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Optimization of cellulase production by *Penicillium oxalicum* using banana agrowaste as a substrate

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The purpose of this study was to produce a higher amount of cellulase by using an alternative carbon source, such as banana agrowaste, and to optimize the fermentation parameters for a high yield. In the present study, cellulase-producing *Penicillium* was isolated from a decaying wood sample. Different nutritional and environmental factors were investigated to assess their effect on cellulase production. The highest crude enzyme production was observed at a pH 6.0 and a temperature of 28°C in a medium that was supplemented with banana agrowaste as the carbon source. Pretreatment with 2N NaOH, at 7% substrate (banana agrowaste) concentration yielded the highest cellulase activity. Further to this, the effect of other parameters such as inoculum age, inoculum size, static and agitated conditions were also studied. It is concluded that *Penicillium oxalicum* is a powerful cellulase-producer strain under our tested experimental conditions using banana agrowaste as the carbon source.

Key Words: alkali pretreatment; banana agrowaste; cellulase enzyme production; *Penicillium oxalicum*

Introduction

Lignocellulosic wastes generated through forestry, agricultural practices and industrial processes are among the causes of environmental pollution. Their conversion into useful products may reduce the problems they cause. Lignocellulose is the most abundant and renewable biopolymer on Earth. It has been used by mankind for centuries; however, its enormous potential as a renewable source of energy was recognized only after lignocellulose-degrading enzymes had been identified (Bhat and Bhat, 1997).

The polysaccharide components of lignocellulosic wastes include cellulose, hemicelluloses and lignin. These macromolecules can be converted into glucose either by chemical, and physical treatments or enzymatic hydrolysis. Currently, there are two major ways of converting cellulose to glucose: chemical versus enzymatic. The enzymatic hydrolysis of cellulose is an important reaction in nature for it marks the first step in the decay of cellulose—the most abundantly occurring organic material. In the early 1970s, the oil crisis generated increased interest in using cellulose as a chemical and energy resource (Coral et al., 2002). Cellulose is a homopolymer consisting of glucose units joined together by β-1, 4 glycosidic bonds. A great variety of filamentous fungi can degrade these macromolecules by using hydrolytic or oxidative enzymes. Cellulases are synthesized by *Trichoderma*, *Fusarium*, *Aspergillus*, *Chaetomium*, *Myrothesium*, and *Penicillium*. Cellulases are the most diverse class of enzymes that catalyze the hydrolysis of a single substrate by three classes of enzymes, endoglucanases (EC 3.21.4), cellobiohydrolases (EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21). Member of all classes are necessary to degrade cellulose (Bhat, 2000). Cellulases are extremely important enzymes, both industrially and in the natural world, because they play a major role in the global carbon cycle by degrading insoluble cellulose to soluble sugars. In the early 1980s, interest in cellulases and hemicellulases increased due to the many potential applications for these types of enzymes; for example, first in animal feed and then in food applications

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(Chesson, 1987). Subsequently, these enzymes were used in the formulation of washing powders, in the textile industry (Cavaco-Paulo, 1998), in the pulp and paper industry (Beg et al., 2001; Ximenes and Filho, 1998), in biofuel (Zaldvas et al., 2001) and to perform enzymatic hydrolysis of the lignocellulosic materials. Enzyme production is closely controlled in microorganisms and for improving its productivity these controls can be ameliorated. Cellulase yields appear to depend on a complex relationship involving a variety of factors like inoculum size, inoculum age, pH, temperature, aeration and others (Immanuel et al., 2006).

Banana is a major cash crop of the Anand district of Gujarat state, India. Banana (*Musa paradisiaca*) is cultivated in an area of 4.796×10^5 ha., yielding 16.37×10^6 t of banana (Shah et al., 2005). Each hectare of banana crop generates nearly 220 t of residual plant waste that mainly consists of lignocellulose material. The main residual waste of the banana crop are leaves and pseudostem. These waste materials are efficient substrates for some fungi, which produce lignolytic and cellulolytic enzymes. Though much work has been done on the production of cellulase from lignocellulosics (Depaula et al., 1999; Kansoh et al., 1999; Solomon et al., 1999), most emphasis has been placed on banana agrowaste.

Filamentous fungi, particularly the *Aspergillus* and *Trichoderma* species, are well known as efficient producers of these cellulases (Peij et al., 1998). Even though there are many reports on cellulase-producing fungi (Shin et al., 2000), only a few have high enough activities for commercial success (Elad, 2000; Kang et al., 1994). In our exploration for cellulolytic fungi, *Penicillium oxalicum* was found to be a high producer of cellulolytic enzyme under suitable nutritional and environmental conditions. The objectives of the present work were to evaluate the capacity of *Penicillium oxalicum* to produce cellulase during growth in liquid media containing banana agrowaste as the carbon source, and also to study the effect of various factors for an improvement of the enzymatic hydrolysis of banana agrowaste.

Materials and Methods

Microorganisms. The fungi used for this study were isolated from two sources: a decaying wood sample collected from the Anand region, and a soil sample from banana farms of the village of Lambhvel, Anand. The fungi were isolated on a modified Czapek Dox Agar medium containing cellulose as a sole carbon source. The master culture was maintained on PDA slant at 4°C and sub-cultured on fresh sterile modified Czapek Dox agar slant and incubated for 5–6 days. The microorganisms were identified in the Division of Mycology and Plant Pathology, I.A.R.I., New Delhi, India.

Banana agrowaste as substrates. The waste substrates used in this study were banana leaves which were collected from a farm near Anand, Gujarat. Banana leaves were thoroughly washed with tap water, drained to remove excess water and dried in an oven at 55°C. Leaves were ground to make powder and sieved by different test sieves (120 μm, 180 μm, and 250 μm) to produce the respective fine powder of uniform particle size used in the study.

Measurement of cellulose, hemicellulose and lignine contents from banana leaves. The cellulose, hemicellulose and lignine contents from banana leaves were determined by the method described by Van Soest (Van Soest et al., 1991).

Medium preparation for Enzyme production. The basal fermentation medium (Mandels and Weber, 1969) used for the production of cellulases contain the following ingredients: $(NH_4)_2SO_4$ 1.4 gm/ltr, KH_2PO_4 2.0 gm/ltr, $CaCl_2$ 0.3 gm/ltr, $MgSO_4 \cdot 7H_2O$ 0.3 mg/ltr, $FeSO_4 \cdot 7H_2O$ 5.0 mg/ltr, $MnSO_4 \cdot 7H_2O$ 1.6 mg/ltr, $ZnSO_4 \cdot 7H_2O$ 1.4 mg/ltr, $CoCl_2$ 2.0 mg/ltr, Proteose Peptone 0.1%, Tween 800.1%, and the pH was adjusted to 5.0 ± 0.2. Trace metal stock solution 1 ml/ltr. 100 ml of liquid medium was placed in a 250 ml Erlenmeyer flask and sterilized by autoclaving at 121°C for 20 minutes. The medium was cooled and inoculated with a culture suspension containing 1×10^6–1×10^8 spores. Samples were withdrawn from the culture at 1-day intervals over a period of 7–9 days.

Enzyme assay. The cellulase activity was determined colorimetrically by measuring the increase in reducing groups by the hydrolysis of a carboxymethyl cellulose (CMC), cotton, salicin and Whatman no. 1 filter paper as the substrate (Jeffries, 1996). Endoglucanase (CMCase) activity was determined by incubating 0.5 ml of supernatant with 0.5 ml of 1% CMC in 0.05 M citrate buffer (pH-4.8) at 50°C for 30 minutes. Exoglucanase activity was determined by incubating 1.0 ml of supernatant with 50 mg of absorbent cotton in 1.0 ml of 0.1 M citrate buffer (pH-4.8) at 50°C for 24 hours. β-glucosidase activity was determined by incubating 1.0 ml of supernatant with 10 mg of salicin in 1.0 ml of 0.05 M citrate buffer (pH-4.8) at 50°C for 30 minutes. Filter paper activity was determined by incubating 1.0 ml of supernatant to 1.0 ml of 0.05 M citrate buffer (pH-4.8) containing 1 cm × 6 cm strip of Whatman no. 1 filter paper at 50°C for 1 hour.

Optimization of Pretreatment of banana agrowaste with NaOH. Alkaline pretreatment was given to the banana agrowaste with a different normality of NaOH, viz. 1, 2, 3, 4 and 5 N. In these solutions 10 g/100 ml of banana agrowaste powder was mixed and incubated at room temperature for 12 hours. This mixture was washed with an acetate buffer to neutralize it. This alkali-treated agrowaste was autoclaved and used in the fermentation medium as the carbon source along with the basal fermentation medium described above, as suggested by Mandels and Weber (1969).

Optimization of substrate concentration. The pre-treated banana agrowaste used were 1%, 3%, 5%, 7%, 9% and 11% in wet weight condition, in 100 ml fermentation media, in 250 ml capacity flasks. The flasks were inoculated with 1×10^7 spores of *Penicillium oxalicum* and incubated at 28° ± 2°C temperature at 120 rpm in an orbital shaker incubator.

Optimization of particle size of banana agrowaste. For the optimization of mesh size, powder was sieved with different test sieves of B.S.S. Mesh no. 60 (particle size 250 μm), and no. 85 (180 μm) and random size particle and were incubated at 28° ± 2°C temperature at 120 rpm in an orbital shaker incubator.

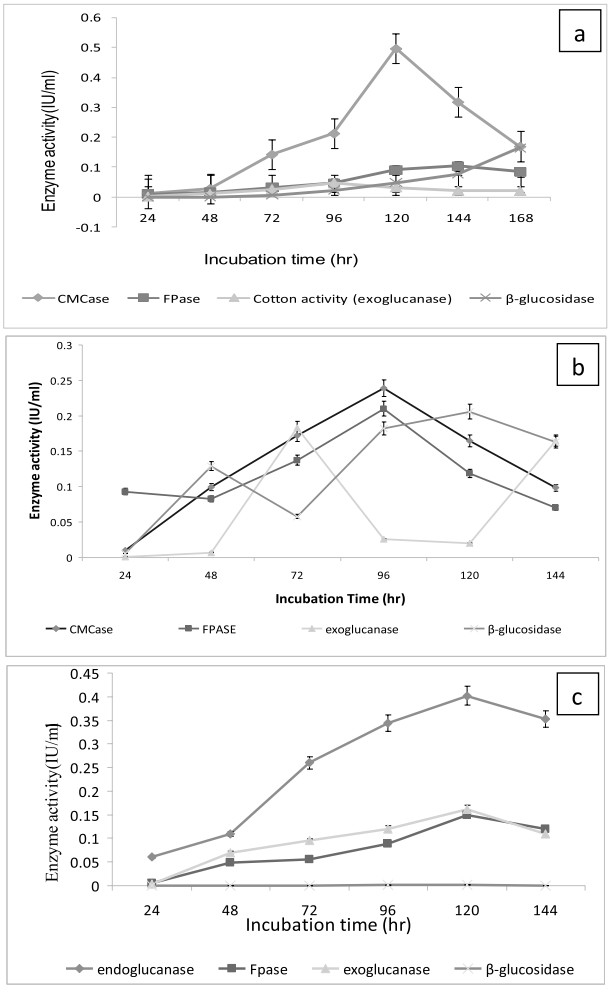

Fig. 1. Cellulase activity of a) *Penicillium oxalicum* b) *Aspergillus niger* and c) *Trichoderma viride* in cellulose media.

Optimization of fermentation time. Inoculated flasks were incubated at 28° ± 2°C temperature for different time intervals of 24, 48, 72, 96, 120, 144 hours and the activity was checked spectrophotometrically.

Optimization of fermentation temperature. The optimization of temperature was carried out by incubating the banana agrowaste powder containing the fermentation medium at 20°, 28°, 37°, 40° and 50°C in an orbital shaker incubator at 120 rpm. At regular intervals, enzyme assays were performed.

Optimization of pH. The pH of the fermentation media was adjusted to various values ranging from 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0 with 0.1N NaOH or 0.1N HCl. The pH was determined using the pH meter. After inoculation, the flasks were kept at 28° ± 2°C temperature in an orbital shaker incubator at 120 rpm.

Optimization of inoculum size. The inoculum size was optimized by preparing the inoculum from a modified Czapek Dox agar medium. The banana agrowaste-containing media were inoculated with 1×10^6 spores/ml, 1×10^8 spores/ml and 1×10^{10} spores/ml. The inoculated flasks were incubated at 28° ± 2°C temperature at 120 rpm in an orbital shaker incubator.

Optimization of inoculum age. Each cellulosic waste was fed with varying ages of inoculum of the organisms. 7-day-, 14-day- and 21-day-old culture spores were inoculated. At regular intervals, enzyme assays were performed.

Effect of static and agitated conditions. Two sets were prepared to check the effect of a static and agitated condition on the enzyme activity. In both the sets, all the conditions (pH, inoculum age, substrate concentration) applied were kept similar. One set was put in an orbital shaker at 120 rpm, while the other set was kept in a static condition. At regular intervals, enzyme assays were carried out.

Statistical analysis. All the experiments were carried out in triplicate. Data regarding the enzyme activities of *Penicillium oxalicum* as (1) affected by different substrates ("C" sources) and time in days, and (2) affected by environmental conditions and time in days, were statistically analyzed using Two Way Analysis of Variance (ANOVA). One way ANOVA was used to estimate the statistical parameters.

Results

Cellulolytic fungi were isolated on a modified Czapek's Dox agar medium. Three organisms showing a clear zone upon the addition of iodine solution were marked as cellulase positive. The isolates were identified on the basis of their cultural and morphological characters; primarily these three fungi were identified as *Penicillium* sp., *Aspergillus* sp., *Trichoderma* sp. The isolates were sent to I.A.R.I., the Division of Mycology and Plant Pathology, New Delhi, India, for identification. I.A.R.I. identified *Aspergillus* sp. as *Aspergillus niger* and *Trichoderma* as *Trichoderma viride*. For *Penicillium* sp. further investigation was suggested. For the identification of *Penicillium* at the species level, the 28s rRNA sequence analysis was carried out. The 28s rRNA sequence of the *Penicillium* isolate was compared with query sequences in the NCBI GenBank by using NCBI BLAST search. From the blast analysis, *Penicillium* showed the highest homology (99% identity similarity) with *Penicillium oxalicum*. The sequence was submitted by us to the Gene Bank under the accession number KC759111.

Measurement of cellulase activity

Cellulase activity of these three cellulase positive organisms were checked further in a fermentation medium proposed by Mandels and Weber (1969). Out of these three isolates, *Penicillium oxalicum* showed better cellulase activity compared with the other two. The maximum endoglucanase, exoglucanase, β-glucosidase and FPase were 0.4965, 0.0473, 0.1643 and 0.1032 IU/ml, respectively, with *Penicillium oxalicum* (Fig. 1). β-glucosidase activity was found to be absent in *Trichoderma* sp. although it showed a higher endoglucanase, exoglucanase, FPase activity. So, for further studies, *Penicillium oxalicum* was selected which showed complete cellulolytic activity.

Measurement of cellulose, hemicellulose and lignine contents from banana leaves

Cellulose, hemicellulose and lignine contents determined from banana leaves agrowaste powder by the method de-

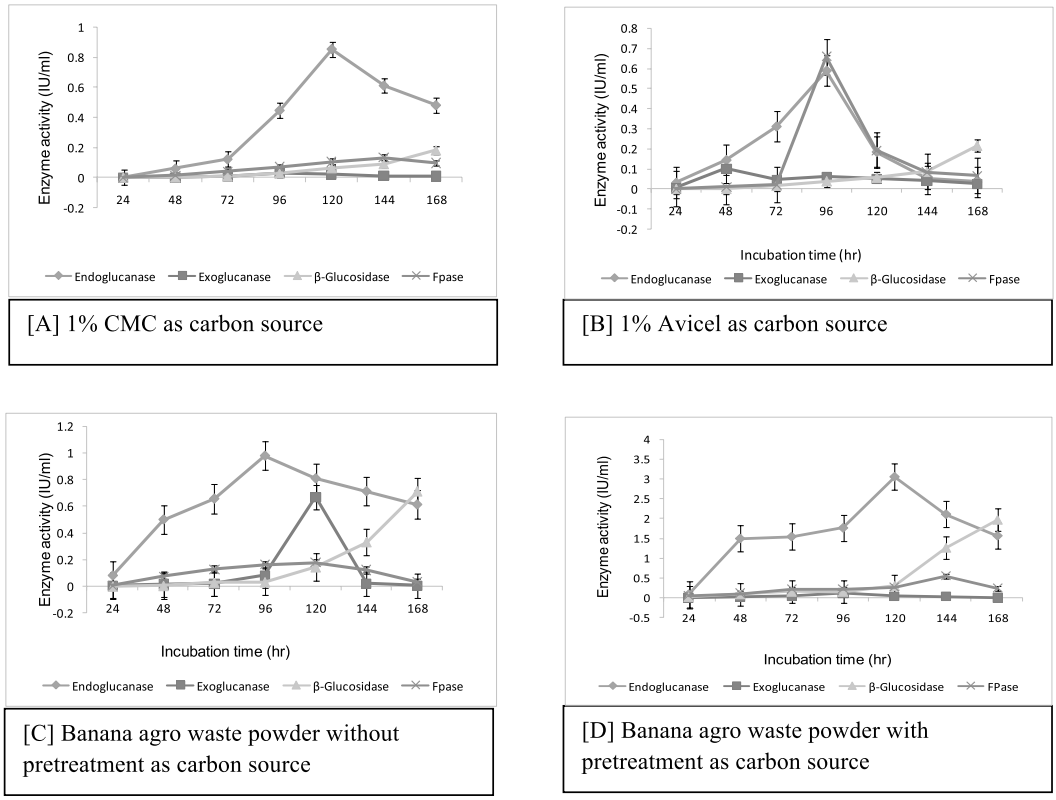

Fig. 2. Effect of different carbon sources on *Penicillium oxalicum* cellulase activity.

Table 1. Analysis of variance (ANOVA) for selected carbon sources.

Source of variation	SS	Degrees of freedom (d.f.)	Mean squares (MS)	F value
Substrates	SSR = 0.16301	Df = (5 − 1)	SSR/4 = 0.04075 (sr)	*Fc_1 = 9.98 (sr/se)
Time in days	SSC = 0.12381	Df = (7 − 1)	SSC/6 = 0.02064 (sc)	*Fc_2 = 5.05 (sc/se)
Error	SSE = 0.09804	(4) × (6)	SSE/24 = 0.004085 (se)	
Total	SST = 0.38486	(5 × 7) − 1		

Numbers represent F-values * = $p \leq 0.01$.

scribed by Van Soest et al. (Van Soest et al., 1991) were 47.09 ± 0.11, 26.16 ± 0.59 and 10.83 ± 0.7 gm/100 gm dry banana leaves agrowaste powder.

Comparison of different carbon sources

The different types of carbon sources used were [A] 1% Cellulose, [B] 1% CMC, [C] 1% Avicel, [D] Banana agrowaste powder without pre-treatment, and [E] Banana agrowaste powder with pretreatment, to study their effect on the production of cellulases by *Penicillium oxalicum* (Fig. 2). Of these different carbon sources used, maximum cellulase activity was observed with banana agrowaste pretreated with NaOH.

The two way ANOVA table (Table 1) shows the construction of the F-statistics:

We have calculated that $Fc_1 = 9.98$. From the table of the F distribution, we found at 5% level of significance and d.f. (4, 6) the tabulated value $Ft_1 = 4.53$ and at a 1% level of significance $Ft_1 = 9.15$. As $Fc_1 > Ft_1$, we concluded that 5 different substrates have a highly significant effect on enzyme activity.

From the F distribution table, we found at the 5% level and the 1% level and d.f. (6, 24) the tabulated values of $Ft_2 = 2.5$ and 3.67, respectively. As $Fc_2 > Ft_2$, we concluded that the incubation time in days had a highly significant effect on the cellulase activities.

Optimization of pretreatment

Banana agrowaste was pretreated with different normalities of NaOH ranging from 1, 2, 3, 4, and 5N NaOH solutions. The highest cellulase activity was observed when banana agrowaste was pretreated with 2N NaOH (Fig. 3). (The one way ANOVA table (Table 2) shows test of significance of pretreatment for cellulase activity.) From the F distribution table (Table 2), at the 5% level of significance and d.f. (4, 10) the tabulated value was found to be 3.48, as Fc > Ft, we concluded that 6 different values of pH of NaOH have a significant effect on the enzyme activity.

Optimization of substrate concentration

Banana agrowaste pretreated with 2N NaOH was used

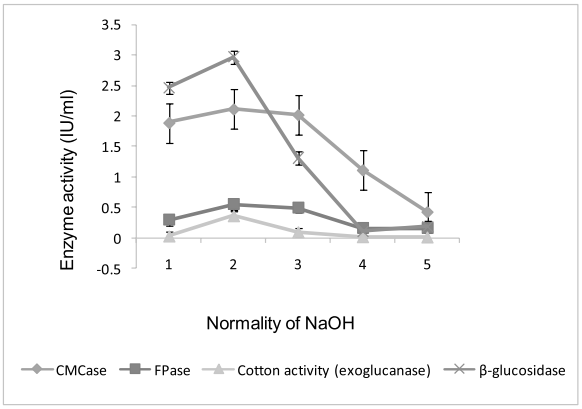

Fig. 3. Effect of NaOH pretreatment on cellulase activity using banana agrowaste as substrate.

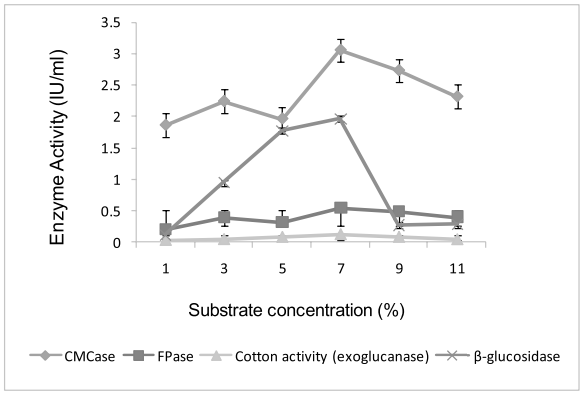

Fig. 4. Effect of substrate concentration on cellulase activity using banana agrowaste as substrate.

Table 2. Analysis of variance and test of significance of pretreatment for cellulase activity.

Source of variation	SS	Df	MS	F value
Within groups	SSW = 0.000102	Dfw = 10	MSW = 0.0000102	9801.47*
Between the sample	SSB = 0.3999	Df(t) = 4	MSB = 0.099975	
Total	SST = 0.400002	N – 1 = 14		

Numbers represent F-values * = $p \leq 0.05$.

Table 3. Effect of particle size of treated banana agrowaste on cellulase activity.

Particle size	Hours	CMCase	FPase	Cotton activity (exoglucanase)	β-glucosidase
180	24	0.0888	0.0555	0.001	0.0086
	48	1.496	0.1074	0.0189	0.0841
	72	1.5435	0.2197	0.0366	0.015
	96	1.7648	0.2221	0.0424	0.1481
	120	2.111	0.2731	0.0484	0.2962
	144	**3.055**	**0.5462**	**0.125**	**1.9629**
	168	1.5665	0.2407	0.003	1.2629
250	24	0.0735	0.0212	0.0005	0.0032
	48	0.1703	0.0166	0.0009	0.0037
	72	1.27	0.148	0.0127	0.0935
	96	1.4814	0.1787	0.0034	0.0972
	120	1.537	0.1064	**0.0152**	0.3425
	144	**2.009**	**0.2314**	0.0114	**0.3842**
	168	1.3265	0.2167	0.0093	0.2912
Random	24	0.0259	0.0111	0.001	0.0032
	48	0.1777	0.0185	0.004	0.0111
	72	0.2148	0.0111	0.0039	0.0222
	96	0.2925	0.0277	0.0054	0.0111
	120	**0.3259**	**0.0283**	**0.0063**	0.0185
	144	0.2263	0.0194	0.0044	**0.0216**
	168	0.1925	0.0143	0.0033	0.0162

for further study. Various concentrations of banana agrowaste ranging from 1, 3, 5, 7, 9 and 11% in a wet weight condition were used and at such substrate concentrations endoglucanase, exoglucanase, β-glucosidase and FPase activities were checked. The 7% substrate concentration showed the greatest cellulase activity. The greatest endoglucanase, exoglucanase, β-glucosidase and FPase activities were 3.055, 0.1255 1.9629 and 0.5462 IU/ml, respectively (Fig. 4).

The obtained enzyme activities indicated that different substrate concentrations of pretreated banana agrowaste have a significant effect on the enzyme activity, as F = 9525.5; $p < 0.05$.

Optimization of particle size

Three different particle size of banana agrowaste pow-

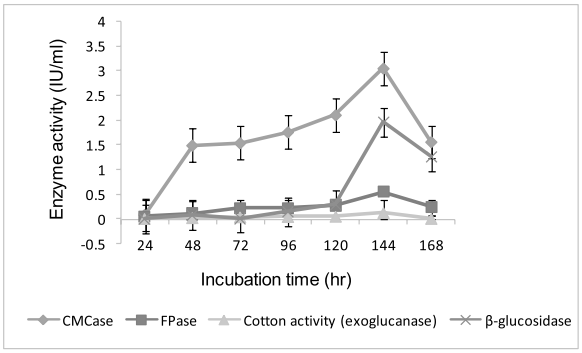

Fig. 5. Effect of fermentation time on cellulase activity using banana agrowaste as substrate.

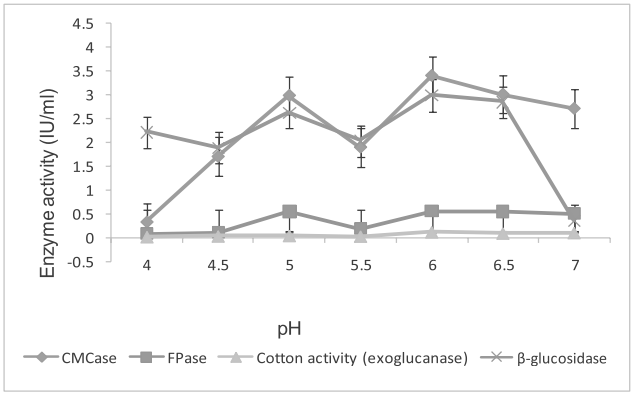

Fig. 7. Effect of pH on cellulase activity using banana agrowaste as substrate.

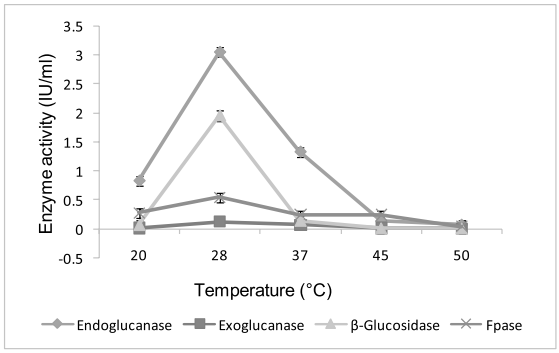

Fig. 6. Effect of temperature on cellulase activity using banana agrowaste as substrate.

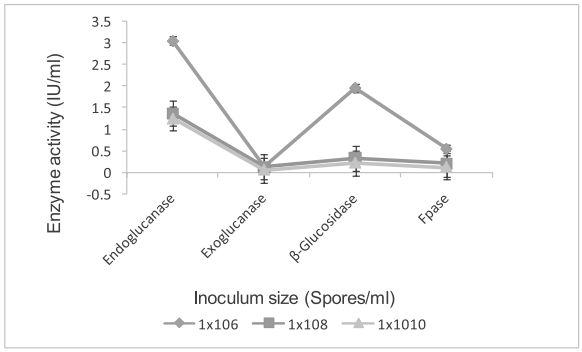

Fig. 8. Effect of inoculum size on cellulase activity using banana agrowaste as substrate.

der compared were 180 μm, 250 μm and random. A particle size of 180 μm showed the greatest cellulase activity compared with the others (Table 3).

Optimization of fermentation time

Fermentation was carried out up to 168 hours. Endoglucanase activity was found to be the greatest (3.0550 IU/ml) at 120 hours. Exoglucanase, β-glucosidase and FPase activities were found to be greatest at 144 hours (0.125 IU/ml, 1.9629 IU/ml, 0.5462 IU/ml, respectively) (Fig. 5).

The statistical analysis by one way ANOVA on the cellulase enzyme activity revealed that different fermentation time was statistically significant ($F = 4329$; $p < 0.05$).

Optimization of temperature

Optimization was carried out by incubating the fermentation flask at different temperatures ranging from 20°C, 28°C, 37°C, 45°C and 50°C, and the cellulase activities were measured. The greatest cellulase activity was observed at 28°C temperature. The endoglucanase, exoglucanase, β-glucosidase and FPase were 3.055, 0.1255, 1.9629 and 0.5462, respectively (Fig. 6).

The stastical analysis revealed that the enzyme activity due to various temperatures was statistically significant ($F = 18372$; $p < 0.05$).

Optimization of pH

The pH is an important factor that influences the cellulase production. The result illustrated by Fig. 7 shows that cellulase production is increased as the pH values increased from 4.0–6.0, with an optimal pH at 6.0. The enzyme production reduced to almost 10% at pH 4 (Fig. 7).

ANOVA showed that the pH variation had a significant influence on enzyme activity ($F = 56805$; $p < 0.05$).

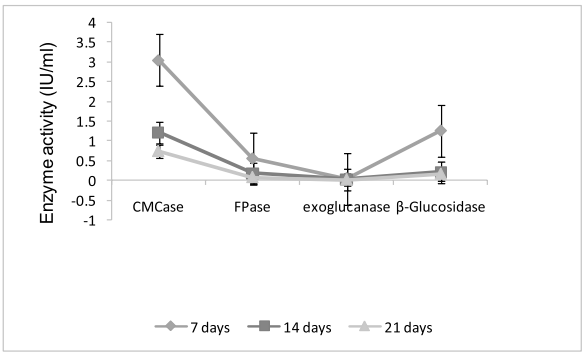

Fig. 9. Effect of inoculum age on cellulase activity using banana agrowaste as substrate.

Optimization of inoculum size

Kocher et al. (2008) reported an initial inoculum of 10^8 spores/ml significantly increased cellulase activities where endoglucanase and exoglucanase activity were 0.103 IU/ml and 0.121 IU/ml respectively (Fig. 8). Also, Zaldivar et al. (2001) reported that optimum cellulase production

Table 4. Effect of environmental conditions on cellulase activity using banana agrowaste.

Environmental condition	Hours	CMCase	FPase	Cotton activity (exoglucanase)	β-glucosidase
Static	24	0.0005	0.0018	0.0003	0.0003
	48	0.0012	0.0037	0.0005	0.0005
	72	0.0074	0.0055	0.0002	0.0010
	96	0.0080	0.0068	**0.0008**	0.0013
	120	**0.0400**	**0.222**	0.0007	0.0190
	144	0.0074	0.0083	0.0004	**0.0256**
	168	0.0062	0.0057	0.0003	0.0250
Agitated	24	0.0888	0.0555	0.0010	0.0186
	48	1.496	0.1074	0.0189	0.0841
	72	1.5434	0.2197	0.0366	0.1500
	96	1.7642	0.2221	0.0424	0.1481
	120	**3.0555**	0.2731	0.0484	0.2962
	144	2.111	**0.5462**	**0.1255**	**1.2592**
	168	1.5665	0.2407	0.0030	1.2490

Table 5. ANOVA for environmental conditions.

Source of variation	SS	Df	MS	F value
Environmental conditions	SSR = 0.14219	Df = (2 − 1)	SSR/1 = 0.14219 (sr)	*Fc_1 = 2.61 (sr/se)
Time in days	SSC = 0.73965	Df = (7 − 1)	SSC/6 = 0.12328 (sc)	*Fc_2 = 2.26 (sc/se)
Error	SSE = 0.32683	(1) × (6)	SSE/6 = 0.05447 (se)	
Total	SST = 1.20867	(2 × 7) − 1		

Numbers represent F-values * = $p \leq 0.05$.

occurred with 5×10^6 spores/ml of *T. aureoviridae*. In our studies, the greatest cellulase activities were obtained with 10^6 spores/ml.

The one way ANOVA showed significant differences between cellulase activities with different inoculum sizes ($p < 0.05$).

Optimization of inoculum age

Inoculum age also affects the cellulase enzyme production. To check the effect of inoculum age, we used 7-day-, 14-day- and 21-day-old slants of *Penicillium oxalicum* and the cellulase activities obtained were 0.5462, 0.1851 and 0.0818 IU/ml, respectively (Fig. 9). The 7-day-old culture gave maximum cellulase activity compared to the other two. The one way ANOVA depicted significant differences between cellulase activities with inoculum age ($p < 0.05$).

Effect of static and agitated conditions

As seen in Table 4, the greatest cellulase activity was obtained in an agitated condition. Cellulase activity was around 0.5462 IU/ml at 120 rpm after a 144 hours incubation period. While in a static condition, the greatest cellulase activity obtained at 120 hours was 0.2220 IU/ml. These results show more enzyme activity as compared with other reported results (Acharya et al., 2008; Ojumu et al., 2003) in which the reported greatest enzyme activity is 0.0925 IU/ml and 0.0743 IU/ml, respectively. The result indicated that an agitated condition had a statistically significant influence ($p < 0.05$) on cellulolytic activity (Table 5).

We have calculated that $Fc_1 = 2.61$. From the F distribution table, we found at a 5% level of significance and d.f. (1, 6) the tabulated value $Ft_1 = 5.99$ As $Fc_1 < Ft_1$, we concluded that 2 different environmental conditions had no significant effect on enzyme activity.

From the F distribution table, we found at a 5% level and d.f. (6, 6) the tabulated value $Ft_2 = 4.28$. As $Fc_2 < Ft_2$, we concluded that the incubation time in days had no significant effect on cellulase activities.

Discussion

The fungus growth, as well as the amount of the enzyme produced, was dependent on many factors such as different substrates, the concentration of the substrates, the particle size of the substrate, the culture conditions, the time, the temperature, and the pH of the culture medium. The result of this work shows that *Penicillium oxalicum* was able to utilize all the substrates used in our study for growth. The cellulolytic enzymes were substrate dependent as reported previously (Jorgensen et al., 2005; Srivastava et al., 1984).

Although *Penicillium oxalicum* was able to produce cellulolytic enzyme utilizing all the tested substrates, noticeable quantities of the enzyme was produced with banana agrowaste as the substrate. The higher result might be due to its cellulose and hemicelluloses content which accounts for 60–80% of the total system. The use of pure cellulose as a substrate is uneconomical for the large-scale production of cellulase. Therefore, freely and easily available banana agrowaste was tested to find out whether it could support the production of cellulase.

Activities of CMCase, one of the components of cellu-

lase, were highest in the presence of treated banana agrowaste. Pretreatment results indicate that not only a difference in substrate produced different amounts of cellulolytic enzymes, but pretreatment with alkali caused a change in the structure of the substrate and that lead to an increase in the level of cellulolytic enzymes. Pretreatment with alkali disrupts lignin-carbohydrate linkage and highly-ordered cellulose, thereby reducing the particle size by increasing the surface to volume ratio, and it causes a reduction in crystallinity, so it becomes easier for fungi to degrade to a less crystalline cellulose. The chemical pretreatment of lignocelluloses causes swelling leading to an increase in the internal surface area, a decrease in the degree of polymerization, a decrease in crystallinity, and the separation of structural linkages between lignin and carbohydrates, thus increasing the cellulose hydrolysis. Pretreating the substrates with sodium hydroxide may have resulted in the swelling of the particles resulting in the easy removal of the lignin and cellulose depolymerisation occasioned by the separation of hydrogen bonds of the cellulose (Damisa et al., 2000). Acharya et al. (2008) reported that *Aspergillus niger* grown on sawdust gave the greatest cellulase activity of 0.1813 IU/ml when the sawdust was pretreated with 2N NaOH. In our studies, *Penicillium oxalicum* showed the greatest cellulase activity of 0.5462 IU/ml when banana agrowaste was pretreated with NaOH. Also, Ojumu et al. (2003) reported that *Aspergillus flavus* grown on sawdust gave the highest cellulase activity of 0.0743 IU/ml at about 12 hours. Damisa et al. (2000) reported that the highest cellulase activities on bagasse, corn cob and corn straw pre-treated with 2 M NaOH by *Aspergillus niger* AH3 were 0.067, 0.049 and 0.504 IU, respectively.

An agricultural waste hydrolysed with alkali produces a variety of sugar and their degradation products. A comparison of the alkali pretreated substrate was made. The 7% substrate concentration gave the greatest cellulolytic activity. Further increase in substrate concentration did not result in a proportionate increase in glucose yield.

Further investigation indicated the influence of particle size, the time, the temperature, the pH, and the inoculum size and aeration of the culture medium on the level of the enzyme production. The results indicate that particle size is extremely important since it affects the surface area to volume ratio of the particle which determines the fraction of the substrate initially accessible to the microorganism. The exposed surface area of cellulose is more important. For a constant geometry, the surface area to volume ratio increases as the particle size decreases (Kishna, 1999).

The fermentation time of the medium has a great effect on enzyme production, as indicated in Fig. 5. The highest CMCase activity was observed after 144 hours of incubation, and thereafter the enzyme activity decreased, while for exoglucanase it was 168 hours and β-glucosidase and FPase were produced at 144 hours. The one reason for a decreased enzyme activity after prolonged incubation might be due to autolysis of the mycelia and/or loss of enzyme stability. It is reported that the highest CMCase activity was achieved at 96 hours of the fermentation period by *Trichoderma harzianam*, *Trichoderma* spp. and *Phanerochaete chrysosporium*, respectively (Khan et al., 2007) and at the 12th hour of fermentation of *Aspergillus flavus* (Kang et al., 1994). Similar results are reported by other authors regarding some fungi in liquid fermentation (Acharya et al., 2008).

Temperature is an important external environmental factor for cellulase production; a temperature higher than 28°C suppressed the production of cellulase activity. These results are in accordance with other results (Acharya et al., 2008). At lower temperatures, the transport of substrate across the cell is suppressed and so a lower amount of enzyme is produced. At higher temperatures, the maintenance energy requirement for cellular growth is high due to the thermal denaturation of the metabolic pathway of enzymes (Aiba et al., 1973) resulting in a minimum amount of product formation.

The pH changes observed during the growth of microbes also effects product stability in the medium (Gupta et al., 2003). Acidic pH was favourable for enzyme production, whereas the enzyme production substantially decreased at a pH above 6.0. The initial pH of the medium had a significant impact on the production of cellulases with pure cellulose as the substrate, and a slightly higher pH for natural cellulosics (Ryu and Mandel, 1980).

When the inoculum size was increased from 1×10^6 to 1×10^{10}, a rapid proliferation of spores occurred that resulted in increased biomass. After a certain time period, enzyme production decreased because of the depletion of nutrients due to the enhanced biomass, which resulted in a decrease in metabolic activity (Kashyap et al., 2002). Decreased cellulase activity with increasing inoculum size might be due to the clumping of cells.

In conclusion, alkali treated banana agrowaste has the ability to induce cellulase production by *Penicillium oxalicum*. The fungus was found to be producing CMCase at a major level than the other two enzymes, exoglucanase and β-glucosidase. The present studies on the optimization of the process conditions reveal that *Penicillium oxalicum* appears to be an industrially-promising cellulolytic fungus since it grows on low cost agrowastes, while findings also indicate the possibility of banana agrowaste as a suitable substrate for large-scale production of cellulase enzymes. Further, strain improvement could lead to an enhancement of enzyme yields. The alkali pretreatment expense and the benefit of enzyme activities have to be economically evaluated.

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References

Acharya, P. B., Acharya, D. K., and Modi, H. A. (2008) Optimization for cellulase production by *Aspergillus niger* using saw dust as substrate. *Afr. J. Biotechnol.*, **7**(22), 4147–4152.

Aiba, S., Humphrey, A. E., and Millis, N. F. (1973) Kinetics. *In* Biochemical Engineering, 2nd ed., Academic Press, New York, pp. 92–127.

Beg, Q. K., Kapoor, M., Mahajan, L., and Hoondal, G. S. (2001) Mi-

crobial Xylanases and their industrial applications: a review. *Appl. Microbiol. Biotechnol*., **56**, 326–338.

Bhat, M. K. (2000) Cellulases and related enzymes in biotechnology. *Biotechnol. Advances*, **18**, 355–387.

Bhat, M. K. and Bhat, S. (1997) Cellulose degrading enzymes and their potential industrial applications. *Biotechnol. Advances*, **15**, 583–620.

Cavaco-Paulo, A. (1998) Mechanism of cellulase action in textile process. *Carbohydr. Polym*., **37**, 273–277.

Chesson, A. (1987) Supplementary enzymes to improve the utilization of pigs and poultry diets. *In* Recent Advances in Animal Nutrition, ed. by Haresign, W. and Cole, D. J. A., Butterworths, London, pp. 71–89.

Coral, G., Arikan, B., Unaldi, M. N., and Guvenmes, H. (2002) Some properties of crude CMCase of *Aspergillus niger* Z10 wild-type strain. *Turk. J. Biol*., **26**, 209–213.

Damisa, D., Ameh, J. B., and Umoh V. J. (2000) Effect of chemical pretreatment of some Lignocellulosic wastes on the recovery of cellulase from *Aspergillus niger* AH3 mutant. *Afr. J. Biotechnol*., **7**, 2444–2450.

Depaula, E. H., Rasmos, L P., and Azeveodo, M. D. (1999) The Potential of *Humicola grisea* var. Thermoidea for bioconversion of sugarcane bagasse. *Bioresour. Technol*., **68**, 35–41.

Elad, Y. (2000) Biological control of foliar pathogens by means of *Trichoderma harzianum* and potential mode of action. *Crop. Prot*., **19**, 709–714.

Gupta, R., Gigras, P., Mohapatra, H., Goswamu, V. K., and Chauhan, B. (2003). Microbial α-amylases: A biotechnological perspective, *Pro. Biochem*., **38**, 1599–1616.

Immanuel, G., Dhanusa, R., Prema, P., and Palavesam, A. (2006) Effect of different growth parameters on endoglucanase enzyme activity by bacteria isolated from coir retting effluents of estuarine environmental. *Int. J. Environ. Sci. Tech*., **3**(1), 25–34.

Jeffries, T. W. (1996) Production and applications of cellulase laboratory procedures. *In* Laboratory Procedures Handbook, pp. 1–10.

Jorgensen, H., Morkeberg, A., Krogh, K. B. R., and Olsson, L. (2005) Production of cellulases and hemicellulases by three *Penicillium* species: Effect of substrate and evaluation of cellulose adsorption capillary electrophoresis. *Enzyme Microbial Technol*., **36**, 42–48.

Kang, S. W., Kim, S. W., and Kimard, K. (1994) Production of cellulases and xylanases by *Aspergillus niger* KKS. *J. Microb. Biotechnol*., **4**(1), 445–743.

Kansoh, A. L., Essam, S. A., and Zeinat, A. N. (1999) Biodegradation and utilization of bagasse with *Tricoderma ressei*. *Polym. Degrad. Stab*., **62**, 273–278.

Kashyap, P., Sabu, A., Pandey, A., and Szakacs, G. (2002) Extra-cellular L-glutaminase production by *Zygosaccharomyces rouxii* under solid state fermentation. *Process Biochem*., **38**, 307–312.

Khan, M. D. M. H., Ali, S., Fakhru'l-Razi, A., and Alam, M. D. Z. (2007) Use of fungi for the bioconversion of rice straw into cellulase enzyme. *J. Environ. Sci. Health Part B*, **42**, 381–386.

Kishna, C. (1999) Production of bacterial cellulases by solid state bioprocessing of banana wastes. *Bioresour. Technol*., **69**, 231–239.

Kocher, G. S., Kalra, K. L., and Banta, G. (2008) Optimization of cellulase production by submerged fermentation of rice straw by *Trichoderma harzianum* Rut-C 8230. *The Internet J. Microbiol*., **5**(2).

Mandels, M. and Weber, J. (1969) The production of cellulases. *Advan. Chem. Ser*., **95**, 391–414.

Miller, G. L. (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem*., **31**, 426–428.

Ojumu, T. V., Solomon, B. O., Betiku, E., Layokun, S. K., and Amigun, B. (2003) Cellulase production by *Aspergillus flavus* Linn isolate NSRR 101 fermented in sawdust, bagasse and corncob. *Afr. J. Biotechnol*., **2**(6), 150–152.

Peij, N., Gielkens, M. M. C., Veries, R. P., Visser, J., and Graaf, L. H. (1998) The transcriptional activator XLnR regulates both Xylanolytic and endoglucanase gene expression in *Aspergillus niger*. *Appl. Environ. Microbiol*., **64**(10), 3615–3619.

Ryu, D. D. Y. and Mandel, M. (1980) Cellulases: Biosynthesis and applications. *Enzyme Microbial Technol*., **2**, 91–102.

Shah, M. P., Reddy, G. V., Banerjee, R., Ravindra Babu, P., and Kothari, I. L. (2005) Microbial degradation of banana waste under solid state bioprocessing using two lignocellulolytic fungi, *Phylostictia* spp. MPS-001 and *Aspergillus* spp. MPS-002. *Process Biochem*., **40**, 445–451.

Shin, C. S., Lee, J. S., and Park, S. C. (2000) Enzyme production of *Trichoderma ressei* RutC-30 on various Lignocellulosic substrates. *App. Biochem. Biotechnol*., **84–86**, 3917–3921.

Solomon, B. O., Amigun, B., Betiku, E., Ojumu, T. V., and Layokun, S. K. (1999) Optimization of cellulase production by *Aspergillus flavus* Linn isolate NSPR 101 grown on Bagasse. *JNSChE*, **16**, 61–68.

Srivastava, S. K., Gopalkrishan, K. S., and Ramochrandan, K. B. (1984) Kinetic characterization of a crude β-D-glucosidase from *A. wentii*. *Enzyme Microbial Technol*., **6**, 508–512.

Van Soest, P. J., Robertson, J. B., and Lewis, B. A. (1991) Methods for dietary fiber, neutral detergent fiber and non starch polysaccharides in relation to animal nutrition. *J. Dairy Sci*., **74**, 3583–3597.

Ximenes, E. I. and Filho, F. (1998) Hemicellulase and biotechnology. *Recent Rev. Dev. Microbiol*., **2**, 165–176.

Zaldivar, M., Velasqez, J. C., Contreras, I., and Perez, L. M. (2001) *Trichoderma aureovinde* T-121, a mutant with enhanced production of cellulolytic enzymes: Its potential use in waste cellulose degradation and/or biocontrol. *Electron J. Biotechnol*., **3**, 160–168.

Zaldvas, J., Nielsen, J. and Olsson, L. (2001) Fuel ethanol production from Lignocellulose: a challenge for metabolic engineering and process integration. *Appl. Microbial Biotechnol*., **56**, 17–34.