PRELIMINARY ISOLATION AND CHARACTERIZATION OF CELLULASES OF TRICHODERMA VIRIDE GROWN ON BANANA STEM WASTE AND BAGGASSE AS SUBSTRATES

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ABSTRACT: *Food and energy shortage are currently the most pressing problems of the world. Experts all over the globe are seriously engaged in exploration of alternative food and energy sources out of which a promising source seems to be transformation of cellulosic wastes into sugars and alcohol by the enzymes cellulases. The work reported in this article was carried to study characterization of cellulase of Trichoderma viride. Trichoderma viride was grown in 5% wheat bran medium containing banana stem waste and baggasse as growth substrates. Production of cellulase and degradation of banana stem and baggasse were monitored. The extracellular cellulase produced, using 5 liters medium was subjected to ammonium sulfate fractionation. An attempt was made to separate cellulase components by binding with DEAE Cellulose` as anion exchanger. The characteristics of the isolated fractions were determined and compared to distinguish them from each other. The results indicate that Trichoderma viride produces three cellulase enzymes i.e. the first fraction and last one are highly active while the second fraction is less active. The comparison of their characteristics indicates that they are different enzymes.*

Keywords: Cellulase, Trichoderma, viride, Substrates, Banana, Waste, Bagasse.

1. INTRODUCTION

Food and energy resources are declining at a very fast rate. Even, if available, there are international constraints on the use of fossil fuels due to liberation of carbon in the global environment that translates into an increase in greenhouse gases and global warming. The research workers particularly from the developing countries are taking these threats seriously and are trying to find and create additional resources of food and energy. The scientists are now focusing on microorganisms such as bacteria, algae and fungi as supplementary sources of food and energy. Microorganisms are capable of producing some useful products from some cheaper raw materials present in environment and thus their transforming activities can be tried to degrade naturally occurring bio-resources to help mankind secure supply of food and energy to fulfill its needs.

Cellulose is the chief constituent of the agricultural wastes and can be degraded by the action of cellulases into fermentable sugars, which can be ultimately converted into alcohol. Glucose is an instant source of energy for human body and alcohol, a source of energy for automobiles and food for humans. An extensive work both at international and national level has been undertaken on cellulases and their effect on cellulose of wood, newspaper waste, wheat straw, rice and cotton husks etc; but effect of cellulases from Trichoderma viride on banana stem is more potent and challenging.

A large number of research studies describe cellulases from different sources but the focus has been mostly on microbial cellulases particularly those from thermophilic fungi due to their characteristic to convert widely encountered cellulose occurring in plants into sugar and alcohol in context of food and energy. The history of studies on cellulose dates back first half of 20^{th} century in attempt to make an active commercial cellulase preparation from Aspergillus niger and other thermophilic fungi and determine their optimal and optimal temperature to have to optimize the yield of the enzyme in commercial context.. The bacterial cellulases were found active at temperatures as high as 70° that was thought to be due to being products of thermophilic fungi. Later studies were extended to study the mechanics of its action and its activity on derivatives of cellulose. Reese, et al (1950) studied the biological degradation of soluble cellulose derivatives and its relationship to the mechanism of cellulose hydrolysis [1]. Mandels and Reese produced cellulase by growing Trichoderma viride and observed that it was influenced by carbon source and metals and was an adaptive enzyme in cultures of *T. viride* [2]. These authors subsequently conducted a study on fungal cellulases and the microbial decomposition of cellulosic fabric, developments [3], Tomita et al studied the chromatographic patterns of cellulase components of *Trichoderma viride* grown on the synthetic and natural media [4]. Okada, et al studied the components of cellulose from Trichoderma viride [5]. Ogawa and Toyama worked on the resolution of *Trichoderma viride* commercial cellulytic preparation into two components to compare their characteristics. After purification, three electrophoretically homogeneous components differing in their tendency to hydrolyze cellulose substrates were reported [6]. Okada continued enquiry ion the cellulose system of T. viride carried out purification and reported properties of two cellulases [7]. Fagerstam et al reported purification of different cellulytic enzymes from *Trichoderma viride* [8]. Håkansson et al reported the purification and characterization of low molecular weight cellulases from *Trichoderma viride* [9]. Toyama, et al studied the production of cellulase of Trichoderma viride in solid (Wheat straw, wheat bran, newspaper, saw dust, filter paper, corrugated card and board and baggasse) and submerged cultures using its different strains [10]. Toyama et al reported the sugar and ethanol production from plant cellulosic resources [11]. Beldman, et al purified and characterized the cellulase of *Trichoderma viride* and compared it with all detectable endoglucanases, exoglucanases and beta-glucosidases [12]. Okada and Tanaka isolated and characterized a novel type of cellulase from *Trichoderma viride* [13]. Chen, et al studied the production of

cellulase on waste paper pulp medium by *Trichoderma viride* [14]. Nuzhat and Khan in Pakistan carried out work on optimization of cellulase production by Ainspergillus niger and digestion of banana waste and wood during the growth of organism [15]. The results indicated almost complete digestion of banana wastes while wood underwent little change. Wyman CE.described ethanol production from lignocellulosic biomass with special reference to: technology economics, and opportunities [16]. Duenas, et al in 1995 studied cellulase production by mixed fungi in solid state fermentation of bagasse [17]. Baht and Baht two year later studied the cellulose degrading enzymes and their potential industrial applications [18].

Parallel studies from other countries in similar context include those by Hawary and Mostafa [19], Saez, *et al*, [20] Juhasz, *et al* [21], Lynd, *et al* [22], Lin, et al, Asgher et al [24], Prassad et al [25], Risheng, *et al* [26], Belal [27] and Acharya *et al* [28].

The research is yet in progress at reasonable pace. Huang, et alin, studied the effect of biomass species and plant size on cellulosic ethanol as a comparative process and also carried out economic analysis [29]. Chandra, *et al* studied cellulase production by six Trichoderma spp., fermented on medicinal plant processings [30]. A number of studies were conducted in 2010**.** Soni, et al attempted bioconversion of sugarcane bagasse into second generation bioethanol after enzymatic hydrolysis with in-house produced cellulase from *Aspegillus* sp [31]. Yoswathana and Phuriphipat conducted. bioethanol production from rice straw [32]. Aslam, et al reported the expression pattern of Trichoderma cellulases under different carbon sources [33]. Li et al studied the enhancement of the cellulase production by *Trichoderma viride* mutated by microwave and ultraviolet [34]. Charithadevi and Kumar Production, Optimization and partial purification of cellulase by aspergillus niger fermented with paper and timber sawmill industrial wastes [35].

One recent study has been carried out in Pakistan by Iqbal, et al [36] and other in Brazil by Andrade, et al in 2011 sound quite relevant for quoting in context of purification of cellulases of Trichoderma viride [37]. Iqbal, et al reported purification and characterization of kinetic parameters of cellulase produced from *Trichoderma viride* under still culture solid state fermentation technique using cheap and an easily available agricultural waste material, wheat straw as growth supporting substrate. Cellulase was purified 2.33 fold. Using carboxymethyl cellulose as substrate, the enzyme showed maximum activity (*V*max) of 148 U/mL with its corresponding K_M value of 68 μM. The purified enzyme activity was inhibited by SDS, EDTA, and Hg^{2+} activated by Co^{2+} and Mn^{2+}.The purified cellulase was claimed to be compatible with four local detergent brands with up to 20 days of shelf life at room temperature suggesting its potential as a detergent additive for improved washing; therefore, it was recommended as potentially useful for industrial purposes particularly for detergent and laundry industry. Andrade, et al also evaluated the production of cellulolytic enzymes by Trichoderma sp. IS-05 strain, isolated from sand dunes, and was grown using wheat bran as the carbon source and peptone as the nitrogen source in different concentrations. The results showed that media containing wheat bran 4.0% (w/v) and peptone 0.25% (w/v) lead to the highest production of cellulase, after 2 days of fermentation [37]. The optimal pH and temperature of the enzyme was reported as 3.0 and 60◦C respectively. The cellulase was most tolerant at 60◦C, retaining more than 59.6% of maximal activity even after 4 hours of incubation. The authors were of the opinion that, "high temperature tolerance, and production of cellulase from agro-industrial residues by Trichoderma sp. IS-05 offers possibilities condition for the biomass hydrolysis process to produce bioethanol."

Bhoosreddy carried out a comparative study of cellulase production by Aspergillus niger and Trichoderma viride using solid state fermentation on cellulosic substrates corncob, cane bagasse and sawdust [38]. Khan, and Amin, in 2012, conducted a study in Pakistan on cellulase of Trichoderma viride for biological degradation of cellulosic wastes [39]. They encountered significant hydrolysis of the different cellulosic wastes by the substrate induced cellulose of the organism. Yasmin and Nehvi1 conducted isolation and characterization of Cellulase from Trichoderma viride and also determined its and molecular weight [40].

The objectives of the work reported here were as under:

1. Finding a useful source of cellulose which may be used in food and feed industry;
2. Decreasing and controlling the pollution problem due to agricultural waste;
3. Providing some extra source of income to local people of different countries, particularly from the developing world,

The main objective of this work is the isolation and characterization of cellulases from Trichoderma viride and to discuss its effect on the degradation of cellulose of banana waste.

2. MATERIALS AND METHODS

The work was carried in the following stages.

1. The organism was grown in 5% wheat bran medium with and without banana waste to produce cellulases with incubation time profile exhibiting different peaks as reported beforeby Nuzhatand Khan [15] and Khan and Amin (2012)
2. The organism was grown in bulk using large number of conical flasks and extracellular cellulase samples under two activity peaks were obtained, filtered and mixed to constitute two bulk samples of extracellular cellulases of Trichoderma viride
3. The bulk samples were subjected to ammonium fractionation.
4. The fractions separated in Step 3 were subjected to ion exchange chromatography, to isolate partially purified cellulases components.

Finally the characteristics of isolated fractions were determined to characterize them and their characteristics were compared to have an idea about their nature.

2.1. Culture and Inoculum

Trichoderma viride strain was obtained from Botany Department, Government College University Lahore. The mold was grown on potato dextrose agar slants prepared as described by Khan and [], inoculated with and incubated at 28^{o}C and transferred weekly. On each transfer, the slants were washed carefully with sterilized distilled water to form a sporal suspension. The spores were centrifuged at 2500 rpm for 20 minutes in a sterilized centrifuged tube. The supernatant was discarded and the pellet was suspended in an adequate volume of sterilized distilled water. The optical density of the suspension was determined in a spectrophotometer. The sporal suspension of the same optical density was transferred for inoculation each time in order to keep the population of spores constant. If the optical density was higher, adequate dilution with sterilized distilled water was made.

2.2. Preparation of Growth Medium

Trichoderma viride was grown in wheat bran as it was considered a suitable substrate for production of extracellular cellulases. The growth medium was prepared by mixing the following quantities per liter:

Wheat bran 50 g, K_2HPO_4 2g, KCl 0.5g, $MgSO_4.7H_2O$, 0.5 g

The ingredients were mixed in distilled water to make 1 L suspension. The suspension was then sterilized for half an hour at 15 psi. The pH of the suspension was noted to be 4.3. 20 g glucose was dissolved in 250 ml of distilled water and was sterilized separately for half an hour. 250 ml of wheat bran medium was taken in different 500 ml Erlenmeyer flasks. To each flask then was added 30 ml of sterilized glucose solution.

2.3. Fermentation

The fermentation was carried in submerged cultures. The flasks containing 250 ml medium and subsequently added 30 ml glucose solution were inoculated using 20 ml of Inoculum and placed in an incubator at 30^{o}C. After 3 or 4 days when the growth of the organism had started, 5ml suspension was

withdrawn. The suspension was filtered and extracellular cellulase activity of filtrate was assayed taking 0.2 ml of the enzyme sample. Later, the course of fermentation was followed by withdrawing 5 to 10 ml of suspension after 1 or 2 days and assaying the enzyme activity as above. The same exercise was repeated by adding in the fermentation medium banana waste and bagasse in consecutive fermentations.

2.4. Assay of Cellulase Activity

The cellulase activity was assayed by the method of Garg and Kantan [41] based on the reaction of the enzyme on cellulose substrate with subsequent determination of reducing sugar spectrophotometrically. 0.2 ml of the enzyme sample was incubated with cellulose substrate and 0.8 ml of citrate buffer (0.05 M) at pH 4.8 for one hour at 37º C. The mixture was heated for 5 min in a boiling water bath and cooled under running tap water. Blank was prepared in another test tube but heat denatured enzyme was used in place of enzyme sample. The optical density of color developed was read at 575nm in a spectrophotometer.

The unit of cellulase activity was defined as the amount of the enzyme that released one micromole of glucose under the assay conditions defined.

2.5. Growth of Trichoderma viride with and without Banana Stem

250 ml of 5% wheat bran medium was taken in each of three 500 ml flasks. In one, no agricultural waste was added, in second 5g banana stem was added while third was used as blank. The flasks except blank were inoculated with 20 ml inoculum and placed in an incubator at 37ºC. The growth was carried out as above and cellulase activity was assayed using 2 ml sample. The fermentation was carried out for 28 days.

Bulk Production of Cellulase of Trichoderma viride

To carry the bulk production of enzymes 500 ml of 5% wheat bran medium was taken in each of ten, one liter flasks and 100 g shredded banana stem was added to each. All the flasks were sterilized, inoculated and fermentation was carried as above. The contents of five flasks were bulked on 8th day and filtered. The volume of filtrate was measured and subjected to ammonium sulfate precipitation for isolation of enzymes. The remaining five flasks were similarly bulked on 21st day and processed as above. Thus over all two samples Sample A and Sample B were obtained which were subjected to ammonium sulfate fractionation and processed further.

2.6. Isolation of Cellulases

2.6.1. Fractional precipitation with ammonium sulfate: To each of sample A and sample B was added a calculated quantity of solid ammonium sulfate to constitute, after dissolution, 10 % ammonium sulfate concentration. As there was no precipitation, to it was further added ammonium sulfate up to 20% concentration. The process was repeated till there was some precipitation at 60% concentration in both. The contents of flasks were kept in a refrigerator for 24 hours and material was centrifuged to separate the precipitates as fraction 1(a) and fraction 1(b). To the supernatant was added more ammonium sulfate to raise the concentration up to 80 %. The precipitates were separated as above to constitute the fraction 11 (a) and 11 (b). The supernatants obtained after separation of precipitates at 80% contained significant cellulase activity, which means that some part of enzymes was still un-precipitated.

The plan was revised due to constraint of time and resemblance of different fractions with each other. The fractions 1(a) and II (a) were mixed and 1(b) and 11 (b) were mixed to constitute two fractions fraction 1 and fraction and II.

2.7. Determination of Enzyme Characteristics

2.7.1. Determination of the effect of temperature: The effect of temperature on the cellulase activity of the enzyme preparation fractions was studied within the range of 30° C to 70°C. 1 ml of the test sample was incubated with 1 ml of substrate in the test tube for one hour at 40º C, 45º C, 50º C, 55º C, 60º C, 65º C, 70º C, 75º C and 80º C. The assay was carried out by the method of Garg and Neela Kantan (1982) as described earlier. The change in optical density was plotted as a function of temperature.

2.7.2. Determination of effect of pH: The effect of pH on the enzyme activity was studied within the range 2.5 to 7.0 using citrate-phosphate buffers of different pHs 0.2 ml of enzyme sample was mixed with 0.8 ml of each buffer containing 50 mg filter paper and the mixture was incubated for one hour at 37º C. The cellulose activity was assayed as usual. The cellulase activity was plotted as a function of temperature.

2.7.3. Determination of thermal stability

To determine the thermostability, 0.2 ml of enzyme taken in different test tubes was subjected to the effect of different temperatures by incubating the sample in thermostatic water bath for 15 minutes. The residual activity of all samples affected by different temperatures was assayed at 37º C using CMC as substrate. The percentage of residual activity was plotted as the function of temperature.

2.7.4. Determination of pH stability

To determine the pH stability, 1ml of enzyme sample was taken in different test tubes. The pH of each tube was adjusted to different values in range 2-12 by the addition of 1N HCl /NaOH. The test tubes were placed in an incubator and allowed to stand for five hours at 37ºC. The pH of all the test tubes was pulled back to 4.5 by the addition of concentrated acid/alkali using a micro-syringe. The remaining cellulase activity was compared with the initial activity to calculate the percentage residual activity. The residual activity was plotted against pH to establish pH stability profile.

2.7.5. Binding with DEAE Cellulose

DEAE is one of the most commonly used anion exchanger. Cellulases have the tendency of binding with the DEAE cellulose like other enzymes. Phosphate buffers of different pHs 7, 8 and 9 were prepared. 100 mg of DEAE cellulose were taken in different flasks and to it were added equal volumes of buffers of different pHs and allowed to equilibrate. The samples of equal volumes of cellulase enzymes were applied and left as such for 24 hours. After 24 hours the solutions were filtered and the activity of filtrate was checked that showed binding with DEAE cellulose.

3. Results and Discussion

The results of the experiments carried out are reported and discussed below.

The variation of cellulose activity during the growth of Trichoderma viride in 5% wheat bran medium at 37^{0}C with

and without banana stem and bagasse as substrates is shown in Fig .1.

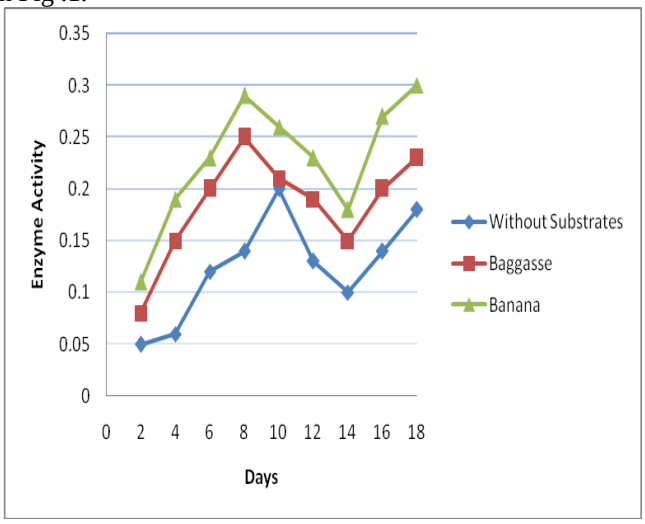

Fig 1: Variation of cellulase activity during the growth of Trichoderma viride in 5% wheat bran medium at 37^{0}C using banana stem and bagasse as substrates and without these substrates

The activity profile makes the following points:

1. The organism is capable of producing significant quantity of cellulase in 5% wheat bran medium. (Curve 1)
2. The cellulose activity is increased in presence of banana waste as well as bagasse substrates.
3. The enzyme activity produced in presence of banana waste is greater than that produced in presence of bagasse.
4. There are two activity peaks obtained in case of banana waste as well as of bagasse one on 8th day and other on 21st day.

The variation in weight both wet and dry biomass during the growth of Trichoderma viride in 5% wheat bran medium at 37^{0}C using banana waste as substrate is shown in Fig 2.

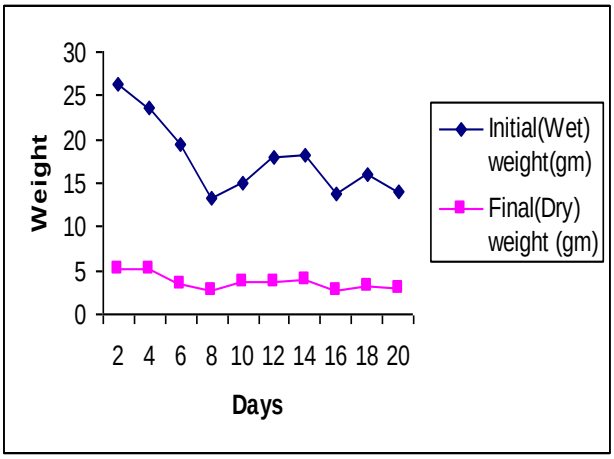

Fig 2: Variation of biomass with incubation time using Banana Stem as substrate

The profiles (Fig, 2) indicate that there is an initial drop of wet biomass (banana waste +cells of T. viride + trapped wheat bran) up to 8th day and after that it starts increasing. It peaks again and between 12 to 14 days after which it starts decreasing again. A third peak is also encountered in the profile. The pattern of variation of dry mass seems to be similar. The comparison indicates that there is around 50% degradation of both wet and dry biomass on 8th day of fermentation after which the weight of banana waste increases with increase in incubation time. This may be due to the increase in the amount of cellulase induced by the substrate with increase in the incubation time up to 8th day (Fig.1) and that may be responsible for extensive degradation of the banana waste and trapped wheat bran to produce fermentable sugar. After that the organism cells start autolyzing to liberate intra-cellar sugar and the cells start growing again due to utilization of fermentable sugar of both origins leading to an increase in overall biomass weight up to day 14 where another peak appears in the biomass (Fig.2) with a gradual decrease in the amount of cellulase (Fig.1). In the later course of the fermentation cells go on forming at slow rate and banana waste degradation continues due to attack of extracellular as well as intracellular cellulase liberated as a result of autolysis.

The variation in weight of wet and dry bagasse biomass during the growth of Trichoderma viride in 5% wheat bran medium at 37^{0}C using bagasse as substrate is shown in Fig 3.

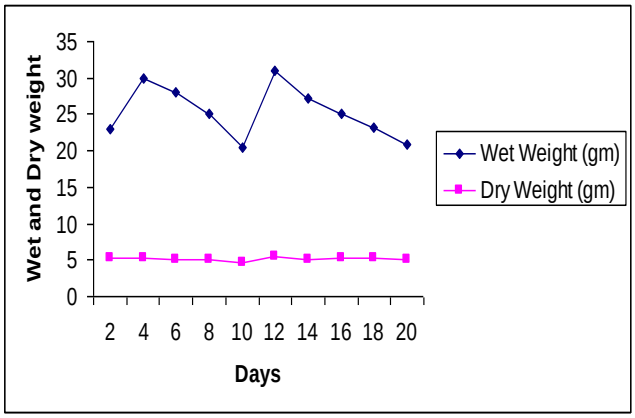

Fig 3: Variation of biomass with incubation time using Baggasse as substrate

The profiles (Fig, 3) indicate that there is an initial 30% increase of wet biomass (banana waste +cells of T. viride + trapped wheat bran) up to 4th day and after that it starts decreasing and falls to minimum on 10th day with 34 % fall. It peaks again with the same rise on 12 day and gradually falls to a similar minimum at end of the fermentation. The pattern of variation of dry mass seems to be similar. The initial rise in the weight of wet biomass may be due to relatively less production of cellulase and less degradation of bagasse and significant growth of the organism translating into more production of biomass. After that the biomass decreases to the minimum due to enhancement in the celluase production (Fig.1) that attacks bagasse and wheat bran trapped in the cells and bagasse waste. This minimum is encountered on 10th day in the profile. Then there is a sudden rise in the bomass and it maximizes on 12th day and subsequently undergoes a gradual decrease to a similar minimum at the end of the fermentation. The peak may be explained in term of the rapid growth of cells due to

utilization of sugar produced as a result of bagasse degradation and cell autolysis.

If the 50 % reduction of banana waste is compared with 34 % of bagasse, it may be concluded that former is subject to more degradation because it is softer and porous and cellulase is capable of attacking it extensively while on the other hand bagasse is comparatively harder and thus is less subject to degradation. Of course, both offer significant degradation and thus it can be said to be capable of disposal and the study presented here is helpful in solid waste management.

As a result of ammonium sulfate fractionation of Sample A and Sample B, two fractions were obtained from each at 60% saturation and 80% saturation respectively. The activities of the ammonium sulfate fractions are reported in Table 1

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Table: 1 Progress of ammonium sulfate fractionation of cellulose of T. viride

Material	Total Enzyme (ml)	Protein (mg)	Cellulase activity (Units)		
			Total	Specific	Yield
Crude Extract	5200	2132	5304	2.48	100%
Fraction (1) 60%	660	343	1669	3.11	20%
Fraction (2) 80%	700	357	1064	2.98	19.6%

The characteristics of isolated cellulases are displayed as profiles in Fig.4 to Fig.6.

Fig. 4 to 6

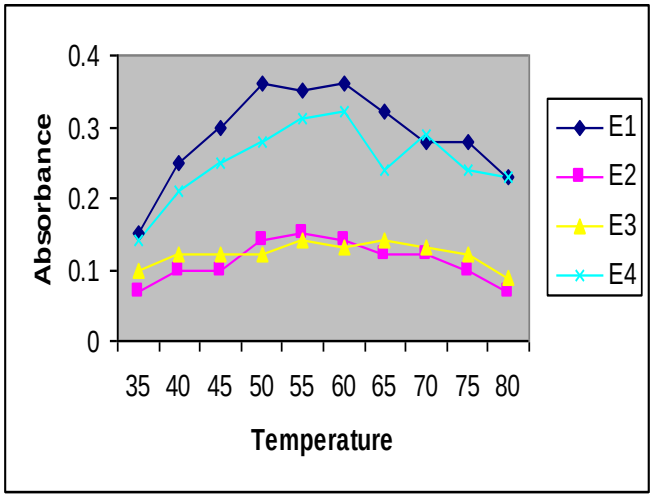

Fig 4: Variation of the enzyme activity of cellulases with change of temperature

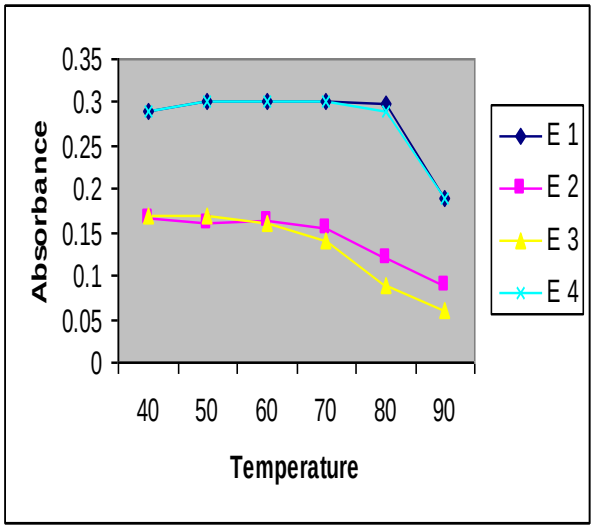

Fig: 5: Temperature Stability of Cellulase Enzymes

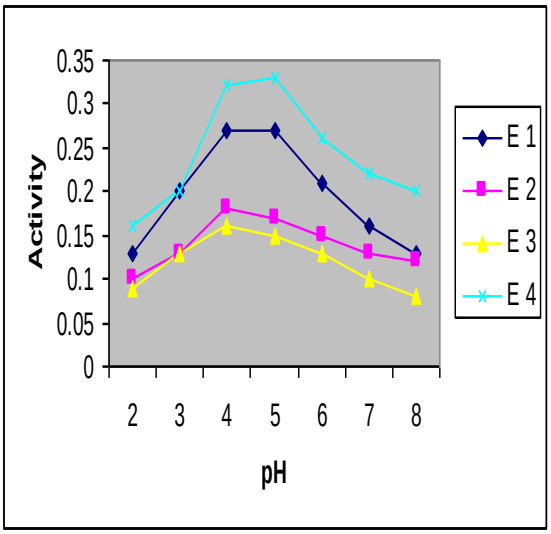

Fig: 6: Variation of enzyme activity of cellulases with pH change.

The percentage binding of cellulase with the DEAE cellulose is shown in Fig. 7

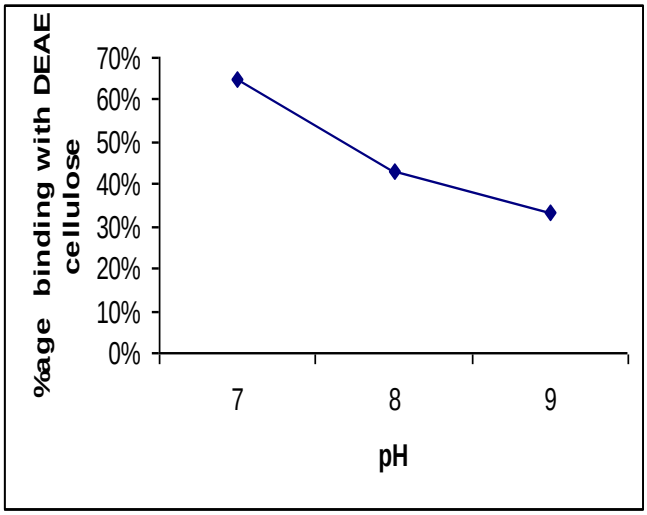

Fig. 7: Percentage binding of cellulase with the DEAE cellulose

The main purpose of experimental work along with the solid waste disposal was to investigate the cause of different activity peaks in the growth profile of *Trichoderma viride* (Cellulase activity-Incubation time) in terms of the nature of the cellulase produced by the organism (Khan and Amin, 2012). Thus it was planned to isolate and characterize the cellulase of *Trichoderma viride*, to examine their nature and relate to the appearance of different peaks encountered in the profiles presented by our predecessors and to see whether different peaks were due to formation of different cellulases or some other factors were responsible for different peaks. Thus, in the first instance, a check experiment was carried out under the standardized conditions. The results indicated that the profile presented by our predecessors were reproducible. Thus our results were quite in agreement with those of Khan and Amin (2012). The maximum yield of cellulase was obtained on 8^{th} day of incubation that was the day of formation of first peak in the profile. The second peak corresponded to 21^{st} day. This formed an adequate basis of bulking the growth samples up to 8^{th} day as sample A and those from to 9^{th} to 21^{st} as sample B, which were subjected to ammonium sulfate fractionation and it's binding with DEAE cellulose.

On subjection to ammonium sulfate fractionation two fractions (Fraction I and Fraction II) were obtained, as the precipitation started at 60% concentration and was completed at 80% concentration. This necessitated the determination of the enzyme characteristics of isolated fractions and subsequent comparison of the determined characteristics to sort out whether these enzymes were absolutely different from each other or some resembled each other and were in the form of mixtures in different fractions.

The characteristics of the fraction E1 indicate that it is clearly distinguished from other three as it exhibits highest temperature optimum 60ºC and is thermally stable up to 80ºC, while all others exhibit temperature optima around 55ºC. Similarly, the enzyme component of the fraction E4 is clearly distinguished from others on the basis of thermostability (up to 60º C). Almost all the characteristics of E_2 and E_3 resemble each other. These seem to be the same enzyme distributed in Fractions I and II, during ammonium sulfate precipitation.

Taking into consideration the points made above, it may be concluded that Trichoderma viride forms three cellulases. All of them differ in their characteristics. That is they are well active in pH-range 4.0 to 6.0 in which are encountered their optima and pH stability.

On comparing with the results of Previous workers i.e. Nuzhat and Khan [15], Khan and Amin [39], our results are in agreement with them. Most of the characteristics of the cellulase are the same as mentioned earlier.

4. CONCLUSIONS

The results and discussion on the production, isolation by ammonium sulfate fractionation and characterization leads to the conclusion that Trichoderma viride extensively degrades agricultural wastes like banana stalk and bagasse and can play an effective role in their disposal. It produces three characteristically different cellulase enzymes that can be further purified and added to the current list of International Enzyme Commission after due numbering. Thus the study adds a significant academic and practical value to the fields of enzymology and Environmental Science. . .

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