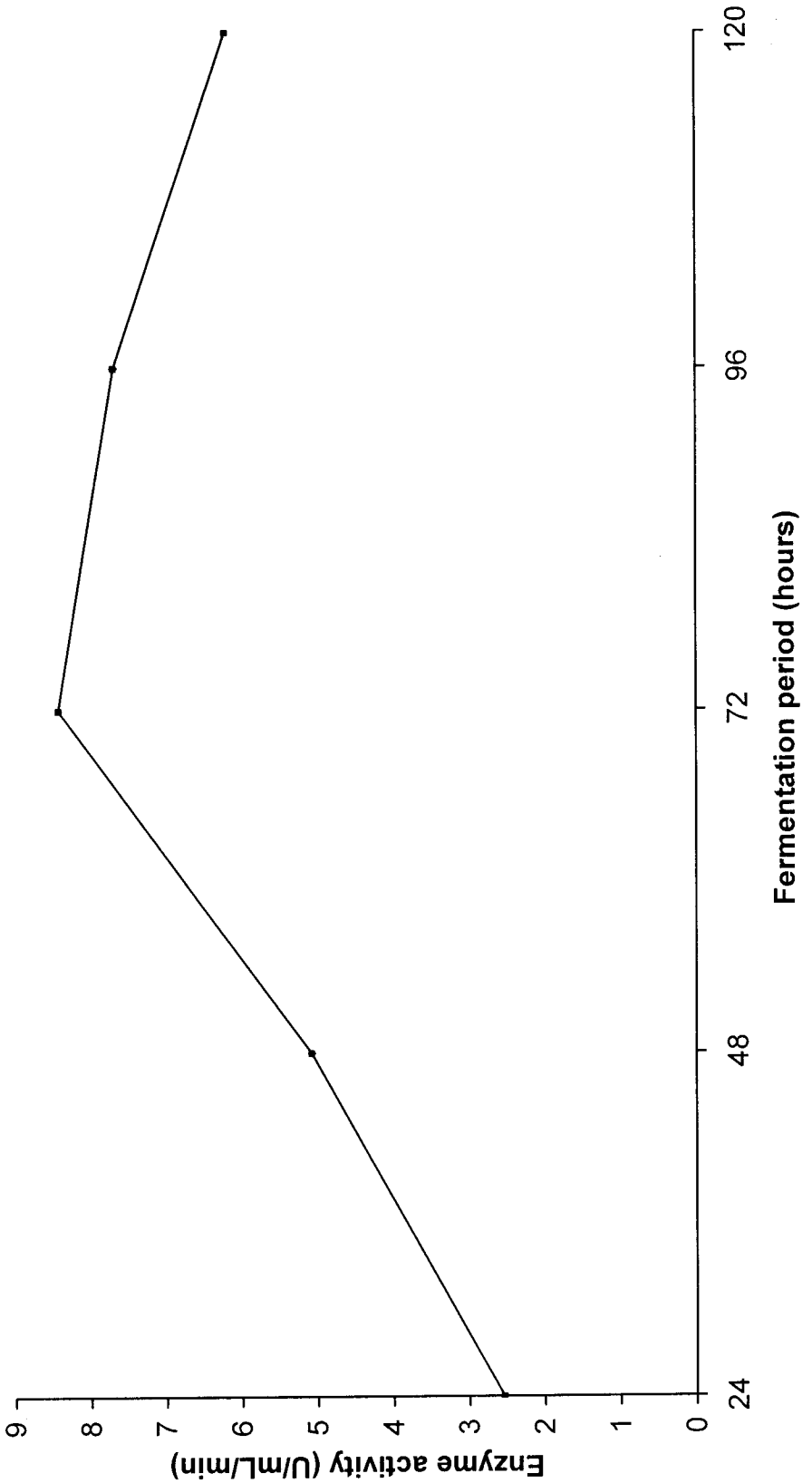

Fig. 1 Effect of fermentation period on pectin lyase production by *T. harzianum*

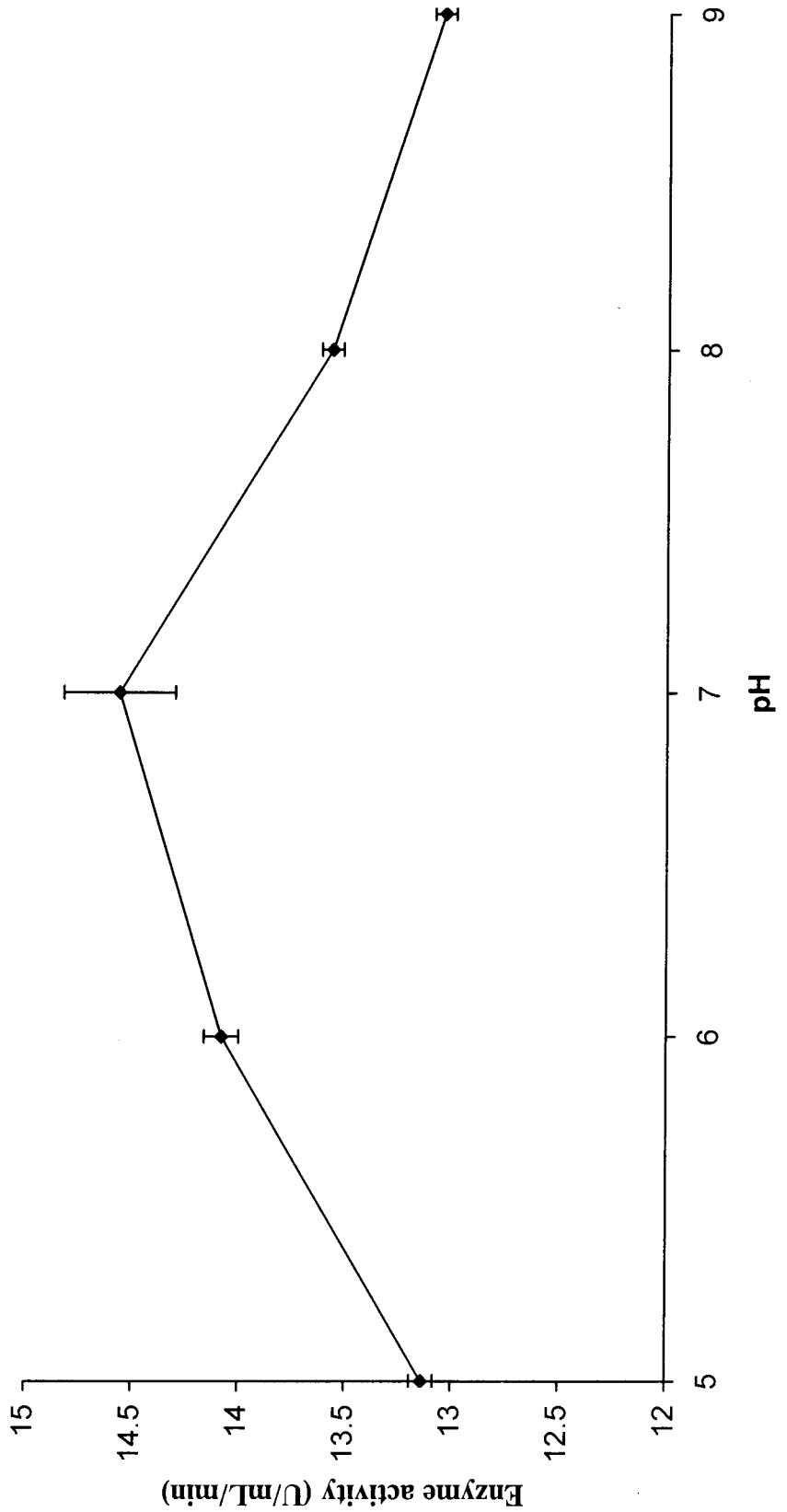

Fig. 2 Effect of pH on the activity of pectin lyase produced by *T. hariznum*.

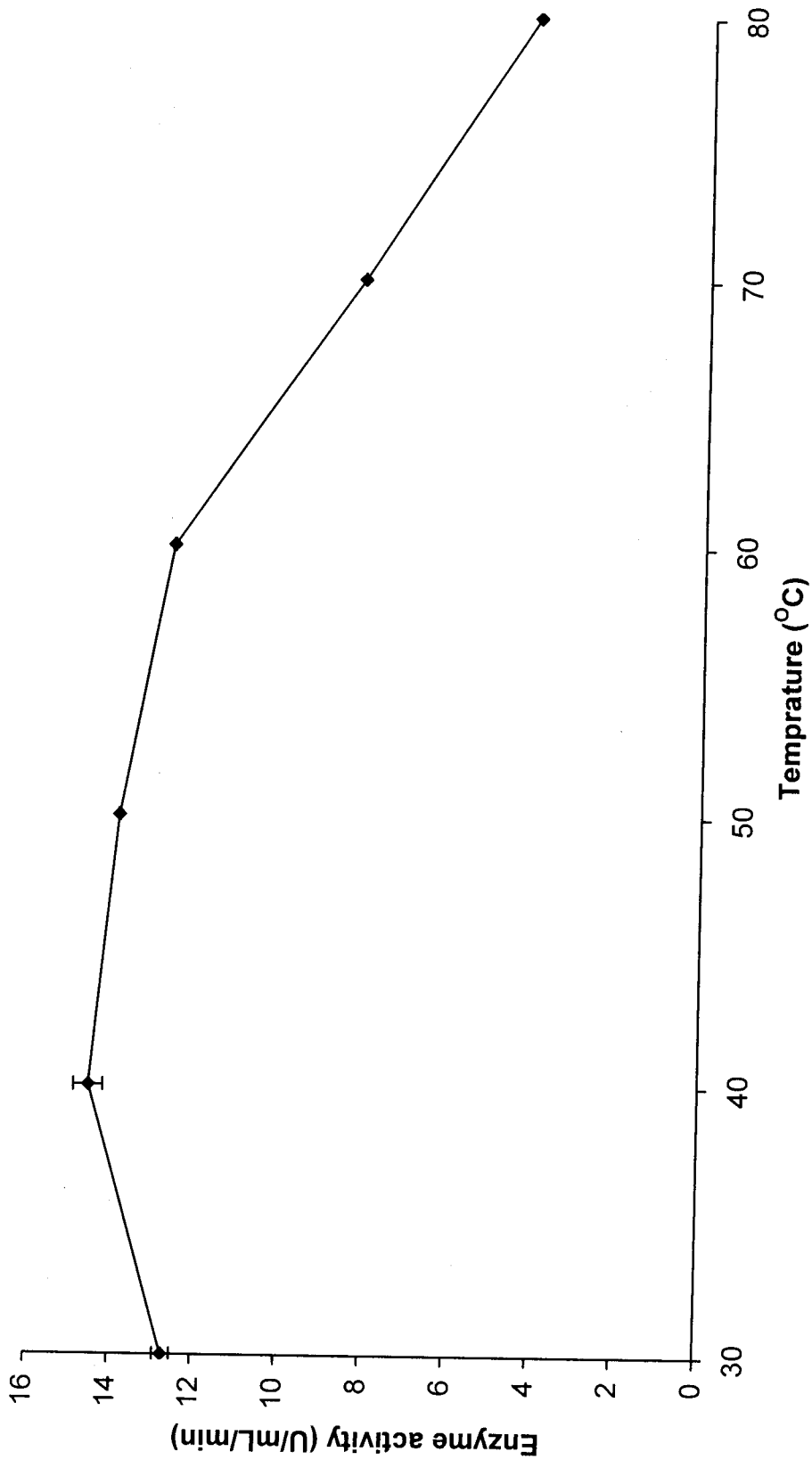

Fig. 3 Effect of temprature on the activity of pectin lyase produced by *T. harzianm*.

Table 7. Activity and specific activity of pectin lyase after $(NH_4)SO_4$ precipitation.

Purification step	Volume (mL)	Enzyme activity (U/mL/min)	Protein conc. (mg/mL)	Specific activity (U/mg)	% yield	Fold purified
Crude enzyme	200	16.20	0.67	24.179	100	1
$(NH_4)_2SO_4$ precipitation (20%)						
Supernatant	50	31.59	0.37	85.3	48.75	3.52
Residue	10	41.745	1.98	21.08	12.88	0.87

Effect of temperature on enzyme activity

Optimal temperature for maximal activity of pectin lyase was determined by conducting the enzyme assay at different incubation temperature (30, 40, 50, 60, 70 and 80°C) at pH 7 (optimum). Pectin lyase activity peaked at 40°C temperature and further increase in temperature showed decreasing trend (Fig. 3). Every enzyme is optimally active and stable upto a certain temperature and gets denatured at higher temperatures (Lehninger *et al.*, 1992) Wei-Chen (1998) observed that pectin lyase produced by *Pythium splendens* showed maximum activity at 50°C at pH 8.

REFERENCES

Acuna-Arguelles, M., M. Gutierrez-Rojas, G. Viniegra-Gonzalez and E. Favela-Torres, 1994. Effect of water activity on exo-pectinase production by *Aspergillus niger* CH_4 in solid state fermentation. Biotech. Lett., 16(1):23-28.

Bardawill and M.M. David, 1949. Determination of serum protein by means of biuret reaction. J. Biochem., 177:751-766.

Blandino, A., K. Dravillas, D. Cantero, S.S. Pandiella and C. Webb, 2001. Utilization of whole wheat flour for the production of extracellular pectinases by some fungal strains. Process Biochem., 37:497-503.

De-Gregorio, A., G. Mandalari, N. Arena, F. Nucita, M.M. Tripoda and R.B. Lo-Curto, 2002. SCP and crude pectinase production by slurry-state fermentation of lemon pulps. Biores. Technol., 83:89-94.

Fujian, Xu, Chen Hong Zhang, Li Zuohu, 2001. Solid-state production of lignin peroxidase (Lip) and manganese peroxidase (MnP) by *Phanerochaete chrysosporium* using steam exploded straw as substrate. Biores. Technol., 149-151.

Galhaup, C., H. Wagner, B. Hinterstoisser, D. Haltrich, 2002. Increased production of Laccase by the wood degrading basidiomycetes. Trametes Pubescens. Enzyme and Microb. Technol., 30:529-536.

Ghanem, N.B., H.H. Yusef, H.K. Mahrouse, 2000. Production of *Aspergullus terrus* xylanase in solid state cultures: application of the plachett Burman experimental design to evaluate nutritional requirements. Biores. Technol., 73:113-121.

Hours, R.A., C.E. Voget and R.J. Ertola, 1998. Some factors affecting pectinase production from apple promace in solid state cultures. Biol. Wastes, 24:147-57.

Krishna, C. and M. Chandrasekeran, 1996. Banana waste as substrate for □-amylase production by *Bacillus subtilis* (CBTK 100) under solid state fermentation. Microbial Biotechnol., 46:106-111.

Latif, F., M.I. Rajoka and K.A. Malik, 1995. Production of cellulase by thermophilic fungi grown on *Leptochloa fusca* straw. World J. Microbiol. Biotechnol., 11:347-348.

Lehninger, A.L., D.L. Nelson and Cox, 1992. Principles of Biochemistry (2nd ed.) Worth Publishers, Inc., New York, pp. 222.

Lo-Curto, R., M.M. Tripodo, U. Leuzzi, U. Giuffre and C. Vaccarino, 1992. Flavonoids recovery and SCP production from orange peel. Biores. Technol., 42:83-87.

Mandels, M. and J. Weber, 1969. The production of cellulase. Adv. Chem. Ser., 95:391-413.

Moharib, S.A., S.T. El-Syed and E.W. Jwanry, 2000. Evaluation of enzymes produced from yeast. Nahrung, 44(1):47-51.

Muniswaran, P.K.A. and N.C.L.N. Charyulu, 1994. Solid state fermentation of coconut coir pith for cellulase production. Enz. Microb. Technol., 29:295-301.

Pandey, A., C.R. Soccol, P. Nigam and T. Soccol, 2000. Biotechnological potential of agro-industrial residues. I: Sugar cane bagasse. Biores. Technol., 74:69-80.

Pereira, B.M.C., J.L.C. Coelho and D.O. Silva, 1994. Production of pectin lyase by *Penicillium griseoroseum* cultured on sucrose and yeast extract for degumming of natural fiber. Lett. Appl. Microbiol., 18:127-129.

Ramesh, M.V. and B.K. Lonsane, 1990. Characteristics and novel features of thermostble □-amylase produced by *Bacillus licheniformis* under solid state fermentation. Starch/Starke, 6:233-238.

Roberta, Hilsdrof, P. Valle, I. Viana, B. Daison elzany Silva and F. Jose Vieira Passos, 2001. Pectin lyase production by *Penecillium griseoroseum* grown in sugar cane juice in repeated batch cultures. World J. Microbiol. and Biotechnol, 17:433-437.

Rodriguez, J.A., J. Echevarria, Z. Rodrigue, N. sierra, A. Daniel and O. Murtinez, 1985. Solid state fermentation of dried citrus peel by *Aspergillus niger*. Biotechnol. Lett., 7(1):577-580.

Solis-Pereira, S., E. Favela-Torres, G. Vinierga-Gonzalez and M. Gutierrez-Rojas, 1993. Effect of different carbon sources on the synthesis of pectinase by *Aspergillus niger* in submerged and solid state fermentation. Applied Microbiol. and Biotechnol., 39(1):36-41.

Tao, S., L. Beithui, L. Zuohu, 1997. Enhanced cellulase production in fed batch solid state fermentation of *Trichoderma viride* SL-1. J. Chem. Technol. Biotechnol., 69(1):429-432.

Tohru, K., N. Higaki, A. Suzumatsu, K. Sawada and H. Hagihara, 2001. Purification and properties of a high molecular weight alkaline exopolygalacturonase from a strain of *Bacillus*. Enzyme and Microb. Technol., 29:70-75.

Vaccarino, C., R.B. Curto, M.M.Tripodo, R.M. Patane, G. Lagana and S. Schachter, 1989a. SCP from orange peel by fermentation with fungi. Sumerged and Surface fermentation. Biol. Wastes, 29:279-287.

Wei-Chen C., H.J. Hsieh and T.C. Tseng, 1998. Purification and characterization of a pectin lyase from *Pythium splendens* infected cucumber fruits. Bot. Bull. Acad. Sin., 39:181-186.