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Original Article

Comparative Studies on Production of Cell Wall-Degrading Hydrolases by *Trichoderma reesei and T. viride* in Submerged and Solid-State Cultivations

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Lignocellulose-degrading organisms have been used for the conversion of lignocellulosic materials into soluble sugars or solvents in several biotechnological and industrial applications. Complete degradation of complex highly structured lignocellosics requires a concerted action of a wide array hydrolytic enzymes. In this study, two newly isolated fungi, *Trichoderma reesei* and *T. viride*, were examined for their ability to produce cellulolytic, xylanolytic and pectinolytic enzymes in submerged and solid-state fermentations. The fungi produced appreciable amounts of the enzymes when grown on potentially low cost lignocellulosic agricultural residues like wheat bran, sugar cane bagasse and corncobs. *T. viride* seems to be superior to *T. reesei* with respect to enzyme productions both in submerged and solid-state cultivations. There was a distinct influence of culture methods on the production of the enzymes by the fungi. The enzyme productions were higher in solid-state fermentations than in submerged fermentations. However, taking into consideration of enzyme yields per gram substrate, it was found that the yields were many-fold higher in submerged cultures than in solid-state fermentations. The recovery of the enzymes from fermented slurries in solid-state fermentations was enhanced by using non-ionic surfactant Tween 80 as leaching agent. The enzymes produced by the fungi displayed optimum activities at pH range between 4.5 and 5.5, and at temperatures between 50 and 55°C. The fungi merit further attention as potential sources of industrial enzymes, as they exhibited some excellent properties including the ability to synthesize a wide array of hydrolytic enzymes while grown on cheap and readily available lignocellulosic residues.

Keywords: Lignocellulosics, cellulase, xylanase, pectinase, *Trichoderma reesei, Trichoderma viride*, submerged culture, solid-state fermentation

Introduction

Because of upward spiral in the price of fossil fuel, coupled with impending shortages of the classical energy sources there has been much interest in investigations of non-conventional energy sources during the last few decades. Lignocellulose is the world's most abundant natural biopolymer and a potentially important source for the production of industrially useful materials such as fuels and chemicals[1-2]. Degradation of the lignocellulosic materials is achieved either chemically, enzymatically, or by the combination of both chemical and enzymatic methods[3-5]. Chemical methods produce more by-products and they are performed at high temperatures compared to the enzymatic hydrolyses, which are technologically important approaches[6-7]. In addition, chemical degradations of the cellulose materials, due to the environmental problems, are unfavourable and uneconomical approaches[5]. Therefore, extensive effort has been made to make cellulose economically hydrolysable under mild conditions[8]. In spite of huge extent of research for finding more active enzyme preparations from a large variety of microorganisms, the enzymatic saccharification of lignocelluloses so far has not been reached to the level of conversion of starch to glucose by the microbial enzymes. Thus much work and research is needed to produce enzymes capable of saccharifying plant materials.

In addition to saccharification process cellulases and hemicellulases have been evaluated for their ability to beneficially modify pulp and paper characteristics[9]. Moreover, cellulase treatment of dried pulp seems to increase the relative bonded area of the fibrous paper network, improving some paper properties[10]. These are some practical examples promoting the usefulness of the enzyme. In every case, the production of hydrolases required to degrade lignocellulosic substrates has been found to be the most expensive step[2,11]. This step takes approximately 40% of the total cost during ethanol production from lignocellulosic biomass[12]. The high cost of the enzyme production limits its industrial use. Therefore, several approaches including random mutagenesis and genetic engineering to obtain

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enhanced hydrolases producing strains have been given considerable priority in the last decade[13-14], and some of them have been successfully used in a number of applications including animal feed, pharmaceutical and textile industries[15-16].

The enzymatic hydrolysis of cellulosic materials is a slow and complex reaction. This reaction correlates with the level of cellulose crystallinity[17]. Moreover, complete enzymatic hydrolysis of the polysaccharides of lignocellulases requires a concerted action of a complex array of hydrolases including cellulase, xylanase, pectinase, and other side-group cleavage enzymes[18-19]. Although a large number of microorganisms are capable of degrading plant cell wall materials, only few of them produce significant quantities of cell-free hydrolases capable of efficiently hydrolysing complex lignocelluloses *in vitro*. The soft-rot fungi, *Trichoderma viride*[20-21] and *Trichoderma reesei*[22-23] are, by far, the most extensively studied, and they have served as a model for fungal lignocellulose degradation. During the past few years, these investigations have made significant progress toward elucidating the enzymology of lignocellulose degradation. In submerged or solid-state culture, most these fungi secrete a complex array of degradative enzymes. The objectives of this study were to evaluate the hydrolase enzyme productions by two newly isolated *Trichoderma, viz*., *T. reesei* and *T. viride*, in submerged and solid-state cultivations.

Materials and Methods

Organisms and growth conditions

The fungal isolates used were isolated from decomposed lignocellulosic matters at the Bangladesh Jute Research Institute, Dhaka, and identified as *Trichoderma reesei* and *Trichoderma viride* by the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands. The cultures were maintained on potato dextrose agar (PDA) at 30°C and stores at 4°C.

Pretreatment of lignocellulosics

Lignocellulosic substrates were pretreated with 1% sodium hydroxide solution (10 ml/g substrate) for 1 h. The treated substrates were streamed by autoclaving at 121°C for 1 h and then washed thoroughly and dried at room temperature. Depending on the types, the treated substrates were ground to fine powder in a grinder.

Inoculum

Fungi grown on PDA for 5 days were transferred into 100 ml Mandels and Weber[24] mineral medium with 10 g of soluble starch as carbon source. After 3-5 days incubation the cultures were homogenized and used to inoculate enzyme production media.

Submerged cultivation

Submerged cultivations were carried out in 250-ml Erlenmeyer flask containing 100 ml Mandels and Weber[24] mineral medium supplemented with 2% lignocellulosic substrate with an initial pH 5.0. The flasks were sterilized at 115°C for 15 min. Urea was sterilized by membrane filtration. Each flask was inoculated with 1 ml of the inoculum. The cultures were incubated on a rotary shaker (120 rpm) at 32°C for 7 day unless otherwise mentioned. The broth after cultivation was used for enzyme studies.

Solid-state cultivation

Solid-state cultivations were carried out in 500-ml Erlenmeyer flask containing 20 g wheat bran as substrate. The substrate was moistened with 50 ml of distilled water and sterilized at 115°C for 30 min. The mineral salt solution without urea was autoclaved to 8-fold concentration. Urea was sterilized at 8-fold concentration by membrane filtration. After sterilization, each flask was supplemented with 10 ml salt solution, 10 ml urea solution and 10 ml inoculum. The final moisture content was about 70-80% w/w. The moisture content was controlled at 24 h intervals.

Enzyme recovery

The culture broth from submerged cultivation was centrifuged at 5,000 rpm for 15 min and the supernatants were used for enzyme assays. The solid cultures were suspended in 100 of distilled water and placed on a shaker for 1 h (unless otherwise mentioned). The suspension was filtered through a nylon cloth, and then centrifuges at 5,000 rpm for 15 min. The filtrates obtained were used for determination of enzyme activities.

Enzyme assays

The activities of 1,4-β-D-glucan cellobiohydrolase (exo-1,4-β-D-glucanase, FPase, EC 3.2.1.91) towards carboxymethylcellulose, and 1,4-β-D-glucan 4-glucanohydrolase (endo 1,4-β-D-glucanase, CMCase, EC 3.2.1.4) towards filter paper (Whatman n° 1) were determined according to the method of Mandels and Weber[24]. For FPase, a rolled 1 x 6 cm strip (50 mg) of the filter paper was dipped into 1 ml of citrate buffer (0.05 *M*, pH 4.8) and incubated with 0.5 ml enzyme solution at 50°C for 1 h. CMCase activity was estimated using 1.0% solution of carboxymethlycellulose (CMC, Sigma, USA) in 0.05 *M* citrate buffer (pH 4.8) as substrate. The reaction mixture contained 1.0 ml citrate buffer, 0.5 ml of substrate solution and 0.5 ml of suitably diluted enzyme solution. The reaction was carried out at 50°C for 30 min. Xylanase (endo-1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8) activity was determined according to Bailey *et al*.[25] using 1.0% birch wood 4-O-methlyglucuronoxylan (Roth, Germany) in 0.05 *M* citrate buffer (pH 4.8). The assay mixture, containing 1.8 ml substrate and 0.2 ml of suitably diluted enzyme solution in the buffer, was incubated at 50°C for 5 min. The enzymic reaction for FPase, CMCase and xylanase were stopped by addition of 3 ml of dinitrosalicylic acid (DNS) reagent. The amount of reducing sugar released in the hydrolysis reaction was measured by the DNS method as described by Miller[26]. One unit of FPase, CMCase, or xylanase was defined as the amount of enzyme that released 1 μmol reducing sugar as glucose or xylose equivalents per min under the assay conditions.

Pectinase (polygalacturonase, EC 3.2.1.15) was assayed essentially as described by Collmer *et al*.[27]. The reducing groups resulting from release of oligogalacturonates from polygalacturonate were measured using Nelson-Somogyi

method[28]. The substrate stock solution was prepared by adding 20 ml of 0.6 *M* NaCl with rapid mixing to 80 ml of a solution containing 75 m*M* sodium acetate (pH 5.3), 7.5 m*M* EDTA and 0.3% polyagacturonic acid (Sigma, USA). The enzyme assay was initiated by mixing 1.0 ml substrate stock solution and 0.2 ml of appropriately diluted enzyme solution in water. The reaction mixture was incubated at 50°C for 5 min, and the reaction was stopped by adding 1.2 ml of copper reagent. The mixture was subsequently incubated in vigorous boiling water bath for 10 min, cooled to room temperature, and 2.4 ml of arsenomolybdate reagent was added to the mixture. After 30 min incubation at room temperature, the assay mixture was centrifuged to remove precipitated residual substrate, and the absorbance of the supernatants was read at 500 nm. The values were used in reference to a D-galacturonic acid (Sigma, USA) standard curve to determine the amount of oligogalacturonic acid formation. One unit of pectinase activity was defined as the amount of enzyme that would produce 1 μmol of reducing groups per min under the assay conditions.

In experiments concerning the pH and temperature profile characteristics, the enzyme assays were performed at various pH values (using 0.05 *M* citrate buffer for pH 4.0-5.0, 0.05 *M* phosphate buffer for pH 7.0-8.0, 0.2 *M* Tris-HCl buffer for pH 9.0, and 0.05 *M* glycine-NaOH buffer for pH 10.0-11.0) and temperatures (30-65°C).

Results and Discussion

There have been considerable efforts to isolate microorganisms with high ability to produce hydrolytic enzymes capable of degrading hard structure of lignocellulosic biomass[2,13-14]. Lignocellulosic material can be converted to fuel or valuable chemical either by thermo-chemical or biological means[3-4,6]. Bioconversion processes can, however, be a more or less difficult task depending on the choice of raw materials[29]. It is widely recognized that the economics of enzymatic processes of lignocellulosic materials will greatly improved if both the cellulose and hemicellulose components are utilized[30]. However, the overall conversion of lignocellulose has been hampered by the low yields of soluble sugars and high cost of microbial enzymes used[31]. Not surprisingly, much research has been aimed at producing the enzyme more cost effective. Notably in this regard have been the isolation of fungal strains, which overproduce cellulases and hemicellulases[31-32], and the selecting of effective but inexpensive and readily available substrates in enzyme productions[12,22].

In this study, two newly isolated fungi, *viz*., *T. reesei* and *T. viride*, were used for cellulases, xylanase and pectinase productions on lignocellulosic substrates in submerged and solid-state cultivations. Both of these species have been currently renamed as *T. longibrachiatum*[33]. Table 1 shows the levels of the enzymes produced by the fungi in submerged cultivations. Both *Trichoderma* species grew rather well in shake-flask cultures on the agricultural residues. Cellulases (FPase and CMCase), xylanase and pectinase were produced irrespective of the substrates. However, the enzyme productions by the fungi were better when wheat bran, sugar cane bagasse and corncobs were used as substrates, while the levels of the enzymes were considerably lower in the medium containing rice straw, jute stick, or saw dust. In case of *T. reesei*, the yield of all the enzymes was highest on wheat bran medium, followed by sugar cane bagasse and corncobs. On the other hand, sugar cane bagasse supported good growth and cellulases and xylanase productions by *T. viride*, while it produced highest level of pectinase on wheat bran.

Table 1. *Extracellular enzyme production by Trichoderma reesei and Trichoderma viride on various lignocellulosic substrates in submerged cultivations*

Substrate (2.0%)	Enzyme production (U/ml) (*T. reesei vs. T. viridae)*			
	FPase	CMCase	Xylanase	Pectnase
Wheat bran	0.33 *vs*. 0.25	0.43 *vs*. 0.39	10.3 *vs*. 19.2	4.1 *vs*. 7.8
Bagasse	0.32 *vs*. 0.38	0.41 *vs*. 0.49	8.1 *vs*. 21.9	4.0 *vs*. 5.0
Corncobs	0.30 *vs*. 0.26	0.35 *vs*. 0.46	7.2 *vs*. 17.5	3.8 *vs*. 6.1
Rice straw	0.18 *vs*. 0.17	0.21 *vs*. 0.19	2.4 *vs*. 8.8	1.9 *vs*. 3.6
Jute stick	0.20 *vs*. 0.16	0.22 *vs*. 0.19	4.2 *vs*. 4.6	2.0 *vs*. 2.1
Saw dust	0.21 *vs*. 0.12	0.25 *vs*. 0.20	2.3 *vs*. 5.7	1.9 *vs*. 3.0

Submerged cultivations of the fungi were carried out under shaking (120 rpm) at 32°C for 7 day.

As shown in Table 1 that there was a distinct difference between the two fungi on their ability to produce the hydrolases on lignocelluloses. *T. viride* was the highest producer of cellulolytic enzymes (FPase 0.38 U/ml, CMCase 0.49 U/ml) and xylanase (21.9 U/ml) when grown on sugar cane bagasse. *T. viride* was also the highest producer of pectinase (7.8 U/ml) when grown on wheat bran. The cellulolytic enzyme activities produced by *T. reesei* (FPase 0.30-0.33 U/ml, CMCase 0.35-0.43 U/ml) were comparable to those produced by *T. viride*, but production of xylanase (7.2-10.3 U/ml) was considerably lower as compared to *T. viride*.

Figure 1 shows the kinetic behaviour of extracellular enzyme productions by the fungi in submerged cultivations using wheat bran as substrate. Enzyme productions by the fungi started after a lag period of 24 h or more, and the activities reached to maximal levels within 5-7 days of incubation. Maximum FPase (0.38 U/ml) and CMCase (0.52 U/ml) were produced by *T. reesei* after 7 days of incubation, while maximum xylanase (23.3 U/ml) and pectinase (10.3 U/ml) was produced by *T. viride* after 5 days of cultivations. The enzyme activities decrease by further incubations.

The results of the kinetic behaviour of extracellular enzyme productions in solid-state cultivations are shown in Figure 2. High enzyme production by the fungi was achieved after 9 days of incubation using wheat bran as substrate. *T. reesei* produced highest level of FPase (0.48 U/ml) and CMCase (0.58 U/ml), while *T. viride* produced highest level of xylanase (26.0 U/ml) and pectinase (10.3 U/ml) in solid-state cultivations.

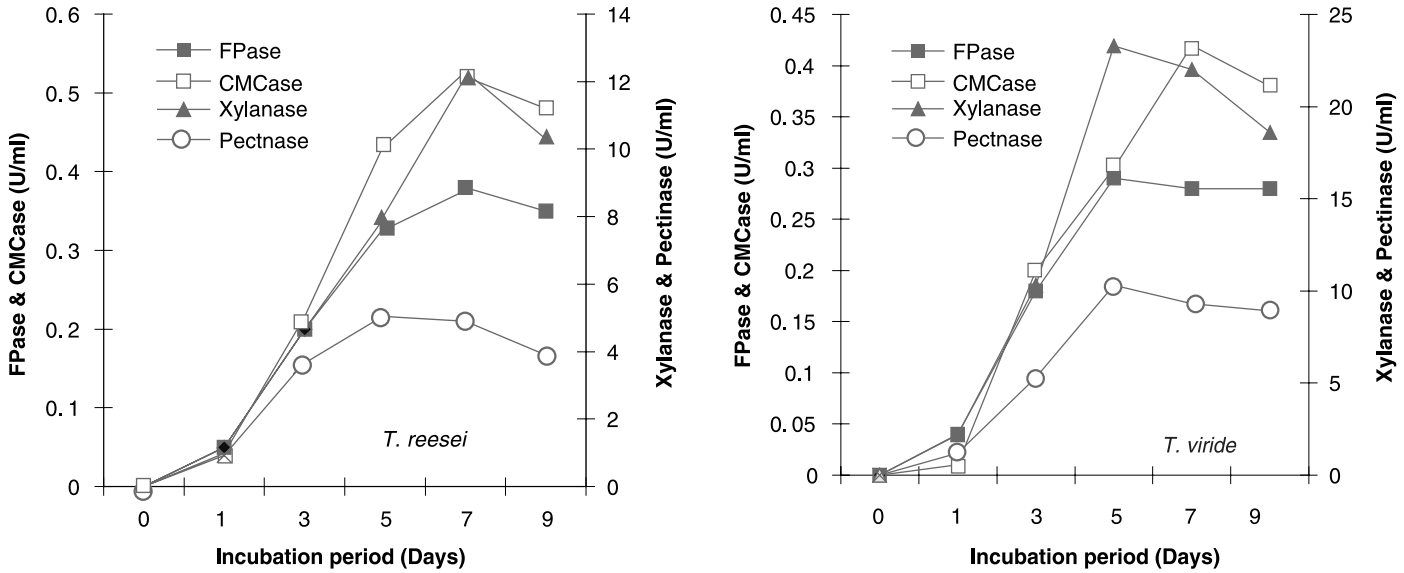

Figure 1. *Kinetic behaviour of extracellular enzyme production by Trichoderma reesei and Trichoderma viride in submerged cultivations on wheat bran as substrate.*

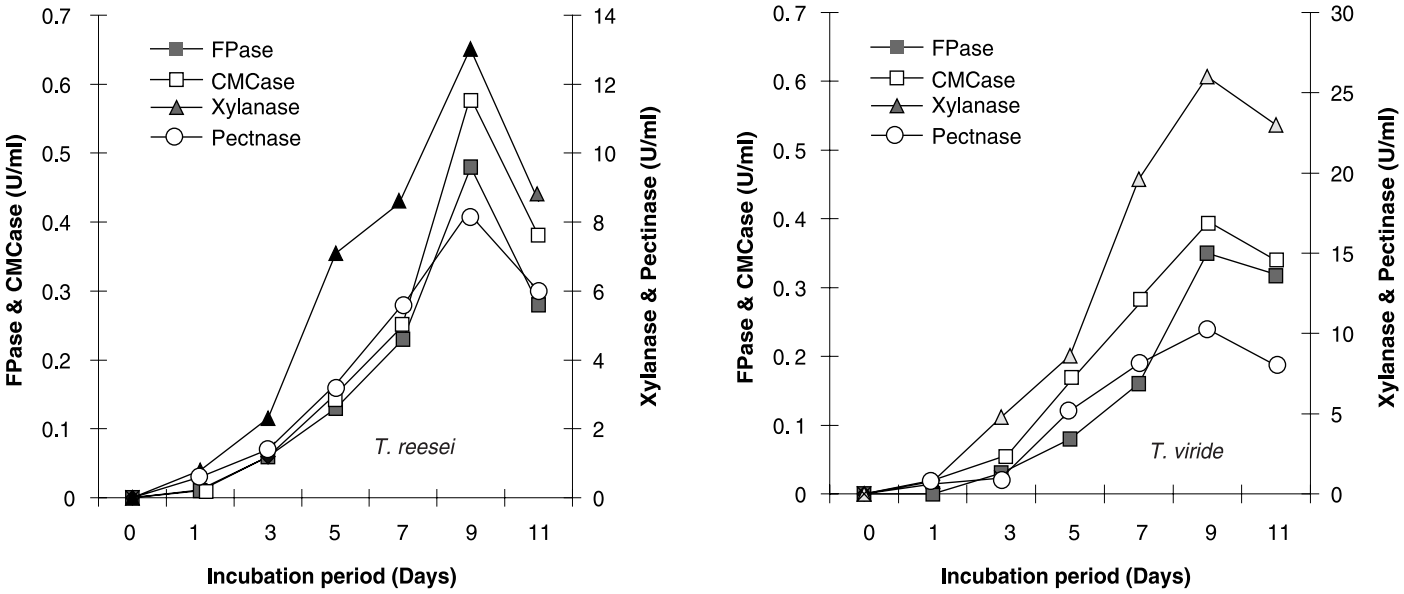

Figure 2. *Kinetic behaviour of extracellular enzyme production by Trichoderma reesei and Trichoderma viride in solid-state cultivations on wheat bran as substrate.*

There was a distinct influence of the culture methods on the production of enzymes by the fungi examined. Both *T. reesei* and *T. viride* produced higher cellulolytic, xylanolytic and pectinolytic activities when grown in solid-state than in submerged cultivations. The comparison of results obtained in submerged and solid-state fermentations is difficult, because of different units of expression of enzyme activity[34]. To eliminate this difficulty it has been proposed to present the results in relation to activities obtained per volume culture[34]. In this study, however, volume was partly equalized with the volume in submerged cultures by adding 100 of water to the fermented slurry in solid-state fermentation. Such presentation of enzyme activities might provide a better orientation in practical productivity of fermentation. Another way of comparison of enzyme yields in submerged and solid-state fermentations is by projecting the yield values in relation to substrate used. As shown in Table 2, the enzyme productions in submerged cultivations were 6.3-10.8-fold higher than those obtained in solid-state fermentations considering the enzyme yields per g substrate used. However, the overall process economy would be cheaper by employing solid-state fermentation for enzyme production using cheap substrates like agricultural residues. Solid-state fermentation (SSF) holds tremendous potential for the production of enzymes. It can be of special interest in those processes where the crude fermented product may be

used directly as the enzyme source[35]. This system offers numerous advantages over submerged fermentation system, including high volumetric productivity, relatively higher concentration of the products, less effluent generation, requirement for simple fermentation equipments, etc.[36-37].

Table 2. *Comparison of yield of hydrolases per gram wheat bran substrate utilized by Trichoderma reesei and Trichoderma viride in submerged and in solid-state cultivations*

Enzyme	Enzyme activity (U/g substrate used)			
	T. reesei		*T. viridae*	
	Submerged	Solid-state	Submerged	Solid-state
FPase	19.0	2.4	14.0	1.7
CMCase	26.0	2.9	21.0	1.9
Xylanase	615.0	65.0	1,165.0	130.0
Pectinase	260.0	41.0	515.0	51.5

One of the major technical difficulties in solid-state fermentation is the extraction or leaching of enzyme from fermented materials, which is critical in determining the economic feasibility of enzyme production[38]. In this study, water, normal saline and non-ionic surfactant Tween 80 were used for separation and recovery of enzymes from the heterogeneous solid-liquid fermented slurries. As shown in Table 3 that the enzyme recovery was enhanced to some extent when Tween 80 was used as leaching agent. This finding is in agreement with other studies[39-40]. The non-ionic surfactant increases extracellular protein accumulation in culture filtrates by enhancing the export of proteins or enzymes through the cell membrane[40]. In this study, no acidic and alkaline leaching solutions were used, since they might adversely affect enzyme activities.

Table 3. *Effect of different leaching agents on extraction of extracellular hydrolases from fermented slurry in solid-state cultivations using wheat bran as substrate*

Leaching agent	Enzyme activity (U/ml)			
	FPase	CMCase	Xylanase	Pectnase
Trichoderma reesei				
Normal saline	0.46	0.52	12.0	11.2
Tap water	0.50	0.53	13.3	11.6
Distilled water	0.49	0.52	13.4	11.3
Tween-80 (0.1%)	0.63	0.68	16.2	15.1
Trichoderma viride				
Normal saline	0.45	0.39	18.8	10.3
Tap water	0.47	0.41	18.6	10.2
Distilled water	0.48	0.46	19.1	9.8
Tween-80 (0.1%)	0.50	0.52	23.2	13.8

Figure 3 shows the effect of pH and temperature on activity of the enzymes produced by *T. reesei*. The optimum pH for enzymatic reaction was different for the various species of enzymes, and it varied between pH 4.5 and 5.5. Cellulases from *T. reesei* work better in more acidic (pH 4.5-5.0) and lower temperature (50°C) conditions than xylanase and pectinase. The latter two enzymes showed maximum activities at pH 5.5 and at 55°C. The enzymes of *T. viride* also exhibited different pH conditions for maximum catalytic activities (Figure 4). The optimum pH for FPase and pectinase was 4.5, while that for CMCase and xylanase was much higher (pH 5.5). However, all the enzymes displayed their optimum activity at 55°C.

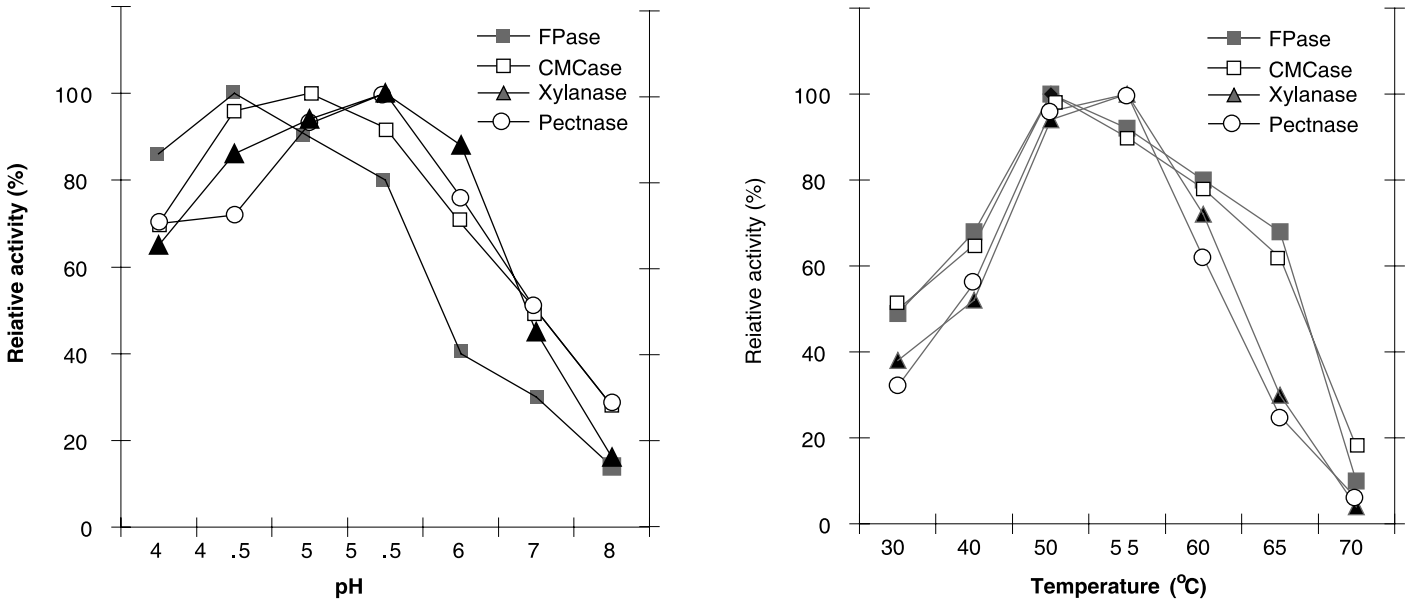

Figure 3. *pH and temperature profile of extracellular hydrolases produced by Trichoderma reesei*

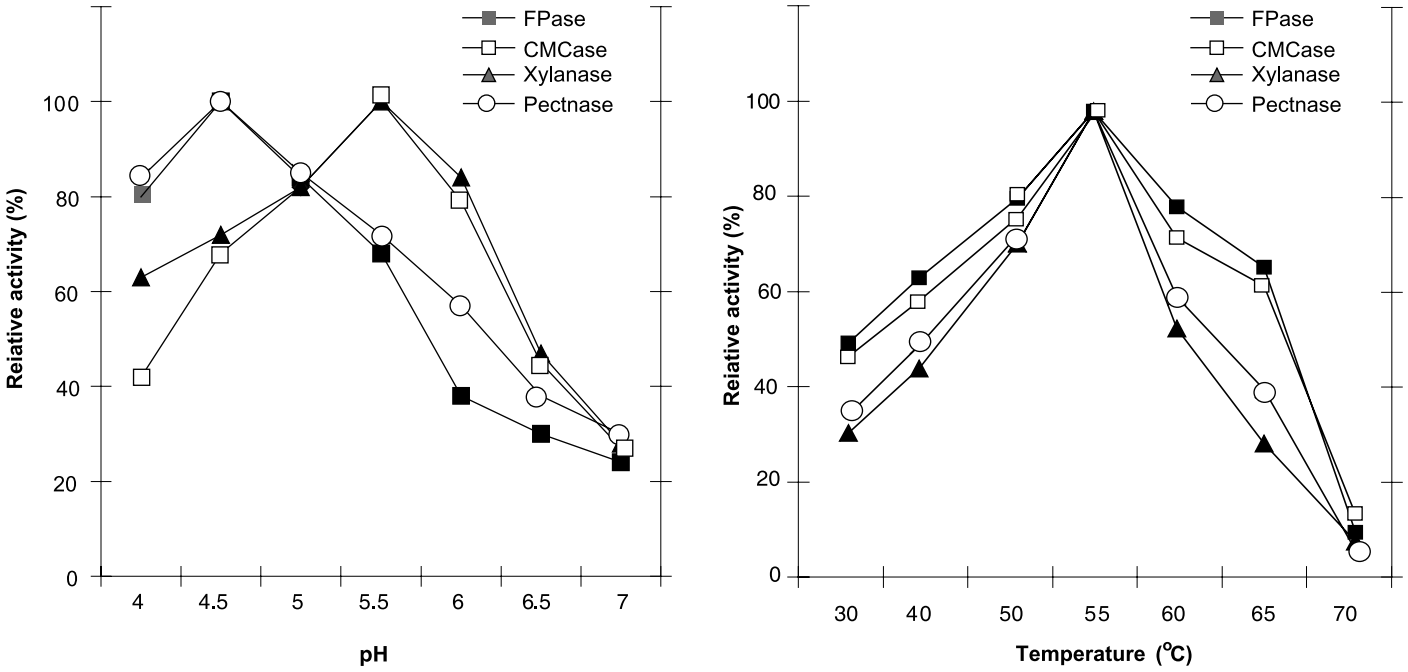

Figure 4. *pH and temperature profile of extracellular hydrolases produced by Trichoderma viride.*

In the case of enzymes produced from *T. reesei* and *T. viride*, the results of this study are in good agreement with published data[20-22]. In view of the results obtained on laboratory-scale, *T. viride* appears to be slightly superior to *T. reesei* with regard to the extracellular enzyme productions in submerged and solid-state fermentations. One fact is worth mentioning that both fungi exhibited a high level of pectinase activity in addition to the key enzymes, cellulases and xylanase, that are required to hydrolyse the major polysaccharide components of lignocellulosic biomass. The global saccharifying ability does not seem to be clearly correlated with individual activities, for which the pectinolytic activity of the preparation appears to play a large part[41]. The fungi used in this study merit further attention as potential sources of extracellular cellulolytic, xylanolytic and pectinolytic enzymes. High levels of the enzymes could be obtained by growing the fungi on potentially low cost lignocellulosic substrates both in submerged and solid-state fermentations, which is invariably a desirable property for large-scale production of the enzymes.

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