PRODUCTION AND PURIFICATION OF CELLULASE FROM *Trichoderma viride* BY OPTIMIZED MEDIUM USING VARIOUS AGROWASTE FOR Poultry FEED SUPPLEMENT

S.Karunakaran, S.Dhanasekaran, R.Roja Blessy, A.Ravulathu Nisha

Abstract—In India, Tamilnadu ranks second in poultry meat and egg production. Poultry feed manufacture has undergone an enormous expansion and development during the past half century throughout the country. The cellulase was produced from *Trichoderma viride* which can degrade cellulose. Then it was given as feed supplementary for the poultry animals. *Trichoderma viride* was isolated from soil and is allowed to grow in Potato dextrose Agar (PDA). For the effective production of T. viride, various agro wastes such as wheat bran, rice straw, molasses, saw dust, and beet root were used. The produced *Trichoderma viride* and cellulase were given as feed supplementary to the chicks in order to increasing their digestibility and growth. The maximum biomass production of *Trichoderma viride* was observed in beetroot media. With these facts in the background, the present study has been designed to explore the technology to synthesize cocktail fibro lytic enzymes at farm gate level and make the farmers as successful entrepreneurs.

Index Terms—*Trichoderma viride*, Cellulase, Gel filtration chromatography, Ammonium precipitation method.

I. INTRODUCTION

Feeding enzymes to poultry is one of the major nutritional advances in the last fifty years. Plants contain some compounds that either the animal cannot digest or which hinder its digestive system, often because the animal cannot produce the necessary enzyme to degrade them. So they need exogenous enzymes in feed to aid digestion. Cellulose is the most abundant biomass on the earth. Cellulose, a crystalline polymer of D-glucose residues connected by \( \beta-1,4 \) glycosidic linkages, being the primary structural material of plant cell wall, is the most abundant carbohydrate in nature. A number of fungi and bacteria are capable of producing multiple groups of enzymes, which are collectively known as cellulases that act in a synergistic manner to hydrolyze the \( \beta-1,4 \)-D-glycosidic bonds within the cellulose molecules. Fungi are the main cellulase-producing microorganisms, though a few bacteria and actinomycetes have also been recently reported to yield cellulase activity. Microorganisms of the genera *Trichoderma* and *Aspergillus* are thought to be major cellulase producers, and crude enzymes produced by these microorganisms are commercially available for agricultural use. The fungus *Trichoderma viride* is a single-celled fungus that can reproduce by splitting them and describe the active cellulose contained in the soil. Some newly developed agro industrial wastes used for cellulase production are banana wastes, rice straw, corn cob residue, rice husk, wheat straw, banana fruit stalk, and coconut coir. One of the main reasons for supplementing wheat- and barley-based poultry diets with enzymes is to increase the available energy content of the diet. Increased availability of carbohydrates for energy utilization is associated with increased energy digestibility. In addition, this review demonstrates that enzymes are a very useful tool in the study of physiological and metabolic mechanisms of poultry animals. Our aim is to convert each and every farmer into an entrepreneur who can be benefited even from the agro waste to produce cellulase using *T. viride* at lower economic cost.

II. METHODS

2.1 Isolation and identification of *Trichoderma viride*

The fungal microorganism *Trichoderma viride* was isolated from the agricultural land soil and serial diluted with sterile saline water. Examination of the shape, size, arrangement and development of conidiophores or phialides provided a tentative identification of *Trichoderma sp*. Characteristics were observed for identification and the plates were stored at 4°C.

2.2 Production of *Trichoderma viride* using various substrates

In this study *Trichoderma sps.* was produced by liquid state fermentation by using five different substrates such as rice brawn, wheat bran, saw dust, molasses, carrot and beetroot. The selection of this substrate is specified because those rich in the source of carbohydrate and it helps in the production of *Trichoderma viride*. These five substrates are stored in sanitary condition after the sterilization in sanitary condition for further use.

2.3 Biomass production

Biomass production was measured as dry weight (mg). After filtering, the substrate and the retained cell mass was dried at 70 °C in an oven until constant weight and then measured.

Manuscript received Jan 20, 2015
S.Karunakaran, Department of Biotechnology, Vivekanandha College of Engineering for Women, Namakkal Dt. Tamilnadu, India
S.Dhanasekaran, Department of Biotechnology, Vivekanandha College of Engineering for Women, Namakkal Dt. Tamilnadu, India
R.Roja Blessy, Department of Biotechnology, Vivekanandha College of Engineering for Women, Namakkal Dt. Tamilnadu, India
A.Ravulathu Nisha, Department of Biotechnology, Vivekanandha College of Engineering for Women, Namakkal Dt. Tamilnadu, India
2.4 Production and extraction of cellulase
After stipulated fermentation time period, cellulase was extracted from the fermented biomass by adding distilled water as extraction solvent in 1:10 (w/v) ratio and the flasks were shaken at 120 rpm for 30 minutes. The contents were filtered through muslin cloth and washed thrice with distilled water. The filtrates were centrifuged at 10,000 × g (4°C) for 10 minutes and carefully collected supernatants were used for enzyme activity determinations and also used for purification purposes.

2.5 Screening of cellulase activity using Congo red clearing zone assay
Grow isolated microbes, which is needed to be screened for cellulase production in CMC agar, (carboxy methyl cellulose it is soluble form of cellulose) NaNO₃, K₂HPO₄, KCl, MgSO₄, yeast extract, glucose. The medium should be solidified using 1.7 % w/v agar. Grow culture 2-3 days at 25-30°C and the screening can be done using congo red clearing zone assay. Congo red clearing zone assay is suitable for qualitative display of cellulase activity. After incubation plates which contain the circular batches of isolated microorganisms are flooded with 0.1% congo red solution and left for 15 min with intermittent shaking. Then destained with 1M NaCl solution and washed with HCl solution. The clearing zone of enzymatic activity will be visible around the batch of growth.

2.6 Protein estimation by Lowry’s method
Different adequate concentration of standard protein solution is taken. The volume in all test tubes is made up to 3ml by using distilled water. Then Folin Ciocalteau reagent is added to each test tube and a standard graph was plotted.

2.7 Enzyme assay
Cellulase activity was measured by DNS method through determination of reducing sugar liberated. A set of five test tube taken, CMC solution, crude enzyme and Citrate was added to the respective tubes to the blank and all the tubes were incubated at 50°C for 30 minutes. After incubation, DNS solution was added to all the tubes. The treated samples were boiled for 15 minutes prior to cool down in cold water for color stabilization. The optical density was read at 540 nm against reagent blank UV-Vis spectrophotometer.

2.8 Purification
The control and UV samples were centrifuged for 10 minutes at 10,000 rpm consecutively at 4°C. The supernatant was collected and enzyme assay was performed as above. Finally, samples were taken for purification by using ammonium sulphate precipitation, dialysis and Gel filtration chromatography.

2.9 Salt precipitation/ salting out
For cellulase, ammonium precipitation was carried out, 33.15 g of ammonium sulphate for 75 ml of control and 30.498 g of ammonium sulphate for 69 ml of test sample was added pinch, under ice cold conditions with continuous stirring on a magnetic stirrer for 45 minutes. After incubation, the precipitated enzymes were centrifuged at 10,000 rpm for 10 minutes. Pellet was collected and dissolved in 10 ml of 10mM Tris –HCl buffer which was later subjected to dialysis. Before the dialysis, the bag was weighed and the value was noted.

2.10 Determination of molecular weight
The protein samples and protein marker (Broad range Marker) were loaded into the wells. The tank was then connected to power supply and the gel was run at 100V until the dye reaches the bottom of the gel. The gels were detached from the glass plates and then subjected for staining. The gels are placed in staining solution for 2 hrs. This was allowed by placing in the de-staining solution for overnight. The protein bands were observed and recorded.

III. RESULTS AND DISCUSSION

3.1 Isolation of T. viride on PDA agar medium:

Figure I - Trichoderma viride
The fungi was isolated from a sample of soil taken from agro-field and grown in a Potato Dextrose Agar plate and incubated at 37°C and green colonies were observed.

3.2 Biomass production of Trichoderma viride observed in various media:

Graph I - Biomass production
Among the five medium 14.32 gram per litre of Trichoderma viride was produced from beetroot.

3.3 Conformation of cellulose activity by Congo red method:

Figure II – Congo red method
Enzyme activity for crude sample:
Table I – Enzyme activity

<table>
<thead>
<tr>
<th>S.NO</th>
<th>OD at 540nm CONTROL</th>
<th>OD at 540nm SAMPLE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>0.454</td>
<td>0.670</td>
</tr>
</tbody>
</table>

Thus the congo red method was performed and the enzyme activity of crude sample was obtained as 0.251micromole/100ml or 1U/100ml

3.4 Lowry method for protein estimation:
From the lowry’s method for protein estimation the concentration was obtained as 46mg of protein in 100ml

Graph II - Protein estimation

Cellulases are the group of hydrolytic enzymes and they are capable of degrading all types of lignocellulosic materials. Cellulases have wide range of applications. Present work focuses on the factors for improvement of enzymatic hydrolysis of various agricultural wastes by using different fungal species. It was the aim to study the Cellulase enzyme production ability of fungal strains against the lignocellulosic bio-waste like Rice husks, Millet husks, Banana peels, Wheat bran, and Coir waste and saw dust.

CONCLUSION
Agricultural waste in the form of cellulose which is the most abundant renewable biomass in the biosphere has been shown to be used in the production of valuable products by microorganism. Some of these agricultural wastes used in this work as fermentation substrate which produced a large amount of cellulase enzymes by *Trichoderma viride*

From the study it was clear that beetroot is the substrate that was yield high amount of of *Trichoderma viride* on liquid medium about 14.32gm/250 ml when compared to other substrates and it shows high enzyme activity. The purified cellulase has molecular weight of 58 kDa with an optimum activity at pH 8 and 55°C.

Our aim of this study involves the cost effective production of cellulase from *Trichoderma viride* by using various agro waste in order to make each and every farmer as an successful entrepreneurs. Then the produced cellulase can given as feed supplement for poultry animals.

REFERENCE