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Production of extracellular pectinolytic, cellulolytic and xylanoytic enzymes by thermophilic mould *Sporotrichum thermophile* Apinis in solid state fermentation

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Among four thermophilic moulds, *Sporotrichum thermophile* produced high titres of xylanases, pectinases and cellulases after 4 days of incubation in solid-state fermentation (SSF). Of the 27 different combinations of agro-residues tried, wheat bran (WB) and citrus pectin (CP) in 1:1 ratio supported a very high production of enzymes. When the mixed substrate at *p*H 7.0 was moistened with tap water (1:2.5 ratio) to a_w of 0.95 and inoculated with 60×10^7 conidiospores (from 5 day-old culture) 10^{-1} g of substrate, *S. thermophile* secreted maximum enzyme titres (xylanase 1900, pectinase 250 and cellulase 42 Ug^{-1} dry mouldy bran in 4 days at 45°C. The mixture of enzymes has been found useful in the treatment of fruit pulps for enhanced juice recovery.

Keywords: citrus pectin, cellulase, pectinase, *Sporotrichum thermophile*, SSF, thermostable, wheat bran, xylanase

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Introduction

In solid substrate cultivation, microbial growth and product formation occur on the surfaces of solid substrates. The solid-state fermentation (SSF) involves the growth and metabolism of microorganisms in the absence or near-absence of any free flowing water. Such a system, being closer to the natural habitats of microbes, may prove more efficient in producing certain enzymes and metabolites[1,2]. Solid substrate is the source of nutrients, mineral salts and trace elements, needed for microbial growth. Low water content of such substrates is a selective factor for mould growth that prevents undesired bacterial contaminations. Therefore, the class of microorganisms that is most commonly used is filamentous fungi. Pure (*Aspergillus awamori*, *A. oryzae*) or mixed (*Trichoderma lignorum* and *Candida lipolytica*) fungal cultures have been used in SSF. Solid substrates may be viewed as three phase gas-liquid-solid mixtures, in which an aqueous phase is intimately associated with solid matrix surfaces in various states of absorption that remain continuously in contact with the gas phase, an external gaseous environment[3].

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The agro-residues can be used for the production of microbial enzymes, such as xylanases, cellulases, amylases, pectinases, proteases and lipases[1,4-8]. Various agricultural products, such as barley, rice, corn, soybean and food processing industry residues mixed with various mineral salts and additives are often used as the traditional substrates for SSF[9-11].

There are numerous advantages of SSF over conventional submerged fermentations (SmF), such as simple and inexpensive substrates, less water requirement, generation of low volumes of effluents and others. Therefore, there has been a resurgence of interest on SSF in the last couple of decades. The present investigation deals with the production of a mixture of xylanases, pectinases and cellulases by a thermophilic mould, *Sporotrichum thermophile* Apinis in SSF using agro-residues, since these enzymes have been found to be useful in fruit juice extraction[12].

Materials and Methods

Microorganisms and Growth Conditions

The cultures of thermophilic moulds, *Malbranchea pulchella* var. *sulfurea, Rhizomucor miehei, Humicola lanuginosa* and *Sporotrichum thermophile* were obtained from the culture collection of the Department of Microbiology, University of Delhi South Campus, New Delhi. The moulds were grown on Emerson's YpSs agar[13] at 45ºC and maintained at 4ºC.

Assay for Polygalacturonase, Xylanase and Cellulase

Polygalacturonase, cellulases and xylanases were assayed by determining the reducing sugars liberated from pectin, xylan and cellulose using dinitrosalicylic acid reagent[14]. The reaction mixtures were incubated in a water bath at 55ºC. One unit of enzyme activity is defined as the amount of enzyme that catalyses the release of 1 μmol of reducing sugars from the respective substrates min^{-1} ml^{-1} under the standard assay conditions.

Processing of Solid Substrate

Wheat bran was washed with tap water followed by hot water, filtered and dried at 80ºC. The pretreatment of wheat bran was carried out to improve its suitability for microbial attack and penetration.

Enzyme Production in Various Agro-residues

10 g of different solid substrates (wheat bran, corn cobs, gram bran, citrus pulp, citrus pectin, rice straw, wheat straw, dried amla, apple pulp, beet root, carrot, oilseed cakes of mustard, cotton seed, sesame) per 250 ml flask were initially used. Based on the enzyme yields, different combinations of agro-residues with/without inducers (carboxymethylcellulose/ cellulose powder/cellobiose/pectin) were employed. Incubation temperature (45ºC), *p*H (7.0), a_w (0.95) and spore density (20×10^6 spores 10 g^{-1} substrate) were kept constant.

The effect of the initial *p*H of the medium on the yield of enzymes was studied by suitably adjusting the *p*H of moistening agent (tap water) with 1 N NaOH/HCl. The effect of temperature on the enzyme production was studied by incubating the Erlenmeyer flasks containing wheat bran and citrus pectin (10 g in 1:1 ratio) at different temperatures (37-60ºC). The effect of other physical parameters like inoculum age (2-6 d), inoculum levels (30, 60, 90×10^6, 20, 60×10^7 and 20×10^8 spores 10 g^{-1} substrate) and incubation time (2-8 d) on enzyme production was also studied.

Different salt solutions, buffer, tap water and distilled water were used as moistening agents. Wheat bran and citrus pectin (1:1) mixture was moistened to attain different a_w values (0.95, 0.92, 0.87, 0.85, 0.80, 0.75)[15]. Different ratios of the substrate to tap water [1:1, 1:2, 1:2:5, 1:3, 1:4 and 1:5] were used for studying the effect of moisture level on enzymes production. The mouldy bran was extracted twice with buffer, and the cell-free filtrate was used for enzyme assays. All the experiments were carried out in triplicate, and the mean values are presented.

SSF in Enamel Trays

200 g of wheat bran (WB) and citrus pectin (CP) mixture was moistened with 500 ml of tap water, and spread in enamel coated metallic trays (26×22.5×5.1 cm) covered with aluminum foil, autoclaved at 121°C (15 lb psi) for 60 min, cooled, inoculated with inoculum level 6×10^7 10^{-1} g substrate and incubated at 45°C for 4 days.

Results and Discussion

Among the four thermophilic moulds, *S. thermophile* was observed to be a potent producer of a mixture of enzymes (xylanase, pectinase and cellulase) (Table 1) and, therefore, this mould was selected for detailed investigation. The production of these enzymes by *S. thermophile* has earlier been reported in SmF[16,17].

The ideal solid substrate is one that provides all the necessary nutrients for the microorganism. Thus, the selection of an appropriate solid substrate plays an important role in the development of efficient SSF processes[18]. WB alone did not support the production of all three enzymes, and therefore, the combination of WB and CP (1:1) was used, which was found to be the best for the production of all three enzymes by *S. thermophile* (Table 2). This could be due to availability of xylan in WB and pectin in the CP and cellulose from both. The supplementation of WB with

Table 1—Production of enzymes with different combinations of substrates in SSF

	Enzyme activity (U/g DMB)								
	Control (WB)			WB+Pectin+FP			WB+Pectin+CMC		
	Xylanase	PGase	FPase	Xylanase	PGase	FPase	Xylanase	PGase	CMCase
H. lanuginosa	1578.5	139.4	—	1040.0	46.9	-	1815.3	67.31	0.78
M. pulchella var. *sulfurea*	350.5	85.3	3.0	300.2	94.9	6.7	15.1	4.7	7.3
R. miehei	2.6	128.5	2.8	11.3	155.2	-	40.6	342.9	3.9
S. thermophile	593.4	65.7	0.95	113.2	21.0	21.2	324.9	302.8	16.8

WB= wheat bran; FP= filter paper; CMC=carboxymethylcellulose; CMCase=carboxymethylcellulase

Table 2—Production of a mixture of enzymes with various combinations of agro-residues in SSF combination

		Enzyme Activity (U/g DMB)		
		Xylanases	Pectinases	FPase
1	WB	334.2	3.0	7.7
2	SOSC	22.6	6.6	12.6
3	CPP	—	5.1	18.5
4	WB + SOSC + CPP (5:3:2)	58.1	3.9	5.4
5	WB + SOSC + CPP (5:2: 3)	75.8	3.2	10.3
6	WB + SOSC + CPP (5:2.5:2.5)	89.6	6.0	6.3
7	WB + SOSC (5:5)	124.1	8.1	11.4
8	WB + SOSC (7.5:25)	77.7	4.2	4.8
9	WB + CMC (9:1)	203.2	117.3	11.2
10	WB + filter paper (9:1)	127.0	90.2	6.6
11	WB + cellulose powder (9:1)	289.5	117.6	7.2
12	WB + cellobiose (9:1)	150.3	88.8	7.0
13	WB + pectin (9:1)	97.8	96.0	7.2
14	WB + cellobiose + pectin (8:1:1)	191.6	221.0	9.4
15	WB + SOSC (5:5)	107.0	115.2	12.0
16	WB + pectin + SOSC (4:2:4)	197.0	119.7	15.6
17	WB +CPP+ cellulose powder (5:2.5:2.5)	300.0	5.2	9.1
18	WB + CPP + cellulose powder (8:1:1)	282.1	8.9	10.0
19	WB + CPP +cellulose powder (4:2:4)	138.6	29.6	6.0
20	WB + CMC + CP (8:1:1)	380.1	59.3	6.9
21	WB + CPP + SOSC (4:2:4)	358.7	169.3	16.5
22	WB + CPP + SOSC (4:2:4)	85.3	110.7	7.4
23	WB + CPP + SOSC (5:2.5:2.5)	196.4	—	8.1
24	WB+ cellulose powder +CP (5:2.5:2.5)	216.6	411.2	40.0
25	WB + CPP (5:5)	1151.0	500.2	44.8
26	WB + CPP (7.5:2.5)	932.8	456.4	33.7
27	WB + CPP + cellobiose (5:2.5:2.5)	631.8	443.7	36.1

WB=wheat bran; SOSC=sesame oil seed cake; CPP=citrus pulp; CMC=carboxymethylcellulose; FPase= cellulase

other solid substrates was found to increase the yield of enzymes. About 41% increase in enzyme production was attained when WB was supplemented with castor seed cake[19]. Wheat bran supplemented with maize bran, rice husk, rice bran and gluten, however, did not support good enzyme titres. The particle size and the chemical composition of the substrate are critical for SSF[7,20]. WP supported high titres of xylanolytic enzymes with low titres of pectinolytic and cellulolytic enzymes. The universal suitability of WB may be due to the fact that it contains adequate amount of nutrients and its ability to remain loose even in moist conditions, thus providing a large surface area[21]. The xylanase, pectinase and cellulase yields by *S. thermophile* were 400-, 200- and 20-fold higher in SSF as compared to those in SmF. A similar increase in yields of enzymes in SSF over SmF has been reported earlier[1,6].

The production of xylanase was optimal at *p*H 4.0 (Fig. 1A), which declined with an increase or decrease in *p*H. While the secretion of pectinase and cellulase was marginally higher at *p*H 7.0 than that recorded at other *p*H. An extrinsic parameter, such as *p*H, acts synergistically with other environmental parameters in addition to being a regulatory parameter in biotechnological processes[7].

The production of enzymes by *S. thermophile* was substantially higher at 45ºC (Fig. 1B). The fungus grew luxuriantly and produced the maximum level of xylanase at its optimal temperature of growth. Temperature control is intimately linked to water activity control, cooling of the system due to

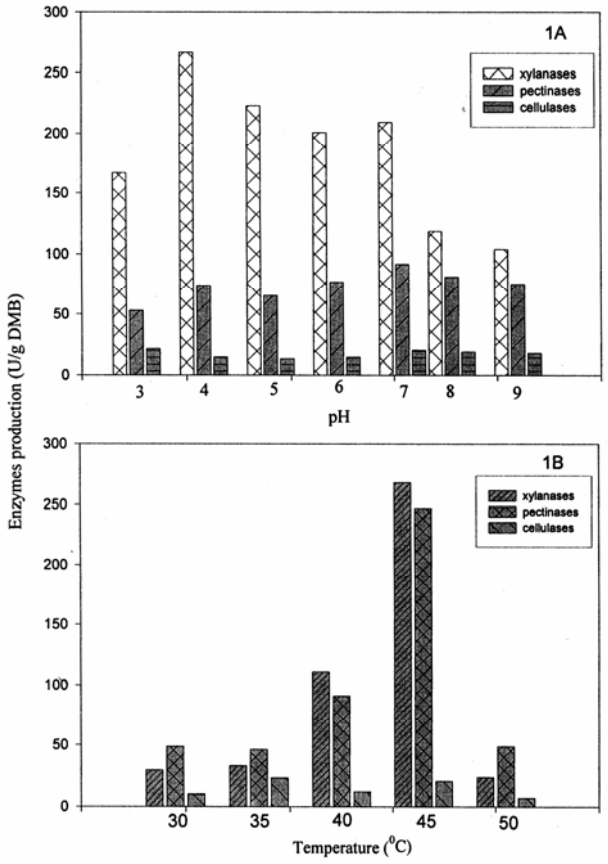

Fig. 1—Effect of (A) *p*H and (B) temperature on enzymes production by *S. thermophile*

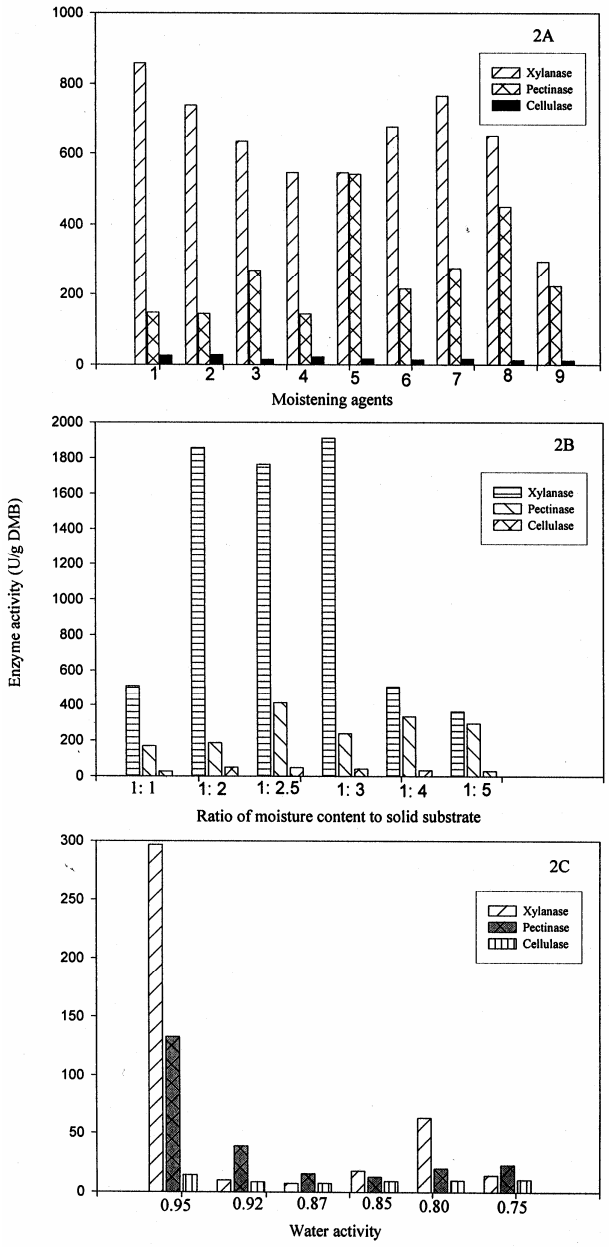

Fig. 2—Effect of (A) moistening agents [g l^{-1}: (1) $(NH_4)_2SO_4$ 4.0, KH_2PO_4 10.0, $CaCl_2$ 0.3, $FeSO_4$ 0.3 and $MgSO_4$ 0.3; (2) $(NH_4)_2SO_4$ 0.4, KH_2PO_4 2.1, $CaCl_2$ 0.3, $FeSO_4$ 0.1, $MnSO_4$ 0.3; (3) $CaCl_2$ 0.1, $MgSO_4$. $7H_2O$ 0.5, $FeSO_4$ 0.1; (4) NH_4NO_3 2.0, K_2HPO_4 6.0, KCl 0.5, $MgSO_4$ 0.5; (5) K_2HPO_4 0.1, $NH_4H_2PO_4$ 1.0, $MgSO_4$ 0.5, $CaCl_2$ 0.1, $FeSO_4$ 0.1, $MnSO_4$ 0.1; (6) Na_2HPO_4 11.0, NaH_2PO_4 6.0, KCl 3.0, $MgSO_4$ 0.1 ; (7) sodium phosphate buffer; (8) tap water; and (9) distilled water], (B) ratio of moisture content to solid substrate, and (C) water activity on enzyme production by *S. thermophile*

evaporation and physiological changes of behaviour of microbes. Among the various moistening agents (mineral solutions, tap water and distilled water), tap water supported high enzyme titres (Fig. 2A). Others have also reported tap water to be a suitable moistening agent in SSF[1,22]. The maximum production of mixture of enzymes was recorded when the substrate to tap water ratio was 1: 2.5 (Fig. 2B). The moisture content in SSF is a crucial factor that determines the success of the process[23,24]. A higher than optimum moisture level causes decrease in the porosity, alteration in the wheat bran particle structure, gummy texture, low oxygen transfer and enhanced formation of aerial mycelia[7,25]. On the other hand, a lower moisture level leads to reduced solubility of the nutrients of the solids substrate, lower degree of swelling and higher water tension[26]. Water activity is an estimate of the proportion of free water that is available for biological and physiological activity. The enzyme titres of *S. thermophile* were consistently high at a_w of 0.95 (Fig. 2C), with a sharp decline at decreasing values of a_w (0.92, 0.80, 0.87 and 0.75), suggesting that this mould has a limited desiccation tolerance. At relatively low moisture

contents, growth and metabolism of the microorganism can be affected. Water activity is known to influence the enzyme activity, protein stability, enzymatic transformations and metabolite production in SSF[23].

The enzymes titres were optimal when the conidiospore suspension prepared from 5-d old culture of *S. thermophile* was used for inoculation (Fig. 3A). The production of enzymes was also optimal when an inoculum density of 60×10^7 spores 10^{-1} g of substrate was used for inoculation (Fig. 3B). In general, the production of enzyme increased with the increase in inoculum density (30×10^6 to 60×10^7 spores 10 g^{-1} substrate), which declined at 20×10^8 spores 10 g^{-1} substrate. However, Raimbault & Alazard[25] reported equal protein synthesis with 4×10^6 to 4×10^7 spores of *Aspergillus niger* g^{-1} of WB that declined with 4×10^8 spores. Low inoculum density might result in decreased amount of biomass, whereas higher inoculum density levels cause fierce competition for nutrients. Further, a high enzyme production was recorded in 4 d of incubation (Fig. 3C). The incubation time is generally dictated by the composition of the substrate and properties of the strain, such as its growth rate, enzyme production profile, initial inoculum and others[7].

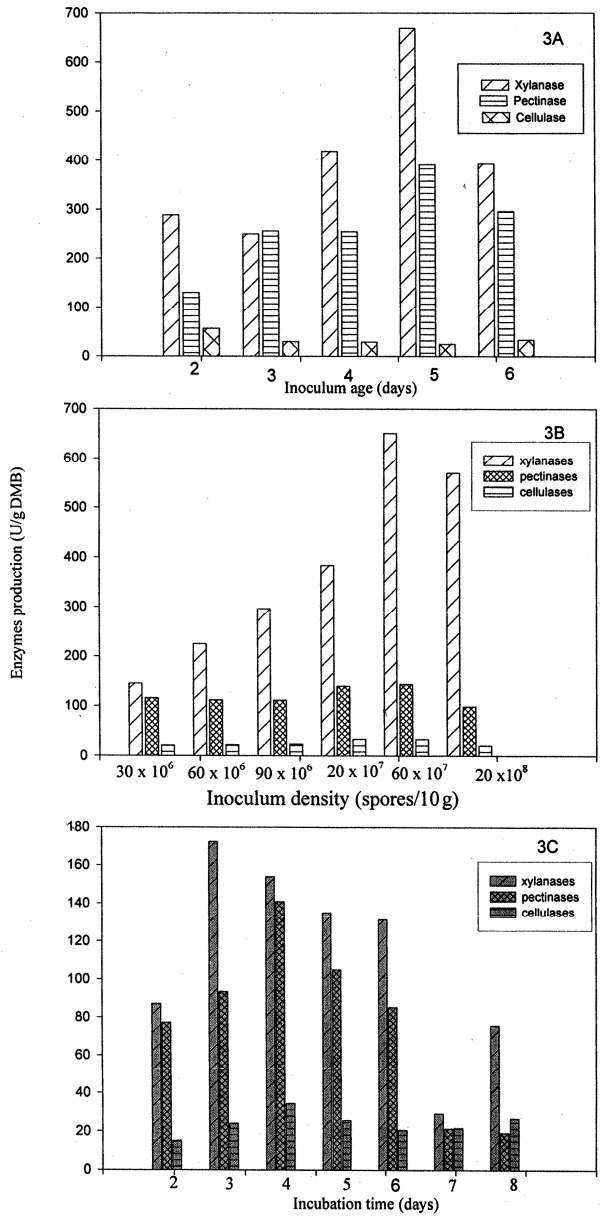

Fig. 3—Effect of (A) inoculum age, (B) inoculum density, and (C) incubation time on the production of enzymes by *S. thermophile*

The production of enzymes by *S. thermophile* in SSF reached a peak in 96 hrs at 45°C followed by a decline. A similar observation was reported in *Melanocarpus albomyces*, which produced a high xylanase titre in 72 hrs at 45°C in SSF[27]. The production of all three enzymes was less in enamel trays than that in flasks. The possible reasons for decrease in enzyme production during the scale up process could be due to reduction in effective aeration and heat transfer. The heat and mass-transfer effects in tray fermentations are directly related to the thickness of solid substrate in the tray, a problem which can be avoided by using shallow trays with substrate either in the form of compressed cakes of thickness less than 10 cm[28,29]. During SSF by *S. thermophile*, reducing sugars were released from lignocellulosic substrates. Since the fermentation process was optimized to produce xylanases, pectinases and cellulases, the reducing sugars released were from the hydrolysis of hemicelluloses, pectin and cellulose. The mixture of enzymes has been found to be useful in the treatment of grapes, apple and banana fruit pulps for enhanced juice recovery[12].

Conclusion

A mixture of xylanolytic, pectinolytic and cellulolytic enzymes could be produced by *S. thermophile* in SSF using agro-residues. A marked increase in the secretion of enzymes was achieved by the optimization of variables in SSF.

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