

Research Article



Enhanced Production of Cellulase on Different Fruit Peel Under Submerged Fermentation

M. Kannahi*, S. Elangeswari

PG and Research Department of Microbiology, Sengamala Thayaar Educational Trust Women's College, Mannargudi, Tamilnadu, India.

*Corresponding author's E-mail: kannahisri79@gmail.com

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ABSTRACT

This study was aimed to evaluate the production of Cellulase by *Aspergillus niger* and *Trichoderma viridae* were isolated and identified from soil and used for the study. *Aspergillus niger* and *Trichoderma viridae* were screened for their cellulase production ability. Cellulase production was analysed in Agro-industrial waste such as Pineapple peel, Orange peel and Banana peel. From this study, *Aspergillus niger* and *Trichoderma viridae* have high enzyme activity in Pineapple peel. The optimization study was performed on pineapple peel the optimum parameters for the isolated organism for cellulase production were studied on varying condition such as P^H, temperature and substrates concentration (Carbon and Nitrogen). The maximum production of cellulase was noticed at temperature 25°C, and PH 6, in pineapple peel for *Aspergillus niger* than *Trichoderma viridae* Finally concluded that the *Aspergillus niger* showed highest level of cellulase production which was recommended for industrial level cellulase production.

Keywords: Cellulase, Agro-industrial waste, Submerged fermentation, *Aspergillus niger*, *Trichoderma viridae*.

INTRODUCTION

Enzymes are proteins that exhibit catalytic activity. The enzyme complex molecular structure consists of one part of protein, but it can be connected to other molecules such as carbohydrates and lipids.

Enzymes are present in all living cells, which exercise the function of catalyst of the reactions that compose the anabolic and catabolic pathway of cellular metabolism.

Cellulase is an important extracellular microbial enzyme, which hydrolyzes cellulose.

It is also one of the most expensive sources of biomass utilized for the production of pressing in the fruit juice industry and other factories via enzyme bioconversion, which proves to have a high industrial value.

A great number of microorganisms, mostly fungi are able to degrade cellulose for their growth and produced a complete set of cellulase for the hydrolysis of cellulose to soluble sugar.

The agro waste such as pineapple peel, rice straw, orange peel, Banana peel etc can be used as the best substrate for bioconversion. Cellulose is commonly degraded by an enzyme called Cellulase.

This enzyme is produced by several microorganisms, commonly by bacteria and fungi.

Fungal genera like *Trichoderma sp* and *Aspergillus sp* and are taught to the cellulase producers and crude enzymes produced by these microorganisms are commercially available for agricultural use.

Cellulases are enzymes that are capable of breaking cellulose glucosidic bonds, resulting in the release of oligosaccharides, cellobiose and glucose.

These hydrolytic enzymes are used in the food, pharmaceuticals, cosmetics, detergents and textile industries.

Their main applications include the bleaching of pulp in the paper industry, the production of dissolved pulp waste water treatment and recycling of paper residues.

Enzyme technology is currently one of the most promising new technologies for the synthesis of valuable compounds.

Industrial processes that employ enzymes in biotransformation processes have a lower environmental impact and concomitantly lower consumption of energy, since they are biodegradable and highly specific which induce undesirable effects.

Cellulase production depends on the type of substrate, pretreatment and strain microorganisms used. Cellulase, it is a multi enzyme complex capable of decomposing plant substrate.

One component of the cellulase enzymes system is β -Glucosidase and it work together with endoglucanase and to break down cellulose in the plant cell walls.

The objective of the present study was to collect the soil sample for isolation and identification of fungi using appropriate method, to screen the cellulase producing fungal strain, to estimate the cellulase productivity using DNS method and to optimize the media formulation for the production.

MATERIALS AND METHODS

Sample Collection

Soil samples were collected from cellulosic waste dumped area at Ullikkottai, Thiruvarur District, Tamil Nadu. It was



thoroughly mixed and brought to the laboratory for further processing.

Isolation and Identification of Fungi^{1,2}

After sample collection the fungal organisms were isolated and identified using appropriate method.

Screening of Cellulase Producing Fungi³

Screening of the isolated organisms for cellulase production was carried out according to the above method. A point of inoculation, the spores of each organism was grown on PDA supplemented with 2% (w/v) Carboxy Methyl Cellulose (CMC). The plates were incubated at 29±1°C for 48 h after which they stained with Congo red stain for 15 minutes. Excess dye was removed by washing with 1M NaCl and the plates were fixed with 1N HCl. The production of extracellular cellulase by the organisms was indicated by zone of clearance around the colony. The organism showing maximum enzymatic activity was identified and used for further studies.

Inoculum Preparation

The culture of *Aspergillus niger* and *Trichoderma viridae* were maintained as stock culture in PDA slants and the spores were harvested aseptically from 5 days old PDA slants. Sterile distilled water (2ml) was added to each fungal agar slant and shaken well which was used as inoculum.

Collection and Pretreatment of Substrate

The pineapple, orange and banana peel were collected from local market in Mannargudi, Thiruvavur district, Tamil Nadu, India. The fruit substrates were washed and peeled. The peel was drained to remove excess water and the latter were then cut into strips and were ground in a blender for 10 minutes.

Ground wet samples were then washed successively with water and sun-dried for about a week or until completely dried. Finally, it was milled into fine powder and it was stored for further processing.

Enzyme Production by Submerged Fermentation⁴

The submerged fermentation was carried out by shake flask method using production medium. The production medium contains KH₂PO₄-0.2g, (NH₄)₂ SO₄-0.14g, Urea-0.03g, MgSO₄-0.16g, ZnSO₄-0.14g, CaCl₂-0.2g, and distilled water. 100 ml of sterile production medium, 2g substrate was added to each flask separately and autoclaved at 121°C for 15 minutes at 15 lbs pressure and finally 1 ml of inoculums was added aseptically. The pH of medium was adjusted to 6.8 and temperature was maintained as 25°C. The inoculated medium was kept shaken at 220 rpm for 48 hours.

Enzyme Extraction

Aspergillus niger and *Trichoderma viridae* were grown in fermented medium (100 ml optimized media with

pineapple substrate) at 37°C for 24 hours individually. After growth, the culture filtrates were collected separately by centrifugation process.

Enzyme Assay by DNS Method⁵

Assay of cellulase done by measuring the Release Sugars (RS) by Dinitrosalicylic acid (DNS) method. It is simple sensitive and adoptable during handling of a large number of samples at a time.

The culture filtrate was collected from the fermentation media by centrifugation.

Exoglucanase and Endoglucanase Activity^{6,7}

Exoglucanase and Endoglucanase activity was assayed by the method reported by Miller, 1996. For this process, enzyme was assayed by measuring reducing sugar as glucose by Dinitrosalicylic acid method. The reaction mixture contain 1 ml crude enzyme.

0.1 ml Phosphate buffer (pH 6.0) 0.03% Sodium Azide and incubated at 35°C for 2 hrs. The reaction was stopped by adding 2 ml DNS reagent.

Then, it was boiled in water bath for 15 minutes, 1 ml of 40% Sodium Potassium Tartarate was added. The Optical Density was noted 570 nm. Enzyme activity was expressed as IU/ml.

Optimization of Cellulase Production

From this study, pineapple waste produced high level enzyme. Hence, the optimization study was performed on pineapple waste as a substrate.

The factors such as pH, Temperature, Carbon and Nitrogen sources were selected for optimization study.

Effect of pH

The optimized media were prepared by using the substrate and the pH was set at different level such as 3, 4, 5, 6, and 7 respectively by adding 1% NaOH and concentrated HCl.

Then the media were autoclaved. Later, they were inoculated with broth culture and were placed in a shaker (150 rpm) at 37°C for 2 days. Simultaneously, for both the organisms and the substrates, assay was carried out separately.

Effect of Temperature

The optimized media were prepared by using the substrate and autoclaved. Later, it was inoculated with broth culture and was set at different temperature 25, 30, 35, 40 and 45°C respectively.

The effect of temperature on the production of cellulolytic enzyme was determined by growing the organisms at the above temperatures. Simultaneously for the organisms and the substrates, assay was carried out.

The enzyme solution obtained from these two (pH and temperature) experiments was individually optimized



based on Dinitro-salicylic acid and filter paper activity methods as described earlier.

Effect of Different Carbon Sources

Different carbon sources (Sucrose, glucose, CMC, maltose and Starch) at concentration of 5% w/v were supplemented as individual components to the fermentation medium containing pineapple waste as substrate. The medium was inoculated and incubated at 30°C for 3 days in an orbital shaker at 120 rpm. The broth was centrifuged and the enzyme assay was carried out.

Effect of Different Nitrogen Source

Different nitrogen sources (peptone, yeast extract, sodium nitrate, ammonium sulphate and ammonium nitrate) at concentration of 5% w/v were supplemented as individual components to the fermentation medium containing pineapple waste as substrate. The medium was inoculated and incubated at 30°C for 3 days in an orbital shaker at 120 rpm. The broth was centrifuged and the enzyme assay was carried out.

Statistical Analysis

Statistical analysis was performed by calculating Mean \pm Standard deviation.

The formula for calculating standard deviation:

$$S.D = \frac{\sqrt{\sum(x - \bar{x})^2}}{N - 1}$$

RESULTS AND DISCUSSION

Table 1: Production of cellulase on various substrates by *Aspergillus niger* and *Trichoderma viridae* under submerged fermentation

| S. No | Substrate | Submerged fermentation (IU/ml) | |
|-------|----------------|--------------------------------|----------------------------|
| | | <i>Aspergillus niger</i> | <i>Trichoderma viridae</i> |
| 1 | Pineapple peel | 0.92 \pm 0.007 | 0.87 \pm 0.05 |
| 2 | Orange peel | 0.30 \pm 0.071 | 0.62 \pm 0.02 |
| 3 | Banana peel | 0.20 \pm 0.05 | 0.55 \pm 0.01 |

Values are represented as Mean \pm Standard deviation

In this study, fungal species were isolated from cellulosic waste dumped soil in Ullikkottai, Thiruvavur District.

Cellulase productivity were estimated in the isolated fungal strain of *A. niger* and *T. viridae* optimized the enzyme productivity using various physicochemical parameters. Bioconversion of renewable lignocellulosic biomass to ethanol as an alternative to liquid fuels has attracted an intensive attention of researches since oil crises is broke out. Cellulase provide a key opportunity for achieving tremendous benefits of biomass utilization⁸.

The isolated colonies were identified by lactophenol cotton blue staining technique. The microscopic result was compared with Standard fungal identification manual. Finally, the isolated fungal species were

confirmed as *Aspergillus niger* and *Trichoderma viridae*. These isolates were used for cellulase enzyme production by submerged fermentation using pineapple peel, orange and banana peel as a substrate. The production of cellulase on different substrates by *A. niger* and *T. viridae* under submerged fermentation represented in the Table 1.

Our study similar⁹ to maximum yield of cellulase were obtained in 1% substrate (cellulose, lactose, cellobiose and glucose) using *T. viridae*. The increase in glucose production until the optimum that was obtained was due to availability of cellulose in the medium while a decrease in production beyond optimum concentration is explained to be as a result of an inhibitory effect of accumulated cellobiose and cellodextrins of low degree of polymerization to the growth medium. It might also be due to the specific binding of the enzymes with the substrates.

Optimization of Cellulase Production

P^H Vs Cellulase Production

The cellulase production was optimized by supplementation using different pH range medium from 3 to 7.

In submerged fermentation, maximum cellulase production by *A. niger* was noticed at pH 6 on pineapple peel (0.84 \pm 0.039 IU/ml) and lowest cellulase production by *T. viridae* was noticed at pH 3 (0.029 \pm 0.002 IU/ml) (Table-2).

pH monitoring is an important variable in the optimal conditions of enzymatic activity, because the fungus has a limited ability to grow under extreme conditions of acidity and alkalinity. In this study, the pH of the medium remained stable.

The temperature is also a very important variable, because it interferes with the optimal conditions for activity⁸. In this study the initial temperature was 28°C.

Temperature Vs Cellulase Production

The production was optimized for pineapple waste using different temperature range of medium from 25°C to 45°C. In submerged fermentation maximum cellulase production was noticed in *T. viridae* was noticed at 35°C (0.52 \pm 0.05 IU/ml) and *A. niger* at 25°C (0.18 \pm 0.04 IU/ml) (Table 2).

Carbon Source Vs Cellulase Production

The cellulase production was optimized using different supplementation medium such as Sucrose, Glucose, Carboxyl Methyl Cellulose, Maltose and Starch.

In submerged fermentation maximum cellulase production was noticed *T. viridae* and *A. niger* for CMC (0.8 \pm 0.05 IU/ml) under submerged fermentation, lowest cellulase production in pineapple peel for CMC (0.9 \pm 0.04 IU/ml) (Table 3).



Nitrogen Source Vs Cellulase Production

The cellulase productivity was optimized using different nitrogen supplementation medium such as Peptone, Yeast extract, Sodium nitrate, Ammonium sulphate and Ammonium nitrate.

In submerged fermentation, maximum cellulase production of *T. viridae* was noticed in pineapple waste and nitrogen source of Ammonium sulphate (0.19 ± 0.05 IU/ml) and *A. niger* using Ammonium nitrate as a nitrogen

source (0.13 ± 0.04 IU/ml) (Table 3).

Thus the present finding is probably the usage of pineapple peel as substrate for cellulase production under submerged fermentation condition, it also confirms the possibility of using cheaper substrate for the large scale production of cellulase enzymes.

The maximum enzyme production was noticed in *T. viridae* at pH (6), temperature (25°C), carbon sources (0.9 ± 0.04 IU/ml) and nitrogen sources (0.19 ± 0.05 IU/ml).

Table 2: Effect of pH on Cellulase production by *Aspergillus niger* and *Trichoderma viridae*

| Substrate | pH | Submerged fermentation (IU/ml) | | Temperature ($^{\circ}\text{C}$) | Submerged fermentation (IU/ml) | |
|----------------|----|--------------------------------|----------------------------|------------------------------------|--------------------------------|----------------------------|
| | | <i>Aspergillus niger</i> | <i>Trichoderma viridae</i> | | <i>Aspergillus niger</i> | <i>Trichoderma viridae</i> |
| Pineapple peel | 3 | 0.06 ± 0.042 | 0.029 ± 0.002 | 25 | 0.18 ± 0.04 | 0.043 ± 0.02 |
| | 4 | 0.76 ± 0.015 | 0.027 ± 0.001 | 30 | 0.12 ± 0.01 | 0.038 ± 0.02 |
| | 5 | 0.79 ± 0.032 | 0.02 ± 0.005 | 35 | 0.09 ± 0.01 | 0.52 ± 0.05 |
| | 6 | 0.84 ± 0.039 | 0.019 ± 0.004 | 40 | 0.06 ± 0.01 | 0.20 ± 0.02 |
| | 7 | 0.82 ± 0.046 | 0.012 ± 0.007 | 45 | 0.03 ± 0.01 | 0.17 ± 0.02 |

Values are represented as Mean \pm Standard deviation

Table 3: Effect of Carbon source and Nitrogen source on Cellulase production by *Aspergillus niger* and *Trichoderma viridae*

| Substrate | Carbon sources | Submerged fermentation (IU/ml) | | Nitrogen sources | Submerged fermentation (IU/ml) | |
|----------------|----------------|--------------------------------|----------------------------|-------------------|--------------------------------|----------------------------|
| | | <i>Aspergillus niger</i> | <i>Trichoderma viridae</i> | | <i>Aspergillus niger</i> | <i>Trichoderma viridae</i> |
| Pineapple peel | Sucrose | 0.5 ± 0.02 | 0.06 ± 0.03 | Peptone | 0.017 ± 0.002 | 0.02 ± 0.03 |
| | Glucose | 0.3 ± 0.03 | 0.01 ± 0.05 | Yeast extract | 0.015 ± 0.003 | 0.06 ± 0.03 |
| | CMC | 0.8 ± 0.05 | 0.9 ± 0.04 | Sodium nitrate | 0.011 ± 0.002 | 0.08 ± 0.03 |
| | Maltose | 0.7 ± 0.08 | 0.5 ± 0.01 | Ammonium sulphate | 0.019 ± 0.002 | 0.10 ± 0.04 |
| | Starch | 0.6 ± 0.01 | 0.03 ± 0.08 | Ammonium nitrate | 0.09 ± 0.05 | 0.13 ± 0.03 |

Values are represented as Mean \pm Standard deviation

CONCLUSION

This study revealed that pineapple peel and pulp, which are examples of domestic and industrial agro-wastes, produce large amounts of cellulase enzymes when hydrolyzed by cellulolytic microorganisms and instead of being left behind for natural degradation can be utilized effectively under these conditions, to produce cellulase.

For majority of commercial enzymes including cellulase complex (CMCase and cellulobiose) used by the industries, India is dependent on imports.

There is an opportunity to exploit fruit waste as a substrate for the microbial production of cellulase complex using *Trichoderma* and *Aspergillus*.

The result of the present study clearly indicates the potential of *T. viridae* can be successfully cultivated under submerged fermentation condition for the production of cellulase using fruit wastes as substrate.

These wastes abundantly available in India as its one of the major producers in world wide.

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