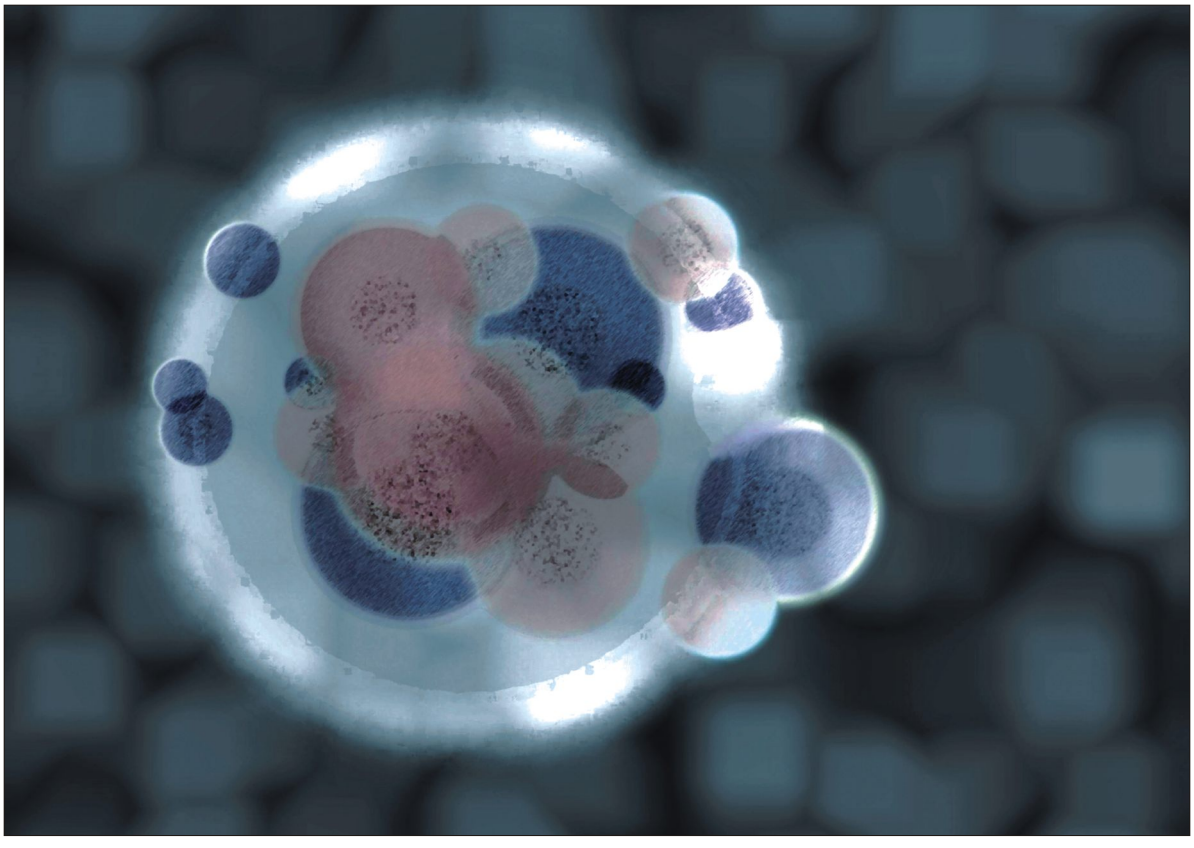

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Production Physiology of Cellulases and β-glucosidase Enzymes of *Aspergillus niger* Grown under Solid State Fermentation Conditions

M. Fadel
Microbial Chemisty Department, National Research Center, Dokki, Cairo, Egypt

Abstract: A new isolate of *A. niger* F-119 was utilizaed to produce endoglucanase (EC 3. 21. 4) exoglucanase (EC 3. 2. 1. 91) and 13- glucosidase (EC 3. 2. 1. 21) adopting the solid state fermentation condition using radicle waste as main substrate. Cultural conditions including moisture level, incubation period, initial pH, incubation temperature and inoculum size were evaluated. The fungus expressed high enzymes production at moisture level 70% (w /w), initial pH 4.5, inoculum size 10% (v/w) at 32°C after 72h which yielded 31.5, 46.0 and 215.2 l U/g original sub st rate. Supplementation of the culture medium with ammonium sulphate (at a level of 20 mg N/g substrate) enhanced enzyme activities. Similarly, wheat bran at 20% (w/w) was the most suitable carbon source for o b taining high enzyme yields. Sucrose at level 1% (w/w) was found to be more effective for enzymes biosynhesis raising the enzymes levels to 59.2, 74.4 and 445 l U/g substrate of FPase, CMCase and β-glucosidase respectively. The distribution of the solid medium at 1.5 cm proved to be suitable for enzymes production wherein the process can be successfully repeated for four different cycles. Partial purification with ammonium sulphate showed that the protein fraction obtained at 20%-40% ammonium sulphate saturation was most suitable for enzymes recovery. The enzymes were found to be active and stable at temperatures 55, 65 and 60°C, p H 4.5, 5.5 and 4.5. The effect of treatment with different concentrations of sodium hydroxide on delignification of wheat straw substrate and thus enhancing the enzymatic hydrolysis of wheat straw was markedly accelerated after treatment with 4.5% NaOH solution.
The partially purified 20-40% ammonium sulphate precipitate enzymes fraction and mouldy substrate were compared with crude enzyme extract to hydrolyze the alkali-treated wheat straw. The highest hydrolysis 82.8% was obtained using mouldy substrate. The enzymatic hydarolyzate containing 68% glucose of total sugars. The enzymatic hydrolyzate of the alkali-treated wheat straw was fermented for 48h by Saccharomyces cerevisiae and hybrid of *S. cerevisiae* has the ability to convert pentoses to enthanol 29.1 and 35.9 gram ethanol/100 g alkali-treated wheat straw were yielded by the two strains respectively.

Key words: *Aspergillus niger,* cellulases and β-glucosidase, production physiology solid state fermentation

Introduction

Cellulose is the most abundant carbohydrate polymer in wastes from forest products agriculture, fruits and vegetables. This cellulose polymer could be converted into simple sugars by enzymic degradation. These sugars can then be used as microbial substrates to produce a variety of fermentation chemicals (alcohols, solvents. etc.) or single cell protein, or they can serve as a base for the manufacture of organic chemicals (Ryu and Mandels, 1980). Recently increasing interest has been expressed in the problem of finding new food sources for our expanding population by growing microorganisms on such materials as certain petroleum fractions and cellulosic wastes. Economical utilization of such lignocellulosic materials is hindered because of the high costs of produce their hydrolytic enzymes such as cellulases. One effective approach to reduce the cost of enzyme production is to replace pure cellulose or lactose with relatively cheeper substrates, such as lignocellulosic materials. There have been reports of successful attempts to produce cellulase on lignocellulosics (Cowling and Kirk, 1976). The development of technology with minimum capital investment is another approach to reduce the cost of cellulase production. This can be accomplished by producing cellulases in a solid-state fermentation (SSF) process that requires relatively inexpensive equipment compared to the conventional fermentor used for liquid-state fermentation (LSF) process. In solid state fermentation insoluble substrate is fermented with sufficient moisture, but without free water.

For complete hydrolysis of cellulose to glucose, cellulase systems must contain endo-1.4 β-glucan (1.4-β-D-glucanohydrolase' EC 3.2.1.4), exo-1.4β-glucan (1.4-β-D-cellobiohydrolase' EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21' 13 -D-glucohydrolase) or cellobiase. Thus the hydrolysis of cellulose is completed by the synergistic action of endo and exoglucanases.

The cellulase systems of hypercellulase producing mutants of *Trichoderma reesei* are generally deficient in β-glucosidase (Duff *et al.,* 1986). During the hydrolysis of cellulose with this type of enzyme system, cellobiose accumulates, which inhibits the action of cellulases (Ryu and Mandels, 1980). Reffering to the reported enzymatic hydrolysis of lignocellulosic materials we can deduce that a cellulase system having a ratio of β-glucosidase activity to FPa se close to 1.0 is necessary to obtain the highest rate of hydrolysis and highest glucose content in the hydrolysate.

The present work was thus conducted in order to elucidat the production physiology of this group of enzymes by a selected strain of *Aspergillus niger* under condition of SSF.

Materials and Methods

Micro-organisms: *A. niger* F-119 was locally isolated from decayed wheat straw and identified by Plant Pathology Departe ment N. R. C, Egypt. The fungus was maintained an potato dextrose agar (PDA) medium. *Saccharomyces cerevisiae* AFZ- 998 was isolated from sugar cane molasses and identified by the author. Fusant of *S. cerevisiae* 5L was obtained from Microbial Genetic Lab N. R. C. Egypt.

Substrate: Radicle, the malt manufacture residue, was obtained from Al-Ahram Company for Beverages, Giza -Egypt. The substrate was dried at 105°C to constant weight and ground to 20 mesh size.

Preparation of fungal inoculum: Inoculum was prepared by scraping the content of one 7 day -old slant with 20 ml sterile 0.05 M citrate buffer pH 4.8, then shaked for 10 minutes at 200 rpm before using for inoculation of the experimental flasks.

Culture conditions: All experiments were conducted in 25O ml conical flasks contained 10 g radicle except in the case of studying effect of the medium depth on the enzyme production which was conducted in 250 ml graduated cylindrical bottles. 0.1 M citrate buffer pH 4.8 was used to moisten the substrate to the desire level. Two buffers namely 0.1 M phosphate buffer and 0.1 M citrate buffer were used to adjust the liquid phase to various pH range between 3.0-7.0, when the effect of pH on enzymes biosynthesis was studied. The experimental flasks containing the growth media were sterilized at 121°C for 30 minute, cooled, then inoculated with spores suspensicn of the fungus. The effect of different concentrations of nitrogen sources as well as the addition of insoluble carbohydrates and soluble sugars were also studied.

Successive reuse of fermented substrate: After enzymes extraction, the residue was oven dried, ground and reused as a medium for fungus cultivation under the optimum culture conditions. The original radicle was taken as control.

Enzym es extraction: The enzymes from SSF were extracted by mixing the whole content of the flask in a total of 100 ml 0.1 M citrate buffer pH4.8 and then shaking for 30 minutes at 150 rpm at 32°C, followed by filtration through glass wool. The filtrate was centrifuged at 5.000 rpm for 15 minutes and clear supernatant was used as a source of the enzymes.

Enzyme assays: Enzyme assays for filter paper activity (Fpase) and carboxymethyl cellulase (CMCase) were performed according to Mandels *et al.* (1974). β-glucosidase activity was determined according to the method described by Kubicek (1981) using cellobiose as substrate.

Protein determination: Total protein in the supernatant was measured by the method described by Lowry *et al.* (1951).

Ammonium sulphate precipitation of enzymes: To aliquots from the culture extract of *A. niger* (100 ml each) ammonium sulphate was added separately with continuous stirring to make 20,40,60 and 80% saturation levels. In another attempt the precipitation was carried out in four steps i.e up to 20, 20 -40, 40, 60 and 60-80% saturation. The saturation were stored over night in refrigerator before separation. The pellet of precipitates in each case were separated by centrifugation and were suspended in 10 ml 0.1 M citrate buffer pH 4.8. The different fractions obtained were stored at 4°C.

Wheat straw delignificattion: Wheat straw was ground and sized to 50 mesh. Suspensions of 5% (w/v) wheat straw were treated with set concentrations of NaOH (1,1.5,3.0 and 4.25%) as described by (Garg and Neelakantan, 1982a, b; Toyama and Ogawa, 1972; Rao *et al.,* 1993). In another experiment suspension of wheat straw 50% (w/v) were mixed with 20% NaOH and incubated at 28°C for 18 h (Rao *et al.,* 1995). All the treatments were neutralized with diluted acetic acid and washed with tap water then dried at 105°C. Dried delignified substrate was ground and sized to 20-40 mesh.

Enzymatic hydrolysis of wheat straw by the fungal enzymes: Hydrolysis of alkali-treated wheat straw was carried out by incubating 5g substrate in the form of 5% slurry with 20-40% ammonium sulphate fraction. In other experiments dried fermented mouldy substrate for alkali-treated wheat straw hydrolysis was carried out in 100 ml capacity conical flask contained 5 g alkali-treated wheat straw in a total volume of 20 ml contained 25 IU FPase and incubated at 50°C for 48 h. Samples were taken after 24 and 48 h to determine both total reducing sugars and glucose. The percent hydrolysis was calculated from the equation according to Szczodrak *et al.* (1984).

$$\%\ \text{hydrolysis} = \frac{\text{Total sugar (g)} \times 0.9 \times 100}{\text{Weight of alkali-treated wheat straw (g)}}$$

Ethan ol production by *S. cerevisiae* grown on enzymatic hydrolysates: Alkali-treated wheat straw was incubated with dried fermented substrate (250 IUFPase) in a total volume of one liter of 0.05 M citrate buffer, pH 4.8, at 50°C for 48 h on rotary shaker 130 rpm. After hydrolysis, the residue was filtered and the supernatant was fermented for 48 h by actively growing yeast wild type *Sacc haromyces cerevisiae* (AFZ-998). In other experiments the genetic fusant of *S. cerevisiae* 5 L was used which can utilize xylose and ribose beside glucose for ethanol production.

Results and Discussion

Production of cellulases and β-glucosidase under various moisture levels: Moisture content in solid substrate affects both aeration and nutrients solubility and suitability to be utilized by microorganisms (Nigam, 1990). Data present in Table 1 show that moisture level has an effect on the enzymes level produced by the fungus, as well as the extra cellular total soluble proteins released. At 50 and 60% (w/w) moisture levels, same amount of protein was released by the fungus, whereas the protein biosynthesis at 60% (w/w) moisture more specific to exoglucanase and endoglucanase activities. On the other hand at 70% (w/w) moisture the specific activities were increased by about 5.9, 11.5 and 11.5% for FPase, CMCase and β-glucosidase respectively. The moisture level demands in solid state fermentation differs according to enzyme to be produced, substrate, microorganism as well as the particle size of the substrate as well as the configuration of the particles (Muniswaran and Charyulu, 1994; Nandakumar *et al.,* 1994; Krishna and Chandrasekaran, 1996; Fadel, 1999). A lower moisture content resulted in a decline in enzyme yield. Perhaps this results may be due to suboptimal growth, less substrate swelling and high water tension during low moisture levels (Lonsane *et al*., 1985). On the dher hand the reduction in enzyme yields at high initial moisture content might be due to the steric hinderance in inter particle spaces and impaired oxygen transfer (Xavier and Lonsane, 1994). In general our results obtained in the present work agree with those reported for cellulase production in SSF by Sharma *et al*.

Table 1: Effect of moisture level on production of cellulases and β-glucosidase by *A. nigar* F-119 on radicle waste under solid state fermentation conditions

Moisture	Protein mg/g	Enzyme activity IU/g substrate			Specific activity IU/mg protein		
% (w/w)	Substrate	Fpase	CM Case	β-glucosidase	Fpase	CM Case	β-gluoss idase
40	123.3	15.7	26.8	165.4	0.13	0 22	1 .34
50	116.7	16.9	27.2	177.4	0.15	0.23	1.52
60	116.7	19.3	30.4	177.6	0.17	0.26	1.52
70	106.7	19.5	31.0	186.8	0.18	0.29	1 75
80	110.0	18.8	28.4	168.4	0.17	0.26	1.53

The enzymes were extracted after four days of growth at 32°C

Table 2: Effect of incubation period on the biosynthesis of cellulases and β-glucosidase enzymes by *A.* niger F-119 cultivated on radicle containing 70% (w/w) at 32°C

Incubation	Protein mg/g	Enzyme activity IU/g			Specific activity IU/mg protein		
time (h)	substrate	Fpase	CM Case	β-glucosidase	Fpase	CM Case	β-glucosidase
24	83.3	18.3	11.8	36.2	0.09	0.17	0 44
48	160.6	16.8	28.6	137.8	0.15	0.26	1.25
72	106.7	19.5	30.4	183.1	0.18	0.29	1.72
96	106.7	19.5	31.0	186.8	0.18	0.29	1.75
120	96.7	15.4	23.6	162.2	0.16	0.24	1.68

Table 3: Effect of initial pH of radicle medium (70% moisture) on the biosynthesis of cellulases and β-glucosidase enzymes by *A.* niger F-119 under solid state fermentation conditions

Initial pH	Protein mg/g	Enzyme activity IU/g			Specific activity IU/mg protein		
	substrate	Fpase	CM Case	β-glucosidase	Fpase	CM Case	β-glucosidase
3.0	75.7	11.3	18.4	128.1	0.15	0.24	1.68
3.5	85.4	14.4	24.0	168.1	0.17	0.28	2 20
4.0	86.1	22.8	31.7	196.2	0.27	0.36	2.28
4.5	98.5	24.4	36.4	211.2	0.28	0.41	2.39
5.0	96.9	23.9	35.4	198.8	0.25	0.38	2.05
5.5	112.4	21.2	32.8	188.0	0.19	0.29	1.67
6.0	106.7	19.5	30.4	156.8	0.18	0.29	1.47
6.5	98.4	17.5	27.2	117.6	0.18	0.28	1.20

The enzymes were extracted after three days of growth at 32°C

Table 4: Influence of incubation temperature on the biosynthesis of cellulases and β-glucosidase enzymes by *A. niger* F-119 under solid state fermentation conditions

Temperature	Protein mg/g	Enzyme activity IU/g			Specific activity IU/mg protein		
°C	substrate	Fpase	CM Case	β-glucosidase	Fpase	CM Case	β-glucosidase
24	81.2	18.9	14.5	110.5	0.23		
28	89.3	23.8	17.2	130.4	0.27		
32	88.5	24.4	36.4	211.2	0.28	0.41	2.39
36	94.9	23.7	31.4	198.6	0.25	0.33	2.59
40	92.01	21.2	17.9	140.4			

The enzymes were extracted after three days of growth on radicle at moisture level 70% (w/w) and initial pH 4.5

Table 5: Influence of inoculum size on the biosynthesis of cellulases and β-glucosidase enzymes by *A.* niger F-119 cultivated on radicle containing 70% (vA) moisture and initial pH 4.5 under solid state fermentation conditions

Inoculumn	Protein mg/g	Enzyme activity IU/g			Specific activity IU/mg protein		
size % (v/w)	substrate	Fpase	CM Case	β-glucosidase	Fpase	CM Case	β-glucosidase
5	81.3	26.4	40.1	177.6	0.33	0.49	2.19
10	88.1	31.5	46.6	215.2	0.36	0.53	2.44
15	88.5	24.4	36.4	212.2	0.28	0.42	2.39
20	89.6	23.9	37.8	189.0	0.27	0.42	2.11
25	87.5	20.7	35.4	180.7	0.24	0.41	2.07

The enzymes were extracted after 72 h growth at 32°C

(1996) and Smits *et al*. (1996). Gupte and Madamwar (1997) and Grajek and Gervais (1987) reported that an increase in initial moisture content of the substrate from 55-74% greatly enhanced the filter paper activity. However, a furthar increase to 80% had detrimental effect on cellulolytic enzymes production.

Effect of incubation period on the cellulases and β-glucosidase enzymes biosynthesis: Short incubation period f o r enzymes production offers the potential for inexpensive production of enzymes (Silva *et al*., 1995). Incubation time necessary for optimal production varied between different enzymes produced from one substrate (Smits *et al*., 1996). Table 2 shows that the production of both FPase and CMCase is increased gradually up to 72 h and a remarked decrease was seen after 120 h. Low activity for β-glucosidase was detected after 24 h, whereas significant increase was noted after 48 h. The low activity for

Table 6: Effect of inorganic nitrogen sources on the biosynthesis of cellulases and β-glucosidase enzymes by *A.* niger F-119 under solid state fermentation conditions

Nitrogen	Source	Protein mg/g	Enzyme activity IU/g			Specific activity IU/mg protein		
Added		substrate	Fpase	CM Case	β-glucosidase	Fpase	CM Case	β-glucosidase
Control 0.0		88.1	31. 5	46.6	215.2	0.36	0 53	2.44
NH_4No_3	2%	91.0	2.00	45.4	219.0	0.27	0.50	2.35
	4%	106.2	6.00	36.2	190	0.24	0.34	1.74
	6%	100.1	23.0	34.2	190	0.23	0.34	1.9?
NH_4 a	2%	115.2	23.6	34.8	205	0.21	0.3?	1.78
	4%	109.2	24.2	36.8	190	0.22	0.34	1.74
	6%	94.1	20.0	34.8	184	0.21	0 37	1.95
$(NH_4)_2HPO_4$	2%	106.2	34.6	50.4	297	0.33	0.48	2.33
	4%	115.3	33.2	48.2	230	0.29	0.42	2.00
	6%	109.2	32.9	3.50	190	0.3?	0.36	1.74
$NH_4H_2PO_4$	2%	85.9	30.8	39.6	144	0.36	0.46	2.32
	4%	88.6	32.2	39.1	144	0.36	0 44	2.25
	6%	109.2	31.5	38.4	187	0.29	0.35	1.71
$(NH_4)_2SO_4$	2%	100.1	38.2	56.6	258	0.38	0.57	2.58
	4%	109.2	38.3	56.9	246	0.36	0.52	2.25
	6%	106	28.2	53.1	224	0.27	0.50	2.11

The organism cultivated on radicle moisture content was 70% (w/w}. initial pH 4.5 with, inoculum size 10% (v/ w). the enzymes were extracted after 72 h of growth at 32°C

Table 7: Effect of supplementation some carbon sources with radicle on the biosynthesis of cellulases and β-glucosidase by *A.* niger F-119 under solid state fermentation conditions

Nitrogen	Source	Protein mg/g	Enzyme activity IU/g			Specific activity IU/mg protein		
Added		substrate	Fpase	CM Case	β-glucosidase	Fpase	CM Case	β-glucosidase
Control		100.1	38.2	56.6	258	0.38	0.57	2.58
Cellulose	10%	96.5	37.2	40.6	246	0.39	0.42	2.55
Powder	20%	94.2	34.2	31.2	226	0.36	0.33	2.40
	30%	88.3	26.2	28.1	205	0.3?	0.32	2.32
Carboxym	10%	116.6	41.7	65.3	305	0.36	0.56	2.62
ethyl cellulose	20%	110.2	38.2	67.1	296	0.35	0.61	2.64
	30%	96.3	31.8	58.4	274	0.33	0.61	2.85
Raw potato								
starch	10%	85.0	31.6	46.5	225	0.37	0.55	2.88
	20%	77.0	29.4	43.3	226	0.38	0.56	2.92
	30%	76.2	24.4	41.5	214	0.32	0.55	2.81
Soluble								
potato starch	10%	94.5	36.3	49.3	294	0.38	0.52	3.16
	20%	96.8	37.2	50.2	313	0.38	0.52	3.23
	30%	99.1	37.7	49.3	310	0.38	0.50	3.13
Ground								
corn cobs	10%	86.4	33.2	48.6	256	0.37	0.56	2.96
	20%	77.6	39.6	46.8	244	0.39	0.60	3.14
	30%	72.9	30.0	44.2	238	0.40	0.61	3.26
Wheat								
straw	10%	79.2	31 6	42.1	256	0.40	0.53	3.23
	20%	76.6	30.2	42.5	243	0.39	0.56	3.17
	30%	73.3	28.9	36.4	216	0.34	0.50	2.93
Corn bran	10%	108.7	49.1	67.1	316	0.45	0.62	2.93
	20%	112.3	54.8	70.9	340	0.48	0.63	3.02
	30%	109.4	48 0	58.2	325	0.44	0.53	2.98
Wheat bran	10%	110.6	49.6	69.6	305	0.45	0.63	2.76
	20%	106.8	52 0	68.7	340	0.49	0.64	3.18
	30%	102.4	49.2	66.2	311	0.48	0.65	3.04

The organism was cu ltivated on radicle at moisture content 70% (w/w). initial pH 4.5, inoculum size 10% (v/w). The enzymes were extracted after 72 h of growth at 32°C

β-glucosidase during first 24h may be due to the fact that original substrate contained no compounds stimulating enzyme production. The action of both endoglucanase (CMCase) and exogluconase (FPase) during the early stages of growth resulted in cellobiose production that can induce β-glucosidase biosynthesis at later stages (Godden *et al.,* 1989). The incubation time required for the formation of such enzyme also varies according to the substrate and micro-organism under study (Deschamps *et al.,* 1985; Ye and Fields, 1989). On the other hand incubation time needed for enzyme production was found to be shorter in solid state fermentation than the submerged culture (Macris *et al.,* 1989; Illanes *et al.,* 1992; Jiafa *et al.,* 1993). Endo glucanase was obtained after 66h by *Trichoderma harzianum* (Deschamps *et al.,* 1985). The high est filter paper activity and carboxymethylcellulase activities of 4.27 and 12.05 IU/g respectively were obtained in 7 days of fermentation, while the maximum cellobiase activity that could obtined after 8 days (Muniswaran and Charyulu, 1994) On the contrary

Dhillon *et al*. (1988) obtained the maximum yield from CMCase, F P ase and cellobiase after 7 days incubation when T. *reesei* QM 9414 was cultivated on rice straw. The fact that cultures of *Asperigillus spp.* produce more beta-glucosidase was reported by many authors (Asquieri and Park, 1992; Gupte and Madamwar, 1997).

Effect of pH variation on the biosynthesis of cellulases and β-glucosidase enzymes: The hydrogen ion concentration has a mar ked effect on enzyme production. This may be to the stability of extracellular enzymes at this particular pH and the rapid denaturation at lower or higher pH values (Kalra and Sandhu, 1986). In SSF process, greater attention is given to optimizing the initial pH value of the moist solid medium (Lonsane *et al.*, 1985). Table 3 shows the effect of initial pH variation on the biosynthesis of cellulases and 13- glucosidase. The enzymes biosynthesis was in hibited at both low pH under 4.0 and at high pH above 5.5. Great enhancement in enzyme biosynthesis was recorded between pH range 4.0 to 5.5 and the optimum was at 4.5. The effect of initial pH of the medium on the r ate of enzyme production was reported irrespective to the type of solid substrate used. The monitoring and control of pH in S SF processes is not usually attempted, probably because of the difficulties in measuring the pH of the moist solids. In fungal SSF processes, the buffering capacity of some medium constituents is employed to eleminate the need for pH control (Chahal *et al*., 1996).

Effect of temperature of incubation on the biosynthesis of cellulases and β-glucosidase enzymes: The selection of incubation temperature is guided by the optimum growth temperature of the culture. Table 4 shows the activity of enzymes produced under five different incubation temperatures ranged between 24-40°C. Data show that biosynthesis of both endoglucanase and β-glucosidase was more inhibited by low and high temperatures (24 and 40°C), than exoglucanase. The optimum temperature 32°C was recorded for the production of the three enzymes in the present study by *A. niger* F-119. The usual temperature maintained in SSF systems, is usually dependant on the growth kinetics of the microorganism employed for the fermentation purposes (Lonsane *et al*., 1985).

Asquieri and Park (1992) found that the optimum temperature for producing CMCase and beta-glucosidase from thermostable *Aspergillus* sp. was produced at 37°C. Maximum cellulases production were observed when the SSF was performed at 35 and 45°C using *Penicillium chrysogen* um (Sharma *et al.,* 1996). Temperature for cellulases production from different fungi on various substrates under SSF were studied in the literature by many investigators (Lonsane *et al*., 1985; Abdullah *et al*., 1985).

Effect of inoculum size: Table 5 shows that the level of enzymes was influenced by the inoculum size. FPase and CMCase were remarkedly affected by inoculum size than β-glucosidase, and the maximal activity of the three tested enzymes was achieved by using inoculum size at 10% (v/w) . Inoculum size controls and shortens the initial lag phase (Sharma *et al.,* 1996). The same authors reported that a smaller inoculum size increased the lag phase, whereas larger inoculum size increased the moisture content to a significant extent. The free excess liquid presents additional diffusional barrier together with that imposed by the solid nature of the substrate and leads to a decrease in growth and enzyme production (Muniswaran and Charyulu, 1994). Observations of the effects of inoculum size on maximal enzyme production in the present study agree with the facts stated above (Fig. 1).

Effect of inorganic nitrogen source: Production of cellulases is sensitive to the nitrogen source and nitrogen level in the medium (Desai *et al*., 1982). Table 6 shows that cellulases and β-glucosidase formation were inhibited by the high levels from all tested inorganic nitrogen sources. More inhibition was seen with ammonium chloride.

Compared to control about 13.6% increase in extracellular soluble protein/g substrate was released when 2% N (w/w) in the form of ammonium sulphate was introduced with the radicle. This increase in soluble proteins was accompanied by increase amounting to resulted in 21.3, 21.5 and 20% in the activity of FPase CMCase and β-glucosidase respectively. In the literature ammonium salts in form of sulphate was shown to facilitate the cellulase and β-glucosidase production in *Penicillium funiculosum, Myrothecium SP, Chaetomium cellulolyticum, Trichaderma reesei A. niger* and *A. terreus* (Harima and Humphrey, 1980; Rao *et al.,* 1995; Chahal *et al*., 1996).

Effect of supplementation with additional carbon sources: Table 7 shows the influence of enzymes biosynthesis by supplementation with additionals carbon source at three levels namely 10, 20 and 30% (w/w). Data show that β-glucosidase was induced in different level by added any substrate except in with the addition of the cellulose powder addition. The highest β-glucosidase was produced when 20% corn bran or wheat bran was added along with radicle. Carboxymethylcellulose (CMC) resulted in about 9.2% increase in FPase production when added by 10% (w/w) and resulted in 15.4-18.6% increase in CMCase when supplemented in 10 and 20% (w/w) respectively, as well as the high increase in β-glucosidase was 16.7% when CMC supplemented level was 20% (w/w). Corn bran and wheat bran at level 20% (w/w) were found to be the most effective nutrient for promoting activity of both cellulases and β-glucosidase as the increases were 40.8, 25.3 and 31.8% for FPase, CMCase and β-glucosidase respectively. When corn bran was used comparable to 36.7, 21.4 and 31.8% for wheat bran. The data showed that t he extracellular protein released in the medium, when wheat bran was supplemented was specifically related to enzymes under study than the protein released when corn bran was supplemented. Supplementation of nutrients for inducing enzymes have been studied by many workers. The universal suitability of wheat bran is apparent from literature. It was reported that wheat bran contain a sufficient amount of nutrients and was found to remain loose even in the moiste state, thereby providing a large surface area (Fenikosva *et al.,* 1960). Higher concentrations of wheat bran were found to inhibit the growth of *A. oryzae* and *A. niger* (Novotel *et al*., 1964). Wheat bran is a familiar as acomplete medium for producing cellulases, amylases and xylanase in SSF (Smits *et al*., 1996). A mixture of wheat straw 80% and wheat bran 20% was used for cellulase production by *T. harzianum* in static and mixed solid state fermentation (Deschamps *et al.,* 1985). Yinbo *et al.* (1996) supplemented 10% (w/w) wheat bran with corn stover for cellulase production by *A. niger* L22, *A. niger* AM- 70 and *T. harzianum* 912.

Table 8: Effect of further supplementation with soluble sugars

Sugar added	Protein mg/g	Enzyme activity IU/g substrate			Specilic activity IU/mg protein		
	substrate	Fpase	CMCase	β-glucosidase	Fpase	CM Case	β-glucosidase
Control	106.4	52.0	68.7	340	0.49	0.64	3.18
Glucose 1%	116.1	59.2	75.3	396	0.51	0.65	3.41
2%	111.2	60.4	78.2	409	0.54	0.70	3.65
3%	108.3	58.1	64.3	376	0.54	0.59	3.47
Cellobiose 1%	112.3	56.1	71.2	415	0.50	0.63	3.70
2%	114.6	58.2	72.1	430	0.51	0.63	3.75
3%	102.3	51.6	62.5	385	0.50	0.61	3.76
Xylose1 %	110.6	47.2	52.6	376	0.43	0.48	3.40
2%	116.0	48.9	54.1	388	0.42	0.54	3.23
3%	116.0	48.6	53.2	385	0.42	0.46	3.32
Maltose1 %	122.1	63.7	76.3	426	0.52	0.63	3.49
2%	118.7	62.2	78.2	430	0.52	0.68	3.62
3%	115.6	56.8	69.5	395	0.49	0.60	3.41
Sucrose1 %	108.9	59.2	74.4	445	0.54	0.66	4.09
2%	119.1	58.6	72.6	410	0.51	0.64	3.60
3%	112.6	54.1	71.8	367	0.48	0.64	3.26

of radicle medium containing 1% N (w/w) in the form of ammonium sulphate and 20% (w/w) wheat bran) on cellulases and β-glucosidase enzymes biosynthesis by *A. niger* F-119 under solid state fermentation conditions. The organism cultivated on radicle moisture content was 70% (w/w). initial pH 4.5 with, inoculum size 10% (v/ w). the enzymes were extracted after 72 h of growth at 32°C

Table 9: Effect of the depth of medium on the cellulases and β-glucosidase enzymes biosynthesis by *A. niger* F-119 under solid state fermentation conditions

Deph	Protein mg/g	Enzyme activity IU/g			Specific activity Ili/mg protein		
mm	substrate	Fpase	CMCase	β-glucosidase	Fpase	CMCase	β-glucosidase
3	108.9	59.2	71.4	445	0.54	0.66	4.09
6	108.1	599	70.1	445	0.55	0.64	4.59
9	106.00	60	73.1	455	0.57	0.60	4.29
12	106.03	60	72.8	448	0.58	0.6	4.22
15	100.1	67.1	65.2	435	0.57	0.59	3.96
18	102.8	57.3	60.1	469	0.50	0.49	3.61
21	84.7	36.0	32.0	236	43	0.38	2.81
24	76.7	28	14 0	149	24	0.18	1.96

*Initial substrate was composed of 80% radicle and 20% wheat bran supplemented with 1% N (w/w) in the form ammonium sulphate. The organ ism cultivated on radicle moisture content was 70% (w/w). Initial pH 4.5 with, inoculum size 10% (v/w). The enzymes were extracted after 72 h of growth at 32°C

Table 10: Successive cycles of cultivation of *A. niger* F-119 on the residue of fungal fermented substrate after enzymes extraction in each cycle

Substrate used	Protein mg/g	Enzyme activity IU/g substrate			Specific activity IU/mg protein			% Utilized medium**
in fermentation	substrate	Fpase	CMCase	β-glucosidase	Fpase	CMCase	Enzyme activity u/g	
Initial Substrate	106.3	61.2	72.8	448	0.58	0.69	4.22	27.6
Residue from cycle No. 1	112.7	62.5	71.9	455	0.56	0.64	4.13	38.4
Residue from cycle No. 2	110.8	60.4	68.6	456	0.55	0.62	4.12	36.2
Residue from cycle No. 3	102.2	56.5	51.4	420	0.55	0.50	4.11	32.3
Residue from cycle No. 4	98.6	41.8	36.2	386	0.42	0.37	3.92	23.8
Residue from cycle No. 5	82.1	28.6	19.6	226	0.35	0.24	2.75	18.2

*Initial substrate was composed of 80% radicle and 20% wheat bran supplemented with 1% N (w/w) in the form of ammonium sulphate.
** utilized medium was calculated on the basis of weight of the medium used in the beginning of each cycle

Effect of supplementation with soluble sugars: Three levels i.e 1, 2 and 3% of five sugars namely, glucose, cellobiose, xylose, maltose and sucrose were tested for promoting cellulases and β-glucosidase by *A. niger* F-119. Results in Table 8 shows that the biosynthesis responded differently to the enzymes additional of sugars as well as the extracellular protein released. Generally, the extracellular protein secreted in the medium was higher than control and the biosynthesis of all tested enzymes were inhibited by xylose addition to radicle medium. On the other hand various increases in the production of the enzymes and specific activity were achieved by addiation of different sugars. FPase production was induced by glucose, maltose and sucrose, whereas, CMCase was induced by glucose and maltose. β-glucosidase was exhibited high levels of activity by addition 1% sucrose than other levels and other used sugars. Taking in consideration the specific activity as a criteria, addition of sucrose at level 1% was more specifically promoting for biosynthesis of both cellulases and β-glucosidase. Many investigators used different soluble sugars for inducing cellulases and β-glucosidase. Bone and Levonen-Munoz (1984) showed that addition of glucose at levels 5 and 10% of straw weight stimulated lignin degradation and cellulose hydrolysis when *Polyporus* sp. A-336 was cultivated on oat straw under SSF conditions. On the other hand Sanyal *et al*. (1988) showed that low level of glucose (1%) did not repress the synthesis of CMCase, but when glucose was added in the growth medium at higher concentrations (10%) further utilization of CMC was prevented and CMCase synthesis was inhibited. Sharma *et al*.

(1996) showed that the presence of glucose in the fermentation medium was found to be the most effective for promoting activity of the glucanase enzyme,as well as sucrose was the most inducing carbon source for cellulases production by T. *viride.*
On the other hand Sharma *et al.* (1996) reported that sucrose induced cellobiase better than glucose, whereas, glucose induced FPase better than sucrose in *P. hrysogenum.* Also cellulases were induced by maltose, whereas cellobiase biosynthels was inhibited by maltose when *P. chrysogenum* was cultured on lignocllulosics under SSF condition.

Effect of the medium depth: Depth is a potential controling factor in SSF (Hansen *et al.,* 1993). It is a key factor in determining the amount of air that travels through the substrate which contributes to heat and mass transport rates, as well as the rate of substrate decomposition (Lynch and Cherry, 1996). Depth may also be important in the production of an industrial enzyme by SSF. The depth of the substrate can affect the magnitude of temperature and oxygen gradients. Such gradients would be smaller in shallower substrate, while more pronounced at greater depths. Moisture gradients may exist in deep substrates, being more dry at the top and more wet at the bottom. There exists the potential for depth to have a major effect on the growth of the micro-organism which in turn would influence the amount produced. Table 9 shows that cellulases and β-glucosidase production have been increased slightly till 12 mm and steady till 18 mm then decreased where the enzyme yield had been reduced to lees than 70% at 24 mm of depth. The data obtained can be discussed on the bases of the moisture and temperature at specific depth are more suitable to prelong the phase in which these enzymes are biosynthesis and released. The optimum depth for enzyme production have been differ according to organism, type of enzyme and its source, substrate, and particle size. The effect of the depth of the subst rate on enzyme production for SSF has not been well studied, and there is a lack of published information on the effects of depth of the substrate. In the examination by Murthy *et al.* (1993) and Rathbun and Shuler (1983) the importance of depth is discussed. Experiments were initially performed on thin beds (0.5 cm) to eliminate heat and mass transport effect were observed to be more prominent in deeper beds. Ridder *et al.* (1997) obtained maximum xylanase when cultivated T. *longibrachiatum* on wheat bran by SSF at 1.5 cm whereas at 0.5 cm the yield was about 75% from its maximum.

Successive cultivation on fermented substrate: Existing literature on the utilization of the cellulosic materials indicates that the problem of effective utilization and conversion of these renewable carbon sources to useful products is not yet solved. This because successful utilization of the lignocellulosics depends upon the development of economically feasible process for enzyme production as well as its utilization. In the process of converting cellulosic materials to useful product, production of enzyme is the most expensive part. Its cost may be considerably reduced using cheaper substrates or efficient fermentation. Table 10 shows the data obtained with recycling the residue of fermented medium after extraction enzymes for five times to obtain better degradation and economic utilization of the substrate for enzyme production using the same fungus *A. niger* F-119. Under these conditions the fungus appears to produce promising quantities of FPase, CMCase and β-glucosidase till the four successive cultivation. Gradual increase in substrate utilization (%dry weight) was noted during each successive cultivation. A little information in the literatures about recycling fermented substrates after enzymes extraction to improve the cellulases and β-glucosidase.
In SSF mixed cultures of cellulolytic fungi was not able to get enhanced enzyme yield (Shamala and Sreekantiah, 1987).

Effe ct of extracting agent on the enzymes recovery from the solid state culture: Table 11 shows the various solvents that were applied for enzymes extraction from fermented substrates under SSF. The efficient solvent to leach enzyme from microorganism substrate may depend on many factors e. g type of enzyme, source of enzymes and substrates used as a medium. Distilled or tap water alone or with glycerin or sodium chloride was found to be best for amyloglucosidase extraction from mould-wheat bran (Ramakrishna *et al.,* 1982), whereas, alcoholic solvents was found to be less efficient than aqueous solvents. Phosphate and acetate buffers (pH 5.9) were found more suit a ble for alpha-amylase extraction (Feniksova *et al.,* 1960; Qadeer *et al.,* 1980). Fernandez-Lahore *et al*. (1998) found that 0.5 M sodium chloride was more suitable for acid protease recovery than distilled water. Shamala and Sreekantiah (1987) soaked the mouldy substrate in water and all owed to stand at room temperature (25 to 28°C) for 1 h for recovery of cellulases and β-glucosidase. In the present study citrate buffer, acetate buffer and sodium chloride can be reported for cellulases and β-glucosidase from mouldy-radicle wheat bran than distilled water, tap water, acetone solution and ethanol solution.

Partial purification with ammonium sulphate: The steps involved in the partial purification of cellulases and β-glucosidase from crude extract of *A. niger* F-119 from solid fermented substrate are shown in Table 12. The total activity in 100 ml crude extract was 413, 486 and 2987 U for FP ase CMCase and β-glucosidase respectively with corresponding specific activities 8.8, 10.3 and 63.4 IU/mg protein of FPase, CMCase and β-glucosidase respectively. Economic and best recovery for the three enzymes activities was obtained by using two steps. The first precipitation with saturation 20% (w/v) ammonium sulphate to get rid of some of unwanted proteins and in hibitors found in crude extract. Second precipitation was accomplished with 40% (w/v) ammonium sulphate. The present method resulted in recoveries of FPase, CMCase and 13 - glucosidase with increase in specific activities from 8 .8, 10.3 and 63.4 IU/mg protein in crude extract to 19.7, 18.8 and 124.5 IU/mg protein dissolved in 0.1 M sodium citrate buffer pH 4.8 for FPase, CMCase and β-glucosidase respectively. On the other hand high recoveries were achieved using 20-40% ammonium sulphate saturation fraction reaching 60.2, 60.0 and 58.4% for Fpase, CMCase and 13- glucosidase respectively. One step was applied for partial purification of cellulases and β-glucosidase by many workers. Eighty percent ammonium sulphate saturation was used for partial purification for cellulase from a strain of T. *viride* (Wood, 1988; Sharma *et al.,* 1991) and the recovery was 70% for CMCase. Ammonium sulphate fractionation (35 to 75%) was applied

Table 11: Comparative effeciencies of different extracting agents on cellulases and β-glucosidase enzymes recovery from the fungai culture grown radicle substrate fermented by *A. niger* F-119 under the optimum cultural conditions

Extracting agent	Protein mg/g substrate	Enzyme activity IU/g			Specific activity Ili/mg protein		
		Fpase	CMCase	β-glucosidase	Fpase	CMCase	β-glucosidase
Distilled water	89.0	49.7	6.20	378	0.56	0.70	4.25
Tap water	115.3	57.5	71.3	390	0.50	0.62	3.38
0.1 M acetate buffer (pH 4.8)	106.2	61.2	68.8	415	0.58	0.65	3.91
0.1 M citrate buffer (pH 4.8)	106.3	61.2	72.8	448	0.58	0.69	4.22
0.1 M citrate Phosphate buffer (pH 4.8)	120.7	59.2	67.7	448	0.49	0.56	3.71
5% acetone solution	100.5	56.3	58.2	424	0.56	0.58	4.21
5% Ethanol solution	101.5	57.7	66.2	436	0.57	0.65	4.30
0.1 M Sodium chloride solution	100.0	59.2	63.8	421	0.59	0.64	4.21

Table 12: Partial purification for cellulases and β-glucosidase activities produced by *A. niger* F-119 on radicle medium SSF under optimal conditions

Purification step	Total volume mL	Total protein (mg)	Total activity (IU)			Specific activity u/mg protein			Recovery %			Purification fold		
			1	2	3	1	2	3	1	2	3	1	2	3
A Experiment No (1) Ammonium sulphate saturation %	100	47.1	413	486	2987	8.8	10.3	63.4	100	100	100	-	-	-
0-20	10	6.5	49.4	60.7	384	7.6	9.3	59.1	12	12.5	12.9	0.91	0.93	0 93
0-40	10	17.0	158	241	1664	9.3	14.2	125.8	38.3	49.6	55.7	1.1	1.39	1.98
0-60	10	26.0	241	256	1670	13.1	17.5	82.6	58.4	52.6	55.9	1.5	1.72	1.30
0-80	0	26.0	241	256	1675	13.1	17.5	82.6	58.4	52.6	56.1	1.5	1.72	1. 30
B Experiment No (2)														
20-40	10	14.0	276	292	1743	19.7	18.8	124.5	60.2	60.0	58.4	2.2	2.1	2.4
40-50	10	5.0	65	82	445	13.0	16.4	89.1	15.7	16.9	14.9	1.5	1.2	1.4
50-60	10	4.0	48	37	257	12.0	9.3	64.3	11.6	7.6	8.9	1.4	.9	1.0

1 = Fpase 2 = CMCase 3 + β-glucosidase
The enzymes were extrated after three days of growth at 32°C

Table 13: Effect of sodium hydroxide concentration in the pretreatment of wheat straw on the extent of enzymatic hydrolysis with crude enzymes

Concentration of NaOH for wheat straw treatment	Hydrolysis of delignified wheat straw			
	24 h		48 h	
	%hydrolysis	% glucose	% hydrolysis	% glucose
Control	5.9	56	8.2	59
1%	7.8	58	15.9	63
1.50%	9.1	58	19.1	61
3.00%	23.2	62	41.8	61
4.50%	65.6	67	80.3	67
20%	18.1	72	29.1	73

% glucose from % hydrolysis

Table 14: Hydrolysis of alkali treated wheat straw with different preparations of cellulases from *A. niger* F-119

Source of enzyme	% hydrolysis	% glucose
Crude extract (supernatant)	80.3	67
Ammonium sulphate precipitate fraction 20-40%	79.1	64
Dried mouldy substrate	82.8	68

% glucose from % hydrolysis

by Garg and Neelakatan (1982b) for partial purification of FPase and CMCase from *A. terreus* GN1.

Characterization of partially purified cellulases and β-glucosidase enzymes of *A. niger* F 119

Effect of incubation temperature on enzymes activities: The effect of incubation temperature ranging from 40 to 90°C on the activity of FPase, CMCase and β-glucosidase was observed and no activity was detected at 80°C. The activity of β-glucosidase exhibited gradual increase from 40°C and the maximum activity was exhibited at 65°C. Sharp decrease in activity was evident above 70°C. where only about 15% was retained at 80°C and the activity was zero at 90°C. The effect of temperature on the cellulases activity was studied by many investigators. Garg and Neelakatan (1982b) reported 55 °C as the optimum for both FPase and CMCase produced from *A. niger* GN1, as well 55°C was the optimum temperature for CMCase of *T. viride* (Sharma *et al*., 1991). The optimum tempratures 55°C was reported for *Sterptomyces* sp. CMCase, whereas it was 60°C for CMCase of *Bacillus* sp. (Dasilva *et al*., 1993).

Table 15: Ethanol production *by Saccharomyces cerevisiae* enzymatic hydrolyzate of alkali treated wheat straw after 48 h at 34 ± 2°C

Source of enzyme	Alkali treated wheat straw (g)	Total sugar produced (g)	Yield of alcohol g/g sugars in enzymatic hydrolyzate	
			S. cerevisiae	*S. cerevisiae* 5 L (fusant)
Crude extract (supernatant)	100	80.3	28.6	35.6
Ammonium sulphate precipitate fraction 20-40%	100	79.1	27.2	32.3
Dried mouldy substrate	100	82.8	29.1	35.9

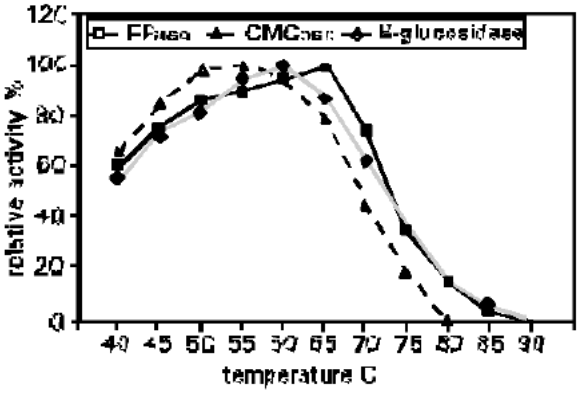

Fig. 1: Effect of temperature on partially purified cellulases and β-glucosidase activities of *A. nigar* F-119 grown on radicle under SSF

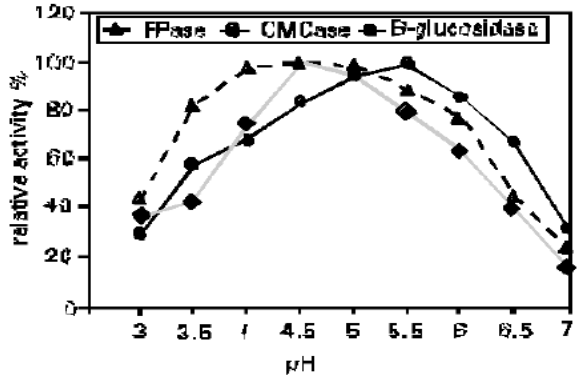

Fig. 2: Effect of pH on pratially purified cellulases and β-glucosidase activities of *A. niger* F-119 grown a radicle under SSF

Effect of pH: The data on the effect of different pH on the F Pase, CMCase and β-glucosidase are given in Fig. 2. FPase had maximum activity at pH range 4.0 to 5.0, peaking at pH 4.5. Whereas, the maximum activity of CMCase was noted at pH 5.5. β-glucosidase expressed its maximum activity at pH 4.5. In the literature many cellulases showed optimum activity in the range between pH 4.5-5.5 (Garg and Neelakatan, 1982b; Sharma *et al*., 1991).

Wheat straw hydrolysis: In developing a technology for cellulose hydrolysis by enzyme, one of the major factors is the production cost of cellulases. In the present work crude enzymes extract (culture supernatant) was applied for hydrolysis of 5% (w/v) alkali-treated wheat straw with different alkali concentrations as mentioned in materials and me thods. Data are given in Table 13 showed that the highest hydrolysis was expressed with alkali-treated wheat straw at 4.5% sodium hydroxide as the hydrolysis was about 80% (w/w). Using low or high concentration of NaOH than 4.5% the hydrolysis was affected greatly. Sodium hydroxide was used to pretreat lignocellulosics to expose the cellulose or hemicellulose for hydrolyzing enzymes (Chahal *et al*., 1996). The hydrolytic potential (80%) of cellulases produced in the present study may be due to high β-glulosidase contents which prevented the accumulation of cellobiose in the hydrolyzate consequently no inhibition took place for cellulase action on the cellulose substrate.

Our fin ding about the concentration NaOH used for wheat straw delignification agree with the results obtained by Chahal *et al.* (1996) and Abraham and Kurup (1997).

Compa rative study on wheat straw hydrolysis with crude enzymes, partially purified enzyme preparation and with mouldy substrate: Table 14 shows the hydrolysis of delignified wheat straw (4.5% NaOH) with crude enzymes and partially purified ammonium sulphate precipitated (20-40%) fraction as well as with mouldy substrate as a source of t he enzymes. Results show that similar hydrolysis could achieved by application of crude enzymes extract, ammonium sulphate precipitated enzymes and mouldy substrate as an enzyme source. The hydrolytic potential of the obtained enzymes could be considered exceeded the reported 80%. Rao *et al*. (1995) used mycelia! biomass of *P. funiculosum* for hydrolyzing alkali treated sugar cane bagasse and obtained similar hydrolyzing power corresponding to that of culture filtrate. Enzyme extract from SSF for cellulase production with *T. reesei* on wheat straw was used for hydrolysis of 5% (w/v) delignified wheat straw in pan bioreactor at concentration of 20 FU FPase/g delignified wheat straw and the hydrolytic potential was 85% (Chahal *et al*., 1996). Cellulase from *T. reesei* and 13-glucosidase from *A. niger* were used to hydrolyze bine barks (from pinus pinaster). About 12% hydrolysis was obtained for orginal substrate, and after delignification treatments the rate of hydrolysis reached 44.4% (Parajo *et al*., 1988).

Under submerged fermentation conditions, the conventional submerged fermentation technique requires strict aseptic conditions and longer incubation time. In addition, the enzymes are produced in diluted solutions. All these requirements are energy demanding processes and consequently increase the cost of production of these enzymes. On the other hand, in cellulase production by solid-state fermentation the expensive steps of extraction and p u rification of enzyme from the solids can be avoided since the cellulase enriched product can be directly utilized for saccharification after air drying at low temperature (40°C). The technique described in this article would help in p roducing enzyme solutions of any desired concentration in a shorter time and consequently may help in reducing the cost of production of these enzymes.

Ethanol production from enzymatic hydrolyzate of alkali-treated wheat straw: Data about the alcoholic fermentation of enzymatic hydrolyzate of alkali treated wheat straw are given in Table 15. Ethanol production from the hydrolyzates by three preparation of enzymes from *A. niger* F-119 i.e. crude enzyme extract, ammonium sulphat precipited enzymes and mouldy substrate are comparable. The lower conversion of sugar to alcohol (34.4 to 35.2% w/w) in three hydrolyzates may be due to the inability of *S. cerevisiae* to convert reducing sugars other than glucose formed during the hydrolysis. More conversion of sugar in hydrolyzates to alcohol (40.8 to 43.4% w/w) by the fusaet *S. cerevsiae* 5 L culture due to the ability of the fuasant *S. cerevisiae* 5 L yeast to convert sugars other than glucose to alcohol. Attempts have been carried out to convert lignocelluloscs to alcohol. Rao *et al*. (1995) could hydrolyze sugar cane bagasse by mycelia! biomass of *P. funiculosum* for alcohol production by *S. cerevisiae.* They obtained 40-45% conversion of sugar to alcohol.

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