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OPTIMIZATION OF CULTURAL PARAMETERS FOR CELLULASE ENZYME PRODUCTION FROM FUNGI

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ABSTRACT

The objective of this study is to reduce the production cost of cellulase by optimizing the production medium and using an alternative carbon source such as agricultural solid waste residue. Present studies describe the optimization of process parameters for the production of cellulases by different fungal species. The fermentation experiments were carried out in shake flasks. Maximum production of cellulases (0.63 U/ml) was observed after a fermentation period of 72 hrs at an temperature of 40° C. Initial pH of the culture medium was also optimized and a pH of 5 was found to support maximum growth and enzyme production (0.9 U/ml) by *A. niger*. Different inorganic nitrogen sources and carbon sources were evaluated for the production of cellulases and ammonium sulphate in case of nitrogen and glucose among carbon sources was found to be the best. Agrobased coconut cake waste gave the best production of cellulases. Cellulase production from *A. niger* and *Penicillium sp* can be an advantage as the enzyme production rate is normally higher as compared to other fungi.

Keywords: Cellulase, Agricultural waste, Fungi, optimization, *Aspergillus niger*, *Penicillium chrysogenum*

INTRODUCTION

Cellulase is the major enzyme used in the sachharification of many natural substrates for production of biofuels. It is widely used for beneficial adulterations of pulp and paper characteristics [1]. Cellulase (E.C 3.2.1.4) refers to a class of enzyme that catalyze the hydrolysis of 1, 4 β-D glycosidic linkages in cellulose are mainly produced by fungi, bacteria and protozoans [2]. Although a large number of microorganisms are capable of degrading cellulose, only a few of these produce significant quantities of cell free enzyme capable of completely hydrolyzing crystalline cellulose [3]. For the production of industrially important enzymes and bioactive secondary metabolites, fungi isolated from soil are known to be potential candidates. These enzymes are mostly ligno-cellulolytic in nature to attain their energy sources[4].

Major constrains in enzymatic hydrolysis of cellulosic materials for the production of fermentation sugar are low productivity and the cost of cellulases [5]. The most abundant renewable carbon source is the cellulosic material. An agricultural waste is a cheap source of cellulose for the production of different useful products all over the world [6]. Cellulase production from agrowastes is economical as compared to production from pure cellulose [7].The hydrolysis of cellulose can be done by

using enzymes to produce glucose, which can be used for the production of ethanol, organic acids and other chemicals [8]. Other applications include cotton processing, paper recycling and as animal feed additives [9]. It is also used for deinking of fiber surfaces in paper industries and to enhance pulp drainage in textile industries [10].

Production of cellulases by the fungal isolates requires optimal conditions for their growth which leads to the release of extracellular enzymes. The growth conditions as well as extracellular enzyme production conditions is likely to vary among isolates. The major components of production medium like carbon and nitrogen sources and physical parameters like temperature, pH and incubation time were found to be critically affecting the cellulase production hence need to be optimized for every isolate [11, 12].

Therefore, the present study aims to investigate high level production of extracellular cellulases through different filamentous fungi and optimizing cultural parameters to enhance cellulase enzyme production.

MATERIALS AND METHODS

Collection of Sample

Soil samples were collected from the Dr. B. Lal Institute of Biotechnology campus by the means of sterilized spatulas and collected in sterile polythene bags. The samples were then brought to the laboratory for microbiological study.

Isolation of Fungi

Soil samples were collected for the isolation of fungi. One gram was transferred to aliquots of 9 mL sterile distilled water in test tube. It was shaken vigorously at constant speed for 15 min. The soil suspension was then subjected to serial dilutions from the appropriate plate in duplicate. The plates were incubated for 5 days at 28°C. The well-grown spread single colonies were picked up and further subcultured on potato dextrose agar slants.

Screening for cellulase enzyme production

Soil associated fungi were tested for their ability to produce cellulase enzyme by the plate assay method using 1% carboxymethyl cellulose in a basal salt media. According to [13] at the incubation period, 0.1% congo red solution was added and counterstained with 1 M NaCl for 15–20 min. The zone of cellulose hydrolysis was appeared as a clear area around the colony.

Production of cellulase enzyme

Strains presenting large clearing zones in congo red test were used for enzyme production on basal salt medium containing 1% cellulose as a sole carbon source [14]. Stationary state technique was used and 150 mL Erlenmeyer flask filled with 50 mL of the medium. Each flask was inoculated with the pre-inoculum of 4-day-old culture fungus actively growing on PDA plates. The flasks were then incubated at 30°C in stationary state.

Cellulase enzyme assay

Filter paper activity (FPase) was performed to determine total cellulase activity in the culture filtrate according to the standard method [13]. Aliquots of appropriately diluted cultured filtrate as enzyme source were added to whatman no. 1 filter paper strip (1 × 6 cm; 50 mg) immersed in one milliliter of 0.05 M sodium citrate buffer of pH 5.0. After incubation at 50 °C for 1 hr, the reducing sugar released was estimated by dinitrosalicylic acid (DNS) method [15]. One unit of filter paper (FPU) activity was defined as the amount of enzyme releasing 1 µmole of reducing sugar from filter paper per ml per min. Cellulase activity (CMCase) was measured using a reaction mixture containing 1 mL of 1% carboxymethyl cellulose (CMC) in 0.5 M citrate acetate buffer (pH 5.0) and aliquots of suitably diluted filtrate. The reaction mixture was incubated at 50°C for 1h, and the reducing sugar produced was determined by DNS method. One unit (IU) of Cellulase activity was defined as the amount of enzyme releasing 1 µmole of reducing sugar per min.

Optimization of culture conditions for cellulase enzyme production

Effect of pH on cellulase enzyme production:
To determine optimal pH, fungus cultures were cultivated in a 150 mL flask containing 50 mL optimized medium with different pH ranges from 5.0 to 11.0. The pH of the medium was adjusted by using 1 N HCl or 1 N NaOH. The flasks were kept in stationary stage at 28°C for 5 days of cultivation.

Effect of temperature on cellulase enzyme production:
In order to determine the effective temperature for cellulase production by the fungal species, fermentation was carried out at 10°C intervals in the range of 25, 30, 40, 50 and 60 ± 2°C.

Effect of carbon sources on cellulase enzyme production:
Effects of various carbon compounds namely, fructose, glucose, sucrose, lactose and maltose were used for studying. The broth was distributed into different flasks and 1.0% of each carbon sources were then added before inoculation of the strain and after culture inoculation, the flasks were incubated for 5 days at 28°C.

Effect of nitrogen sources on cellulase enzyme production:
The fermentation medium was supplemented with organic and inorganic compounds (ammonium sulphate, urea, yeast extract and peptone) replacing the prescribed nitrogen source of the fermentation medium.

Effect of agricultural waste on cellulase enzyme production:
In the present study, we aim to determine the appropriate concentration of municipal solid waste residue for cellulase production by the fungal sp. The fermentation medium was supplemented with agricultural waste residue such as Groundnut Cake, Coconut Cake, wheatbran and Soya cake, replacing the prescribed carbon source of the fermentation medium.

Statistical Analysis

Data presented on the average of three replicates (±SE) obtained from their independent experiments.

RESULTS AND DISCUSSION

Screening of fungi for cellulase enzyme activity

Screening of fungi for their cellulase activity was carried out by the hydrolysis of substrate incorporating in the basal salt medium. After an

Fig 1: Effect of pH on Enzyme Production

incubation period, enzyme activities were detected by the appearance of zones either by substrate clearances or coloration and discoloration around the fungal colonies. Four fungal isolates i.e. *Aspergillus sp, Penicillium sp, Fusarium sp and Microsporium sp* showed the highest zone around the colony, were used for further study. All the fungal isolates exhibited cellulase activity; fungi of the genera *Aspergillus and Penicillium* have been reported as good cellulase producers [17, 18].

Optimization of culture conditions for cellulase enzyme production

Effect of pH on cellulase enzyme production: Among physical parameters, pH of the growth medium plays an important role by inducing morphological changes in microbes and in enzyme secretion. The pH change observed during the growth of microbes also affects product stabilty in the medium. The optimal pH varies with different microorganisms and enzymes. All the four isolates were allowed to grow in media of different pH ranging from 5.0 to 11.0. Maximum enzyme activity was observed in medium of pH 5.0 in case of *Penicillium chrysogenum* (0.95 U/ml) followed by *Aspergillus niger* (0.85 U/ml) (Fig 1). Beldman *et al*. [19] also reported that *Aspergillus* species grow and metabolize well in acidic pH medium betwen pH 3.0 –5.0. Their study investigated that maximum cellulase production from *A. oryzae* was reported when the pH of the medium was

Fig 2: Effect of Temperature on Enzyme Production

Fig 3: Effect of Carbon sources on Enzyme Production

Fig 4: Effect of Nitrogen sources on Enzyme Production

Fig 5: Effect of Agricultural waste on Enzyme production

adjusted to 5.0. The activity reduces with increase in pH from 5 to 11. Similar observation was reported for cellulase production by *A. tereus* QTC 828 by Ali *et al*. [20] and *Trichoderma resei* by Dopelbauer *et al*. [21].

Effect of temperature on cellulase enzyme production:

Incubation temperature plays an important role in the metabolic activities of a microorganism. Even slight changes in temperature can affect enzymes production. Presently, the optimal temperature for maximum cellulase production was at 28ºC with production decreasing at higher temperature. Since enzyme is a secondary metabolite produced during exponential growth phase, the incubation at high temperature could lead to poor growth and thus a reduction in enzyme yield [22]. The effect of temperature on cellulase activity was determined by incubating the flask at a range of 25°C, 30°C, 40°C, 50°C and 60°C. The results of the test made at different temperatures value showed that the optimal temperature for cellulase activity (0.63 U/mL) produced by *A. niger* at 40°C (Fig 2). Many researchers have reported different temperatures for maximum cellulase production either in flask or in fermentor studies using *Aspergillus* sp. and T*richoderma* sp. suggesting that the optimal temperature for cellulase production also depends on the strain variation of the microorganism [23, 24]. Our results also agreed with Immaneul *et al*., (2007) [25] who reported the optimum temperature for cellulase enzyme production by *A. niger & A. fumigatus* at 40^0C.

Effect of carbon sources on cellulase enzyme production:

Various sources of carbon such as Starch, fructose, maltose and sucrose were used to replace glucose which was the original carbon source in growth media. Results obtained showed that *Aspergillus niger* in presence of glucose, fructose and maltose brought about the maximum cellulase production compared to other carbon sources (Fig 3). Vinod Kumar Nathan *et al* (2014)[1] observed the similar results with glucose as carbon source.

Effect *of nitrogen sources on cellulase enzyme production*:

Results indicate that the sources of nitrogen greatly affected the production of cellulase enzyme. Ammonium sulphate (AS) was the best nitrogen source for *Aspergillus niger* (Fig 4). It was reported that good cellulose yield can be obtained with ammonium compound as the nitrogen source. Our results are in accordance with the work of Enari *et al*. who reported that good cellulase production can be obtained with the organic nitrogen sources such as yeast extract and peptone [26]. But enzyme production was remarkably decreased in presence of urea (U) a report contrary to that of by *Aspergillus niger* [27]. Many papers have reported that ammonium compounds are the most favorable nitrogen sources for protein and cellulase synthesis.

Effect of agricultural waste on cellulase enzyme production:

The bioconversion of agro waste based lignocellulosic material to energy has gained much interest during the recent past. Low cost of enzyme production improves the economics, as the cost of enzymes constitutes a major part of the total cost of hydrolysis [28]. The enzymatic degradation of waste cellulose by fungal enzymes has been suggested as a feasible alternative for the conversion of lingo cellulosic material in to fermentable sugars and ethanol [29, 30]. The cellulolytic enzyme complex when incubated with agro waste released sugars. The degree of saccharification was assayed on the basis of release of reducing group. The amount of reducing sugar increased with time of incubation in the presence of enzyme. The maximum amount of percent saccharifaction was found to be 0.60 U/ml (*P.chrysogenum*) and 0.52 U/ml (*A. niger*) for coconut cake (Fig 5). Enzymatic conversion of cellulose to food, fuel and chemical feedstock is a well-established process. However, high cost of cellulases production has hindered use of this enzyme in industry. The enzymatic conversion of the carbohydrate part of lignocellulosic material has received considerable interest during recent years. This source of raw material is available in abundance and generally free of cost.

CONCLUSIONS

The cost-effective technologies are needed for economical production of cellulases using Agobased waste residues as substrate. Major parameters affecting the fermentation process for enzyme production were studied and optimal levels were identified. 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, pH value, temperature, presence of inducers, medium additives, aeration, growth time, and so forth.

The need for utilizing renewable resources to meet the future demand for fuel has increased the attention on cellulose, the most abundant and renewable resource in the world. Presently our studies investigated the superiority of *Aspergillus niger* over the other tested fungal cultures for production of extracellular cellulases. It is concluded from the findings that the strategy to produce cellulose from coconut cake waste was successful as it resulted in a considerably good amount this enzyme produced under laboratory conditions. Furthermore, evolutionary operation factorial-design technique could be considerably effective in maximizing the yield of enzyme but all the parameter was optimized by one at a time method. The high activity and stability of cellulase enzymes will be of use in various industrial and biotechnological applications.

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