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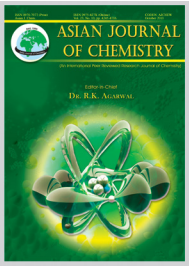

Cellulose of *Trichoderma viride* for Biological Degradation of Cellulosic Wastes

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Microbial cellulase is under intensive investigation due to its expected use as a tool for biological degradation of cellulosic wastes. The production of cellulase by *Trichoderma viride* in 5 % wheat bran medium with glucose as the carbon source was studied. The work was extended to investigate the biological degradation of cellulosic wastes such as banana stem, waste newspaper, waste plane paper, *etc.*, during the growth of the organism due to the production of the cellulase activity. The results indicated that the organism produced two cellulases, one in the earlier phase and the second in the later phase. The cellulase produced in the earlier phase was in significant quantity and was subject to induction by the cellulosic substrates included in the medium. The banana stems, particularly, in the shredded form underwent a large degradation, as this material after inclusion in the medium, underwent 90 % loss in weight. The results will hopefully help the world in production of sugar and ethanol from no-cost solid wastes and will offer partial solution to the ongoing food and energy crises along with an effective disposal of solid waste to keep environment clean.

Key Words: Cellulase, *Trichoderma viride*, Biodegradation, Cellulosic, Waste.

INTRODUCTION

Cellulase is an important enzyme that hydrolyzes cellulose. The scientists are investigating it all over the world and also in Pakistan in context of techno-economic disposal of agricultural wastes with their utilization as a source of food and energy. Cellulose is a structural polysaccharide that constitutes the structural backbone of the plant kingdom. Its structure has been described by Khan[1] with the evidence on which it is based. The detailed structure of cellulose has been developed through a large number of research studies such as Cowling and Brown[2], Emert *et al.*[3], Sihtola and Neimo[4]. Food and energy are the basic need of humanity both in development and welfare contexts. Cellulase is capable of hydrolyzing cellulose component of these wastes to glucose that is a source of instantaneous food, which can be injected into the human body for instantaneous supply of energy. Because the process was of applied interest, the cellulase applications were successively reviewed by a number of experts at early stage of its history including Cowling and Brown[2], Emert *et al.*[3], Linko[5], Ryu and Mandels[6]. The sugar produced as a result of hydrolysis is fermentable to ethanol that apart from being a starting material for the synthesis of a countless number of organic compounds is under active consideration as a motor fuel substitute.

The research studies lodged to achieve the said purpose have been mostly focused on the microbial sources of cellulase as this option is favourable because the cellulase-producing organisms that are mostly the fungi grow well on cellulosic substrates the dominant components of the agricultural wastes. The studies that may be quoted as examples include those conducted by Mandals, *et al.*[7], Herr[8], David and Thiry[9], Wyk[10], Wyk and Mohulasti[11] and Cysewski and Wilke[12]. Some organisms including *Aspergillus niger* and *Trichdema viride* have responded well to these studies. The basic studies were on the investigation of the microbial sources of cellulase and on the characterization of the cellulase produced by different organisms. The later studies including the current ones are mostly on the transformation of cellulosic wastes intact with the growing cells of the cellulytic organisms. Many studies present a lot of versatility in approach; some from the point of view of types of waste subjected to degradation, some from that comparison between chemical and biological hydrolysis of cellulose and others. The studies that can be narrated in context of cellulysis of waste newspaper include those conducted by Wilke[13], Sharma[14] and Grethlein[15]. The experts compared the economics of acid and enzymatic hydrolysis of newsprint and other wastes. Youssef *et al.*[16], have attempted to improve the biodegradation of cellulosic wastes by acid pretreatment. The studies on the improvement of energy efficiency of the process was conducted by Erickson[17] and the status of transformation of biomass into biofuels has been reviewed by

Lee *et al.*[18]. who examined ethanol produced from cellulosic biomass as large scale transportation fluid and highlighted its desirable features.

The process has been of global importance and is expected to receive focus of attention in future. To put the concept into practice, the microbiological production of ethanol from biomass is being modeled in United States[19]. The contracts have been signed because biotransformation of biomass into ethanol is expected to cause significant cost reduction as compared to the thermo-chemical hydrolysis of cellulose. US Department of Energy (USDE)[20] reports that its National Renewable Energy Laboratory claims improvements in production of cellulase enzymes for the conversion of biomass to biofuels and chemicals. Maureen[21] has reported production of cellulase in plants and genes for conversion of biomass into sugar for energy *via* ethanol. FAO Corporate Document Repository (FAO)[22] has recently documented alcohol production using an integrated pilot plant. The process involves preparation of cellulase biologically that is separated and used to cause saccharification of biomass. Unfortunately, the desired successes have not yet been achieved and thus the authors of this article yet feel that there is a lot to be done on the fundamental level. The examples may be the production of cellulose from biomass by changing different classes of organisms (Fungi, Bacteria and others), species, even different mutant strains by dragging in this field the discipline of Genetics. Some work, of course, has been conducted in this context by Ostrikova and Konovalov[23].

Pakistan, being an agricultural country, is naturally rich in agricultural wastes in the form of stalks, stems and husks. Besides natural production of these wastes, man also supplements them with municipal solid waste including paper waste, textile off cuts, garbage, *etc.* and agricultural wastes including crop waste, animal waste and forestry waste. Thus, some significant work has been carried out in Pakistan to transform different agricultural wastes into sugar for subsequent transformation to alcohol by Khan and Jamil[24]. Special attention has been paid to the biological transformation of banana waste into sugar by *Aspergillus niger* and this organism has been reported to cause extensive degradation of banana waste into sugar in surface culture but the sugar yield is low[25].

Here, the work extended to increase the yield of sugar by substituting *Trichoderma viride* for *Aspergillus niger* for growth under the same conditions to investigate biological degradation of cellulosic wastes such as banana stems, printed newspaper, plane newspaper, *etc*. The inductive effect of the cellulose present in these wastes as substrate was also studied.

EXPERIMENTAL

Culture and inoculum: *Trichoderma viride* was grown on solidified potato-dextrose-agar slants at 301 K. For the preparation of culture medium, 200 g potato slices were boiled for 1 h in about 500-700 mL distilled water. The resulting thick syrup was drained through a clean muslin cloth. To this was added glucose and agar. The final volume was made up to 1000 mL. The mixture was cooked for 1 h in a water bath. The pH of the medium was maintained at 4.5. *Ca.* 10 mL of medium was poured in each test tube. All the test tubes were cotton plugged. The medium was sterilized in a pressure cooker at 15 psi for 15 min. The medium in test tubes were allowed to set for 24 h to prepare the slants. The slants were inoculated with a sterilized needle loop and incubated at 303 K. The growth was allowed to occur for 4-6 days. The slants were preserved in a refrigerator. The culture medium had the following composition:

Potatoes	200 g dm^{-3}
Dextrose	20 g dm^{-3}
Agar	15 g dm^{-3}
pH	4.5

To prepare the inoculum, the slants were washed carefully with sterilized distilled water and a sporal suspension was obtained. The spores were centrifuged at 2500 rpm for 20 min in a sterilized centrifuge tube. The supernatant was discarded and the pallet was suspended in an adequate volume of sterilized distilled water. The optical density of the suspension was determined in a spectrophotometer. The suspension of the same optical density was transferred each time to keep the total population of spores constant; 10-20 mL of sporal suspension was transferred to each of the flasks containing 250 mL wheat bran medium and 30 mL glucose solution.

Fermentation medium: Wheat bran was chosen for the growth of *Trichoderma viride* as it was considered to be a suitable medium for the production of extra-cellular cellulase. The growth medium for *Trichoderma viride* was prepared by mixing the following quantities of ingredients per dm^3.

Wheat bran	50 g
K_2HPO_4	2.0 g
KCI	0.54 g
$MgSO_4 \cdot 7H_2O$	0.5 g

The above quantities of ingredients were mixed in distilled water to make one-liter suspension. The pH of the suspension was adjusted to 4.5. The suspension was then sterilized for half an hour at 15 psi. 20 g glucose was dissolved in 250 mL of distilled water and was sterilized separately for 0.5 h.

250 mL of wheat bran medium was taken in different 500 mL conical flasks. To each flask then was added 30 mL of sterilized glucose solution to make final concentration of glucose 1 %. The flasks were cotton plugged and were ready for inoculation.

Fermentation: *Trichoderma viride* was grown by surface culture technique. The flasks were inoculated using 10 mL of inoculum and subsequently incubated in an incubator. The growth temperature was 303 K. After three or four days when the growth of the organism had started, about 5 mL suspension was taken out. The suspension was filtered and the enzyme activity of the extra-cellular cellulase present in the filtrate was assayed taking 0.2 mL filtrate.

Assay of cellulase activity: The cellulase activity was assayed by the Method of Garge and Kantan[26] based upon the reaction of the enzyme on cellulose substrate and subsequent determination of reducing sugar colorimetrically.

To assay, 0.2 mL enzyme sample was incubated with 50 mg filter paper substrate and 0.8 mL of 0.05 mol citrate buffer in a 25 mL test tube for 1 h at 313 K. 1 mL dinitro-salicylic reagent was then added to 1 mL of the mixture. The mixture

was heated for 5 min in a boiling water bath to stop the enzymatic reaction and cooled under cold running tap water and filtered. A blank was prepared in another test tube as above but heat denatured enzyme prepared by heating in boiling water for 0.5 h or by direct heating on a low flame for 5 min was substituted in place of the enzyme sample. The optical density of the colour developed was read in a spectrophotometer at 575 nm. The unit of cellulase activity was defined as the amount of the enzyme that released one micromole of glucose under the assay conditions defined.

Enzymatic digestion of banana stem and printed newspaper during growth: In these experiments, 250 mL of wheat bran medium was taken in three 500 mL flasks. In the first flask, no agricultural waste was added; in the second flask 5 g banana stem and in the third flask 5 g printed newspaper was added. All the flasks were inoculated with 20 mL inoculum and the fermentations were carried as described above. 5 mL of samples were withdrawn and assayed for cellulase activity.

At the end of the fermentation that was carried for 20 days, banana stem was removed, washed, dried and weighed. The difference between initial and final weight was compared.

Enzymatic digestion of plane newspaper during growth: In this experiment, 250 mL of 5 % wheat bran medium was taken in each of two 500 mL flasks. In the first flask, neither glucose nor any agricultural waste was added, while in second flask 2 g plane newspaper was added. Both flasks were inoculated and fermentation carried as above for 20 days. 5 mL samples were withdrawn after 2-3 days and celllase activity was assayed.

Enzymatic digestion of different weights of banana stem during growth: Here, 250 mL of 5 % wheat bran was taken in each of four 500 mL flasks marked as 1, 2, 3 and 4. To these flasks were added 5 (Shredded), 10, 15 and 20 g (Non-shredded) banana stem, respectively. All the flasks were inoculated and fermentations were carried for 20 days as above. The cellulase activity was assayed as usual. The banana stems were removed after 20 days, washed, dried and weighed to find the differences in initial and final weights.

RESULTS AND DISCUSSION

The variation of cellulase activity with incubation time during the fermentation of *T. viride* in presence of banana stem and printed newspaper and in the absence of these substrates is shown in Fig. 1.

The profile indicates that significant quantities of cellulase are produced when the organism is grown in 5% wheat bran medium with glucose as major source of carbon and energy. The profile bears two peaks indicating the likelihood of the production of two cellulases. One is produced in the earlier phase, while second is produced in the later phase.

The shapes of profiles in presence of printed newspaper and banana stem resemble the profile discussed above. Of course, the heights of the first peaks in presence of these substrates are increased, while those of the second peaks remain almost the same. This means that the presence of the substrates induces the synthesis of cellulase produced in the early period while it does not affect the cellulase produced in the later period.

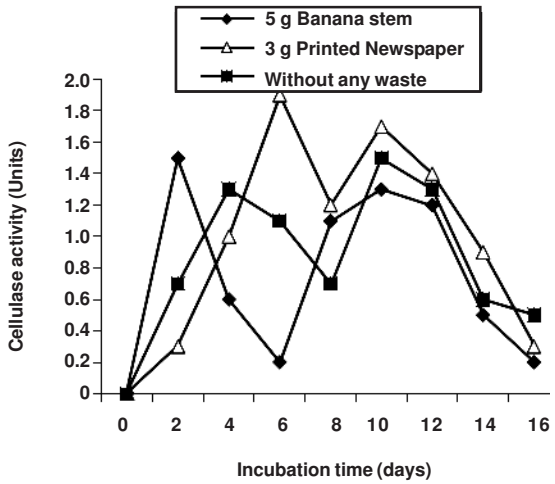

Fig. 1. Variation of cellulase activity with incubation time during the growth of *Trichoderma viride* in presence and absence of cellulosic wastes at 303 K

These results, when compared to the results of Khan and Jamil[27] are entirely different. The predecessors have reported the production of one cellulase in culture of *Aspergillus niger*. The reasons may be as under: (1) The physiology of *Aspergillus niger* may be different from that of *Trichoderma viride.* (2) The predecessors started analysis after the growth had significantly appeared (after 8-10 days) and thus they might have missed the enzyme produced in the earlier phases of microbial growth.

The variation of cellulase activity with incubation time during the course of fermentation of *Trichoderma viride* grown on wheat bran as sole source of nutrition and the variation of cellulase activity in presence of plane newspaper is shown in Fig. 2.

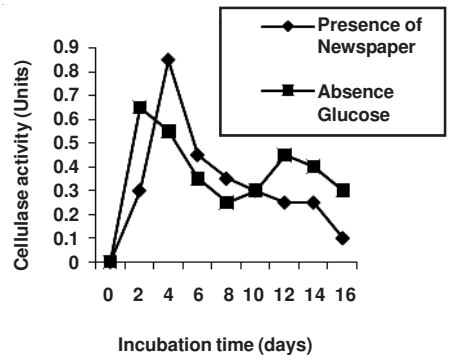

Fig. 2. Variation of cellulase activity with incubation time during the growth of *Trichoderma viride* in absence of glucose as carbon source at 303 K

The comparison of the profiles suggests that little cellulase activity is produced when the organism is grown in absence of sugar. Of course, some increase in its production during the early period of fermentation occurs. It may be due to less growth of the organism because the lack of the instantaneous carbon and energy source does not go in favour of cell multiplication. All substrates may contain some sugar but that may be in very small quantity as compared to supply of glucose as an instantaneous source of carbon and energy.

The variation of cellulase activity with the incubation time during the growth of *T. viride* in medium containing different weights of banana stem is shown in Fig. 3.

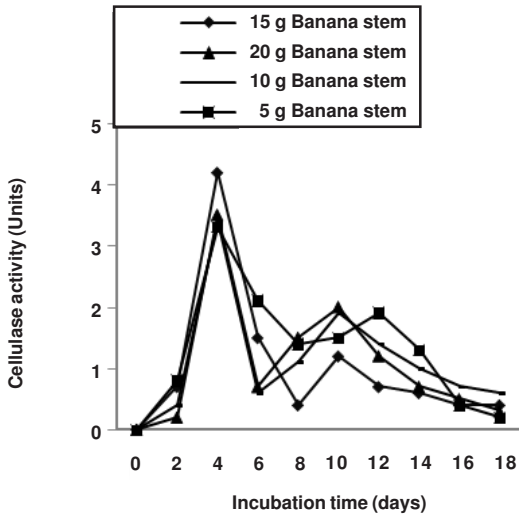

Fig. 3. Variation of cellulase activity with incubation time during the growth of *Trichoderma viride* in presence of different weights of banana stem at 303 K

Two peaks indicating the production of two cellulases as noted earlier are clearly visible in the profiles. Moreover, the production is almost double as compared to that in fermentation in which banana stem used was put in the medium as single piece without shredding (Fig. 1).

An increase in enzyme activity with the increase in the weight of the shredded banana stem can also be marked (Fig. 3, 5 g shredded banana stem curve)

The variation of reducing sugar in the fermentation medium with incubation time during the course of fermentation of *Trichoderma viride* in presence of banana stem and printed newspaper is compared in Fig. 4.

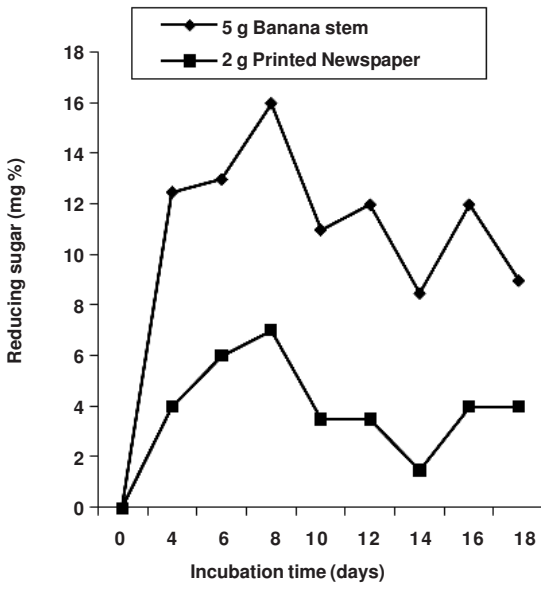

Fig. 4. Variation of reducing sugar concentration with the incubation time during the growth of *Trichoderma viride* in presence of different cellulosic wastes at 303 K

The comparison of profiles indicates that more reducing sugar is produced in presence of banana stem than that of printed newspaper. The sugar produced is far less as compared to the amount of substrates hydrolyzed. The low sugar level in spite of extensive cellulysis may be due to its immediate mobilization by the organism as immediate source of carbon and energy.

The weights of the banana stem pieces before and after fermentation are compared in Table-1. The high weight loss in banana stem pieces indicates extensive cellulysis during the fermentation of *Trichoderma viride* due to production of high amounts of cellulase activity. The maximum sugar produced in different fermentations in presence of different substrates is compared in Table-2.

TABLE-1
COMPARISON OF DIFFERENT WEIGHTS OF BANANA STEM DURING THE GROWTH OF *T. viride*

Substrates	Initial weight (g)	Final weight (g)	Loss (%)
Banana stem as a single piece	5	0.35	93.01
Banana stem in shredded form	5	0.18	96.4
Banana stem in shredded form	10	0.48	95.2
Banana stem in shredded form	15	0.86	94.0
Banana stem in shredded form	20	1.3	93.5

TABLE-2
MAXIMUM SUGAR PRODUCED DURING FERMENTATIONS WITH DIFFERENT SUBSTRATES

Substrates	Maximum sugar (mg %)
Banana stem-5 g	15.3
Shredded stem-5 g	30.6
Printed paper-2 g	6.3
Plane newspaper-2 g	12.9

The highest sugar is produced in case of shredded banana indicating that the shredded banana stem is hydrolyzed more than the other substrates. The increase may be due to increase in the area of the substrate to the action of the organism and subsequent enhanced substrate induction.

The comparison of results with previous reports shows that the potential of transformation of banana waste into sugar for onward conversion into alcoholic substitute for petrol or diesel along with its disposal to solve environmental problem also exists for other cellulosic wastes such as news paper, printed plain paper, *etc*.

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