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Optimization of SSF parameters by OFAT for biosynthesis of cellulase using isolated *Aspergillus niger*

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Cellulases represent a major group of the industrially significant enzyme. The present study deals with cost effective production of cellulase (CMCase and FPase) using isolated *Aspergillus niger* with the employment of a horticultural waste banana peel (BP). Optimization of environmental and nutritional parameters has been done by the classical one factor at a time method (OFAT) followed by chemical characterization of banana peel by EDX and visualization of the topology of banana peel after fermentation by SEM. By applying OFAT methodology for optimization of physical and nutritional parameters, the CMCase and FPase activity is found to increase about 5.21 and 1.97 fold for (111.05 U/gds and 9.45 U/gds) CMCase and FPase, respectively compared to the control (21.3 and 4.8 U/gds for CMCase and FPase, respectively). EDX analysis shows that the banana peel contain high amount of carbon and that is the reason why no additional carbon supply is essential for the growth of the isolated fungi. SEM micrograph illustrates asymmetrical and micro porous morphology of BP and uniform growth of the fungi on the surface of the peel after fermentation.

Keywords: CMCase, FPase, *Aspergillus niger*, EDX, SEM, SSF

The importance of microbial cellulases has increased in current scenario due to its enormous biotechnological applications mainly in food, animal feed, detergent, paper and pulp industries as well as for bioconversion of lignocellulosic waste into value-added products. Research on cellulase has progressed very hastily in the past few decades, the emphasis being on enzymatic hydrolysis of cellulose to glucose. The process of hydrolysis requires the employment of consortium of free cellulases which comprise of endoglucanases (EC 3.2.1.4, carboxymethyl cellulase), exoglucanases (EC 3.2.1.91, cellobiohydrolase) and cellobiases (EC 3.2.1.21, β-1, 4-D-glucosidase)[1]. Microbial enzymes have the enormous advantage of being able to be produced in large scale by established fermentation techniques. Enzyme production can be closely controlled in microorganisms and consequently, to improve the productivity of the enzyme, these controls can be exploited and customized. Cellulase yield appears to depend on an intricate relationship involving a variety of physical and nutritional factors which includes *p*H, temperature, incubation period, involvement of metal salts, carbon, and nitrogen supplements in the fermentation medium[2,3]. To establish a successful fermentation process, it is necessary to obtain a potent microorganism for overproduction of the desired metabolite. An elaborate investigation is therefore, required to establish the optimum conditions required for the large scale production of enzyme[1]. The aim of this work was to (i) to determine the optimum conditions for production of cellulase from the isolated strain following one factor at a time methodology (OFAT) [4]. (ii) Optimization of nutritional parameters for cellulase production by OFAT. (iii) Visualization of the morphology of banana peel before and after growth of *Aspergillus niger* by SEM. (iv) Chemical characterization of BP by EDX analysis which was selected as substrate for SSF utilizing our isolated *Aspergillus niger*.

Experimental Section

Chemicals

Whatman No 1 filter paper was procured from Sigma Aldrich. Carboxymethylcellulose (CMC) was procured from LOBA chemicals. 3, 5 Dinitrosalicylic acid, Folin-Ciocalteu, carbon sources, nitrogen sources, surfactant and metal salts were procured from Merck. Horticultural waste banana peel (BP) was collected from local sources for solid state fermentation. For biomass estimation, Ehrlich reagent and acetyl acetone reagent were prepared following the composition mentioned[5].

Microorganism

The isolated *Aspergillus niger* was maintained on PDA (Potato Dextrose Agar) and stored at 4°C. The genotypic identification of the strain was done by GCC Biotech, India Pvt. Ltd.

SSF

SSF was carried out in 100 mL Erlenmeyer flasks containing banana peel as substrate moistened with distilled water (w/v). The flasks were sterilized by autoclaving at 121°C for 15 min. The flasks were inoculated with spore suspension (10^9 spores/mL) made with sterilized distilled water. The contents of the flasks were mixed well with sterilized glass rod to distribute the inoculum throughout the substrate and incubated at desired temperature for desired period of time following OFAT methodology.

Extraction of enzyme

The enzyme was extracted from the solid moldy medium by mixing the medium with (1:10 w/v) distilled water. Dampened cheese cloth was used to filter the extract and pooled extracts were centrifuged at 8000 rpm for 5 min at 4°C[6]. The clear supernatant was used as a source of crude extracellular enzyme[7].

Enzyme assay

The filter paperase (FPase) and carboxymethylcellulase activity (CMCase) was determined by the method of Mandels, Weber[8] and Ghose[9], respectively. For the FPase assay, the reaction mixture contained 50 mg of Whatman No.1 filter paper strips as a substrate, 1 mL 0.05M sodium citrate buffer (*p*H-5) and 1mL enzyme. This concoction was incubated for 1 h at 50°C in a water bath. For CMCase assay, 1mL of 2% carboxymethylcellulose (CMC) was used as a substrate. The reaction mixture was incubated for 30 min at 50°C in a water bath. For both the cases, the reducing sugar liberated was estimated by spectrophotometer at 540 nm after addition of DNS. One Filter paper unit (FPU) is defined as the amount of enzyme in the filtrate releasing 1 µmol of reducing sugar from filter paper/mL/min[10]. One CMCase unit is the amount of enzyme releasing 1 µmol of reducing sugar from carboxymethyl cellulose/mL/min[10]. The enzyme activity was expressed as U/gds (Unit per gram dry substrate). The dry weight of the samples was determined by drying them in a hot air oven at 80°C to a constant weight.

Protein estimation in crude enzyme

An aliquot of this culture filtrates was used for extracellular protein estimation according to the method of Lowry[11]. Bovine serum albumin was used as the standard.

Biomass estimation

Fungal biomass estimation was carried out by determining the N-acetyl glucosaminereleased[5] by acid hydrolysis of the chitin, which is one of the constituents of the fungal cell wall. The results are expressed as mg glucosamine per gram substrate (g)

Optimization of environmental parameters of SSF

Cellulase production under SSF was optimized by altering the cultural conditions. The optimal level of one factor was determined by varying its level, while keeping other factors in the medium constant[12]. SSF was done with the hydration of substrates with distilled water in the ratio of 1:1, 1:2, 1:3, 1:4, 4:1, 3:1, 2:1 (w/v) and enzyme assay was done to optimize the hydration ratio on enzyme production. The effect of incubation time on the production of cellulase was determined by monitoring the fermentation for (0-7 days). 3, 5, 7, 9, 11 and 13g of the dry substrate was taken in 100 mL Erlenmeyer flask and fermentation was carried out at 30°C under stationary condition. In each case, enzyme activity was measured to get the optimum substrate amount for better production of the enzymes. The fermentation was done at 23, 30, 37, 43 and 50°C and after fermentation enzyme activity was measured to determine the optimum fermentation temperature. Fermentation was also carried out with the spore suspension of 1, 3, 5, 7 mL followed by enzyme assay to get the highest yield of enzymes. The production of cellulase was also monitored for inoculums age (0-7 days).

Optimization of nutritional parameters influencing cellulase production by SSF

For optimization of nutritional parameters, the medium was supplemented with glucose, fructose, lactose, CMC, maltose, sucrose, sorbitol, starch, sodium acetate and xylose as carbon source (1% w/w) and yeast extract, peptone, glycine, sodium nitrate, gelatin, ammonium sulfate, ammonium chloride, ammonium heptamolybdate, casein and urea as nitrogen source (0.5%w/w). Studies were also carried out with NaCl, $MgSO_4$, KCl, $PbSO_4$, $ZnSO_4$.$7H_20$, $MnSO_4$. H_2O, Fe (II) SO_4, $BaCl_2$, $FeCl_3$ and $Cacl_2$ as metal supplements (0.05%w/w) and triton X-100, tween 80, SDS and EDTA as surfactant sources (0.3% w/w/v/v). After fermentation, enzyme assay was done for each case to optimize the enzyme production.

Chemical characterization of banana peel (BP) by Energy-dispersive X-ray spectroscopy (EDX) and SEM analysis of BP before and after fermentation utilizing isolated *Aspergillus niger*

Energy-dispersive X-ray spectroscopy is an analytical technique used for the elemental analysis of a sample[13]. It relies on the investigation of an interaction of a sample and a source of X-ray excitation. Its characterization capabilities are due in large part tothe prime principle that each element has an exclusive atomic structure that allows the formation of a unique set of peaks on its X-ray spectrum[13]. EDX micrograph of banana peel was done to determine the percentage weight of chemical composition available on the peel. Besides that, scanning electron microscopic images of banana peel before and after the isolated microorganism's growth were taken on JEOL-JSM 6360.

Results and Discussion

Optimization of physical parameters for production of cellulases

The significance of moisture level in the SSF media and its influence on microbial growth and product biosynthesis can be attributed to the impact of moisture on the physical properties of solid waste used for fermentation. The cellulase production and supernatant protein concentration were seen maximum at the substrate to moisture ratio 3:1(w/v) at the level of 24.6 U/gds and 5 U/gds for CMCase and FPase respectively (Fig. 1). A low moisture content is known to reduce the solubility of nutrients leading to low substrate swelling and higher water tension[14]. The moisture ratio of 1:2 (w/v) was observed optimum for production of cellulase under SSF when *Aspergillus niger* was used as cellulase producer and coir waste was used as a substrate for fermentation[15]. Previously, it was reported that

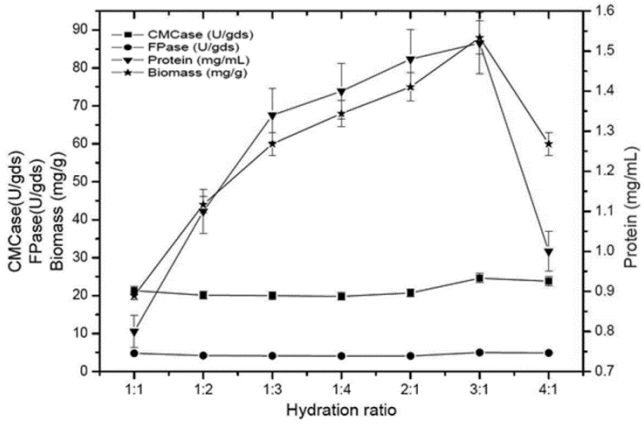

Fig. 1 — Effect of hydration ratio on production of cellulase from isolated *Aspergillus niger* (error bar shown with 5% error)

enzyme activity of alpha amylase was found to be maximum at 0% moisture level followed by 10% level when *Aspergillus niger* strain SK01 was used as the enzyme producer under SSF[16]. The CMCase and FPase production was monitored at regular intervals for up to 7 days of incubation using banana peel as solid support in a 100 mL Erlenmeyer flask, the enzyme production increased with an increase in incubation time and reached maximum on the 5th day of incubation period at the levels of 28.6 U/gds for CMCase and 5.4 U/gds for FPase (Table 1). After 5th day of incubation, CMCase and FPase production decreased. Denaturation of enzyme, substrate inhibition and substrates nutrient availability are reported to be the reasons for the observed decrease in cellulase activity[14]. Maximum cellulase production was reported on 5th day of fermentation using *Trichoderma*sp. on apple pomace[17]. The volume of inoculum is important for SSF process. The maximum enzyme activity 35.5 U/gds and 6.6 U/gds for CMCase and FPase respectively was observed when 5 mL of inoculum containing 10^9/mL spore suspension was used for solid state fermentation (Table 1). The optimum inoculum level is an important consideration for SSF processes since an overcrowding of spores can inhibit the growth and development of isolated microorganism[14]. An increase in inoculum level generally improves the growth-related enzyme activities of the organism up to a certain level, beyond which there can be nutrient limitation resulting in a reduction in microbial activity[14]. In an earlier study, maximum cellulase activity with an inoculum size of 8% was reported[18] .The importance of age of inoculum for microbial fermentation process is widely accepted. It has an optimal value that not only depends on the microbial species but also on the fermentation system. The production of cellulase increased with increase in age of inoculum and reached maximum on the 5th day at the levels of 44.4 and 6.7 U/gds for CMCase and FPase respectively as shown in Fig. 2 beyond 5th day the production declined, this reduction in activity may be due to the entry of the organism into the decline phase of growth curve. Maximum cellulase production at the levels of 59.1 U/gds and 7.35 U/gds for CMCase and FPase respectively was obtained when 5 g of the substrate (banana peel) was used for SSF (Table 1). Earlier it has been reported that substrate concentration is a dynamic influencing factor that affects the enzyme yield and the initial hydrolysis rate of cellulose[19]. A high substrate

Table 1 — Effect of time of incubation, volume of inoculum and amount of substrate on production of CMCase and FPase under SSF

Parameters	Enzyme activity CMCase	(U/gds) FPase	Protein (mg/mL)	Biomass (mg/g)
Time of fermentation (days)				
0	11.9±0.1	2.1±0.13	0.1 ±0.12	28±0.1
1	15.2±0.15	2.4±0.25	0.32±0.1	40±0.09
2	18.6±0.1	3.2±0.1	0.46±0.06	43±0.15
3	19.9±0.14	3.8±0.16	0.58±0.1	55±0.18
4	20.5±0.13	4.5±0.12	0.73±0.13	61±0.12
5	28.8±0.13	5.4±0.15	0.8±0.07	83±0.15
6	21.3±0.1	5.2±0.1	0.76±0.1	78±0.12
7	18±0.07	4.8±0.09	0.54±0.21	70±0.15
Volume of inoculum (mL)				
1	19.6±0.26	4.4 ±.02	0.8±0.1	44±0.1
3	31±0.1	4.55±0.1	0.75±0.02	54±0.1
5	35.5±0.1	6.6±0.1	1.09±0.01	86±0.1
7	33.3±0.01	3.44±0.02	0.95±0.02	59±0.01
Amount of substrate (g)				
3	44.4±0.2	4.86±0.12	0.52±0.2	50±0.1
5	59.1±0.9	7.35±0.1	0.94±0.1	93±0.1
7	31.7±0.1	2.4±0.1	1.42± 0.02	76±0.01
9	27.2±0.2	1.82±0.036	1.4±0.1	70±0.15
11	24.1±0.11	1.73±0.09	1.27±0.01	67±0.1
13	22.2±0.15	1.7±0.1	1.5±0.1	56±0.1

*Each value is a mean of triplicates ± standard deviation, "U/gds" Unit per gram dry substrate, "mg/g" Milligram per gram of substrate.

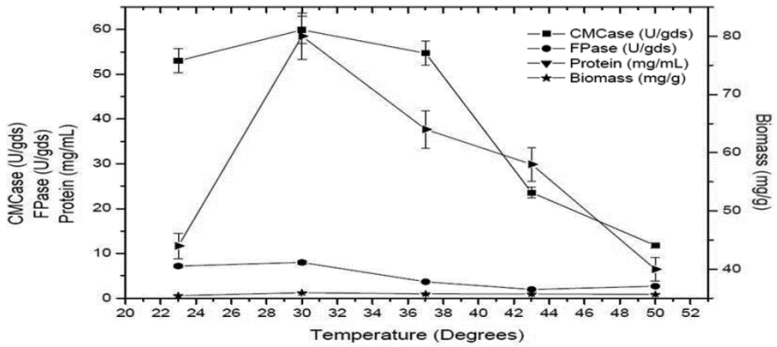

Fig. 2 — Effect of age of inoculum on production of cellulase from isolated *Aspergillus niger* (error bar shown with 5% error)

concentration can cause substrate inhibition, which can substantially cause reduction in enzyme formation[20]. The thickness of the substrate layer also influences the porosity and aeration of the substrate. Maximum production of cellulases was reported with 5 g of waste cabbage using *Penicillium notatum NCIM NO-923* as cellulase producer[21]. Temperature is one of the important factor, which strongly affect the SSF process[12] as cell growth, production of enzymes and metabolites are usually sensitive to temperature. Figure 3 shows the influence of temperature on cellulase production. The activities of cellulases were assessed at different temperatures varying from 23°C to 50°C. Maximum CMCase and FPase yield was obtained at 30°C at the levels of 59.9 U/gds and 8 U/gds

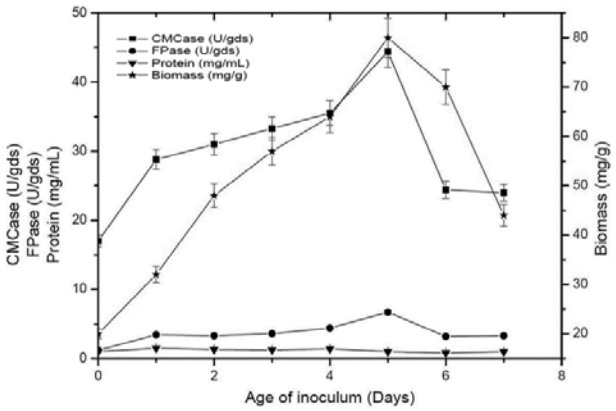

Fig. 3 — Effect of temperature of fermentation on production of cellulase obtained from isolated *Aspergillus niger* (error bar shown with 5% error)

respectively, earlier maximum cellulase production was reported at 30°C when *Trichoderma reesei* was used as the cellulase producer[12].

Optimization of nutritional parameters for production of cellulases

Extracellular enzyme production depends significantly on the composition of the medium. Various carbon sources were tested for their effect on cellulase production. The influence of the carbon sources on cellulase production by *Aspergillus niger* is depicted in (Table 2), none of them could impart a positive effect on cellualse production as the banana peel itself is reported to be rich in carbon[12,13] as a result of which additional supplement of carbon was not needed[13].

Table 2 — Effect of different nutritional supplements on production of CMCase and FPase under SSF

Nutrients (w/w/**v/v**)	Cellulase activity CMCase	(U/gds) FPase	Protein (mg/mL)	Biomass (mg/g)
Carbon source (1%)				
Control (BP)	60±0.01	8.01±0.03	0.8±0.1	96±0.1
Sorbitol	50.3±0.01	4.5±0.01	0.71±0.02	98.1±0.1
Glucose	59.6±0.1	6.8±0.03	0.82±0.1	100.1±0.1
Carboxymethylcellulose	59.8±0.03	6.16±0.1	0.85±0.1	100.7±0.01
Sodium acetate	47.2±0.1	6.3±0.01	0.889±0.1	100.3±0.1
Sucrose	58±0.1	7.7±0.01	0.68±0.01	99.8±0.01
Fructose	55.5±0.01	5.96±0.02	0.72±0.1	99.2±0.01
Starch	45.2±0.03	4.05±0.1	0.83±0.15	100.2±0.2
Xylose	49.3±0.1	4.01±0.1	0.78±0.01	97.6±0.01
Nitogen source (0.5%)				
Control (BP)	60±0.01	8.01±0.03	1.5±0.1	96.3±0.1
Yeast Extract	71.9±0.1	8.1 ±0.01	1.67±0.1	99.32±0.15
Sodium nitrate	74±0.1	8.32±0.1	1.83±0.02	100.6±0.12
Ammonium sulphate	65.7±0.01	8.06±0.02	1.68±0.1	100.2±0.1
Glycine	49.3±0.03	6.2±0.1	1.54±0.01	99.1±0.1
Ammonium chloride	61.6±0.02	5.3±0.02	1.65±0.15	95.4 ±0.1
Ammonium heptamolybdate	56±0.01	3.49±0.01	1.38±0.1	99.3±0.1
Urea	63.7±0.15	8.19±0.01	1.27±0.14	100.3±0.15
Caesin	59.6±0.1	7.09±0.01	1.35±0.3	98.3±0.1
Gelatin	57.5±0.1	4.6±0.1	1.45±0.1	99.5±0.15
Peptone	69.8±0.01	8.12±0.01	1.61±0.02	100.5±0.1
Metal salt (0.05%)				
Control (BP+0.5%$NaNO_3$)	74.1±0.1	8.32±0.01	1.95±0.02	100.6±0.1
$CuSO_4$	48.1±0.1	5.3±0.1	1.65±0.1	98±0.1
$MgSO_4$	85.7±0.1	9.04±0.01	1.98 ±0.1	100.8±0.1
$BaCl_2$	64.9±0.15	5.7±0.15	1.87±0.1	97±0.02
$MnSO_4$	76.7±0.01	8.4±0.1	1.77 ±0.1	97.5±0.1
$FeSO_4$	48.5±0.03	5.1±0.01	1.73±0.01	99.1±0.1
$Fecl_3$	53.8±0.15	5.8±0.01	1.83±0.1	98.3±0.15
$ZnSO_4$	75±0.02	8.38±0.1	1.88±0.15	99.5±0.16
NaCl	77.2±0.01	8.5±0.1	1.72±0.1	100±0.15
KCl	78.1±0.1	8.75±0.1	1.61±0.01	98±0.1
$PbSO_4$	63.1±0.02	6.2±0.1	1.32 ±0.1	98.4 ±0.01
$CaCl_2$	63.7±0.1	6.58±0.15	1.31±0.1	97.5±0.1
Surfactant (0.3%)				
Control (BP+0.5%$NaNO_3$+0.05%$MgSO_4$)	85.8±0.02	9.05 ±0.1	1.65±0.1	98 ±0.15
Tween-80	100.7±0.1	9.11±0.1	2.02 ±0.03	101.5±0.02
Triton X-100	111±0.01	9.45±0.1	2.15±0.01	104.6±0.1
EDTA	98.6±0.2	9.25±0.15	2.13±0.02	98.7±0.15
SDS	94.5±0.1	9.14±0.1	1.8±0.1	99.5±0.2

*Each value is a mean of triplicates ± standard deviation, "U/gds" Unit per gram dry substrate, "mg/g" Milligram per gram of substrate.

Different nitrogen sources have shown variable effects on cellulase production (Table 2). Inorganic nitrogen source sodium nitrate was found to produce maximum CMCase and FPase at the levels of 74 and 8.32U/gds for CMCase and FPase respectively. Our results are in agreement with the information obtained earlier[22]. Some studies have shown that inorganic nitrogen source resulted in enhanced enzyme production compared to organic nitrogen source as reported by Kalogeris *et al.*[23]. Presence of metal ions influenced enzyme production by the microorganism in solid media. Various metal ions were used as additives in the basal medium to establish their stimulatory or inhibitory effect on enzyme production (Table 2). An increase in CMCase and FPase production was observed when the production medium was supplemented with 0.05 % $MgSO_4$ (85.76U/gds and 9.06 U/gds) (Table 3) which is in agreement with the report obtained by Fawzi *et al.*[24]. The supplementation of surfactants in the production medium of hydrolytic enzymes is well recognized[25]. Triton X-100 maximally enhanced CMCase (111 U/gds) and FPase (9.45 U/gds) production at the concentration of 0.3% (w/w) (Table 3). Such compounds are reported to augment the permeability of the microorganism's cell membrane, permitting rapid secretion of enzymes[26]. These results are in accordance with studies of Deswal *et al.*[27].

EDX and SEM analysis

Figure 4 shows the EDX analysis of banana peel. The percentage weight of chemical compositions

Table 3 — Optimization of amount of $NaNO_3$, $MgSO_4$, Triton X-100 on production of CMCase and FPase under SSF

% of Nutrient (w/w)	Cellulase activity CMCase	(U/gds) FPase	Protein (mg/mL)	Biomass(mg/g)
$NaNO_3$				
0.1	45.2±0.02	6.3± 0.1	1.45±0.1	96.8 ±0.15
0.3	67.8±0.1	7.2±0.15	1.53±0.02	97.5±0.1
0.5	74±0.1	8.35±0.1	1.62±0.1	98.5±0.1
0.75	66.8±0.01	5.75±0.02	1.60±0.02	98.2±0.1
1	64.1±0.1	4±0.1	1.59±0.1	97.6±0.1
$MgSO_4$				
0.01	75.3±0.01	8.42±0.02	1.63±0.15	98.7±0.15
0.03	78±0.1	8.8±0.01	1.76±0.1	99±0.15
0.05	85.76±0.1	9.06±0.01	1.98±0.1	100.7±0.1
0.075	83±0.01	9.01±0.1	1.85±0.1	99.8±0.1
0.1	79.5±0.15	8.76±0.1	1.79±0.1	97±0.1
Triton X-100 (v/v)				
0.1	109±0.1	9.13±0.1	2.14±0.15	101±0.01
0.3	111.05±0.01	9.4±0.1	2.16±0.01	104.7±0.1
0.5	108.5±0.1	9.36±0.1	2.06±0.01	102±0.01
0.75	105±0.02	9.28±0.15	2.02±0.1	98.8±0.1
1	101.2±0.1	9.08±0.01	2.04±0.1	97.2±0.1

*Each value is a mean of triplicates ± standard deviation, "U/gds" Unit per gram dry substrate, "mg/g" Milligram per g of substrate.

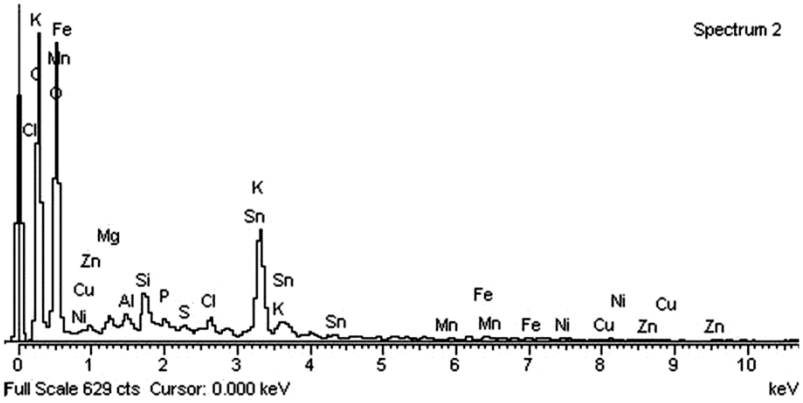

Fig. 4 — Energy-dispersive X-ray spectroscopic analysis of Banana peel

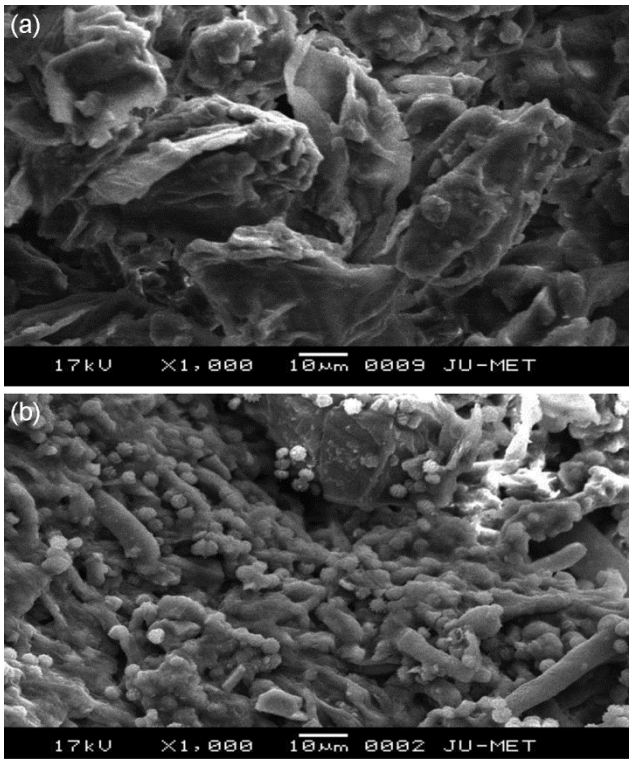

Fig. 5— (a) Scanning Electron Micrograph of BP without the growth of isolated *Aspergillus niger* (b) BP with the growth of *Aspergillus niger* on it.

available on the surface of banana peel were as follows (%): C : 41.86 , O: 45.89, Mg: 0.44, Al : 0.46, Si: 1.29, P: 0.26, S: 0.18, Cl: 0.69, K: 6.59, Mn: 0.24, Fe: 0.32, Ni: 0.09, Cu: 0.30, Zn: 0.15. Our results were similar to the previous findings[28] . On the other hand Fig. 5a and 5b shows the SEM micrograph of banana peel before and after fermentation which represented the porous and irregular surface of the peel and presence of spores and hyphae of *Aspergillus niger*.

Conclusion

The present investigation is aimed at optimization of environmental and nutritional parameters for solid state fermentation utilizing banana peel (BP) as substrate. It is evident that BP can be used as an economically feasible substrate for fermentation as it could increase the yield of industrially important enzyme cellulase up to 5.2 fold for CMCase and 2 fold for FPase. Furthermore, no additional carbon source is required as the peel itself is found to be rich in the supplement. Therefore it can be used resourcefully as a solid substrate for supporting the growth of isolated *Aspergillus niger* for fermentation. If the production is studied using the statistical approach such as Response surface methodology, which uses combinatorial relations of culture conditions, it may result in enhanced production.

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