Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

Original Research

Influence of medium composition and physical factors on enhanced production of endoglucanase by locally isolated fungal strain in solid state fermentation

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Received November 3, 2017; **Accepted** April 15, 2018; **Published** April 30, 2018

Doi: http://dx.doi.org/10.14715/cmb/2018.64.5.9

Abstract: Endoglucanase is one of the most important enzymes of the cellulase group. Endoglucanase are involved in the catalytic hydrolysis of cellulose and plays a pivotal role in different sectors like pharmaceutical, textile, detergent, and food processing as well as paper and pulp industry. With consumers getting more and more aware of environmental issues, industries find enzymes as a better option over other chemical catalysts. In the current research different thermophilic fungal strains were isolated from the different sources. Qualitative screening was carried out on the basis of cellulose hydrolysis zone. The quantitative screening was carried out employing solid state fermentation. The fungal culture, showing highest EG potential was selected identified and assigned the code *Aspergillus fumigatus* BBT2. Different fermentation media were evaluated and M 2 containing wheat bran gave maximum EG production. The maximal enzyme productivity was recorded in 72 hours, 40°C, pH 5, inoculum size 1.5ml, and moisture content (1:1). Glucose (1%) and peptone (1%) were optimized as best carbon and nitrogen sources, respectively.

Key words: Thermophilic fungi; *Aspergillus*; Endoglucanase; Solid state fermentation.

Introduction

Endoglucanase (EC 3.2.1.4) is one of the most important enzymes of the cellulase group. Cellulases are the group of enzymes, including many enzymes such as endoglucanase, exoglucanase and β-glucosidase etc. These three enzymes are involved in the catalytic hydrolysis of cellulose (1). Cellulases act on the cellulose, which is a major polysaccharide and the main constituent of plant cell wall (2). The structurally endoglucanase (EG) consists of the catalytic domain (CD) and cellulose binding module (CBM). Catalytic domain helps in the catalysis process while, binding module aids the enzyme in the binding process. The catalytic domain (CD) has a short poly linker region to its N-terminal that joins cellulose binding module (CBM) with it, CBM contains short chain of 35 amino acids, excess amount of serine and threonine are present in its linker(3). Endoglucanase acts at a random fashion and breaks the inner O-glycosidic bonds of its substrate by acting on the non-crystalline sites and converting them into glucan chains of varying lengths e.g. glucose, oligosaccharides or polysaccharides. Endoglucanase has enzymatic activity for the conversion of the complex structure of cellulose to the simple sugar (4). In the hydrolysis reaction, the EG initiate the enzymatic process and break down the β, 1-4 glucosidic linkages of the cellulose polymer by cleaving it at internal amorphous site

Endoglucanase can be produced by fungi as well as bacteria. Fungi are preferable over bacteria for biosynthesis of EG, because it has cellulosomic system, elongated hyphae for penetration into the substrate and stability in the harsh environments. In addition to this the endoglucanases obtained from fungi has ability to act both on the amorphous as well as crystalline structure of cellulose as compared to bacteria (5).

EG have a wide spectrum of applications in different industrial sectors such as the pharmaceutical, textile, detergent, and food processing as well as paper and pulp industry. EG is also being used for refining of biomass, extraction of fruit juices, manufacturing animal feed, waste water treatment, pollution control, and green chemistry. In fact, EG is the third largest industrial enzyme by dollar volume and accounts for approximately 20% of the total enzyme market in the world (6).

Generally, there are two types of fermentation techniques used for EG biosynthesis. EG was traditionally produced using the submerged fermentation. However, Recently SSF has got the recognition because it is an environment friendly process and various agricultural by products and industrial residue can be utilized as substrates (7). SSF requires lower energy requirements, produce less wastewater and resolve the problem of solid waste disposal (8). In addition to this it reduces the production cost of enzyme by improving the titre of enzyme production. Enzymes produced by SSF are less susceptible to substrate inhibition problems and also have greater stability to changes in temperature and pH (9).

Optimization of SSF conditions may further improve the overall production economics and moreover can make it an attractive technique for enzyme produc-

tion. SSF aids the fungi in achieving all the nutritional requirements by the solid substrate and making the contact with the insoluble substrate to enhance the enzyme production (10).

Materials and Methods

Isolation of thermophilic fungi

Isolation of different thermophilic fungal strains was carried out from different sources like animal dung, Fruits, barks and soil. The serial dilution method was used for isolation of fungi from each sample (11). Primary screening was carried out using the Bergs medium plates on the basis of cellulose hydrolysis zone (12). The colonies showing large clear zones of cellulose hydrolysis were chosen and subjected to secondary screening through solid state fermentation.

Pretreatment of substrates

Pretreatment of different substrates for the solid state fermentation like sugar cane baggase, tree bark and kanna etc was carried out according to Acharya *et al.*(1)

Inoculum preparation

For the preparation of inoculum10ml of saline autoclaved water was added in the 3-5 days old slant having abundant fungal growth**.** Mix the fungal spore/conidia with the help of inoculating water and vortex the test tube for making uniform suspension.

Fermentation process

1ml of conidial / spore inoculum was transferred in 10g sterilized pretreated substrate moistened with 10ml of sterilized mineral salt solution. All the flasks were kept in incubator at 40^{o}C for 72h After a fixed time interval add 100ml of phosphate buffer (pH7) and placed all the flask in shaking incubator at 160 rpm for I hour . The fermented broth was filtered using Whatman filter paper1. The filtrate was used for the estimation of endoglucanase (CMCase and Fpase).

Fermentation media

Six different fermentation media with different chemical composition and substrates were used.

M1: 10g of wheat straw moistened with 10 ml of 0.9 % (w/v) ammonium sulphate (13); **M2:** Wheat bran 10 g **;** 10ml trace elements solution containing (g/l) ammonium sulphate 10; potassium dihydrogen phosphate 3g; magnesium sulphate 0.5; calcium chloride 0.5(14); **M3:** Pretreated sugarcane baggase 10g; 10ml of mineral salt solution contained(g/l) ammonium sulphate 1.4g; potassium dihydrogen phosphate 2; calcium chloride 0.3; magnesium sulphate 0.2; peptone 50; yeast extract20; urea 3; Tween-80 10; iron sulphate 50; zinc sulphate. (15); **M4:** Pretreated Kana grass, 10g; 10 ml of trace metal solution comprises (g/l) magnesium sulphate 0.1; ammonium sulphate 1; potassium dihydrogen phosphate 2; dipotassium hydrogen phosphate 7; sodium citrate 0.5(1); **M5:** Pretreated bark 10 g; 10ml of Media containing (g/l) potassium dihydrogen phosphate 2; magnesium sulphate 0.5; urea 3.8(16); **M6:** 10g of rice bran, 10ml of Mineral salt solution containing (g/l) ammonium sulphate 5; potassium dihydrogen phosphate 0.25; calcium chloride 0.13; magnesium sulphate 0.63; yeast 4; urea 4(17); **M7 :** Pretreated sugarcane baggase 10g ; 10ml of trace metal solution containing (g/l) ammonium sulphate 10; potassium dihydrogen phosphate 3; magnesium sulphate 0.5; calcium chloride 0.5(1).

Estimation of CMCase

Estimation of CMcase was performed according to Gao *et al*.(19). 0.5 ml of crude enzyme was added in 0.5 ml of 1% CMC prepared 0.1 M citrate buffer (pH5). Blank was also run parallel. The enzyme substrate mixture was incubated at 60 ^{o}C for 30 min. The reducing sugar was measured at 546nm according to Miller (19).

"One unit of CMCase activity was defined as the amount of enzyme required to liberate 1 µmol of glucose from the appropriate substrate per ml per min under standard assay conditions."(20) .

Estimation of FPase

Estimation of FPase was performed according to Gao *et al*.[18]. 0.5 ml of filtrate was added in 0.5ml of 0.1M citrate buffer (pH 5.0) along with the addition of 50 mg of 1×6 cm strips of Whatman filter paper No. 1. Incubate this complex for 30 min at 60^{o}C. The reducing sugar was determined at 546nm according to Miller (19)

One unit of FPase activity is defined as the amount of enzyme required to liberates 1 µmol of glucose from the appropriate substrate per ml per min under standard assay conditions(20)".

Estimation of total protein

Total proteins were measured according to Bradford (21).

Statistical analysis

All the data were subjected to statistical analysis for determination of significance by using ANOVA in SPSS 16.0 software.

Results

Isolation and screening of fungal strains

Twenty five different fungal strains were isolated and then screened out on the basis of the zone of cellulose hydrolysis. All these strains were tested to find their ability for endoglucanase production. Among all the strains, strain No.2 gave the best CMCase (4.64 U/ml/min) and FPase (4.24 U/ml/min) production (data not shown.) The selected strain was identified as *Aspergillus fumigatus* according to McClenny (22) and designated as *Apergillus fumigatus* BBT-2.

Screening of fermentation media for endoglucanase production

Figure 1a illustrates the screening of seven different fermentation media for endoglucanase production by *Aspergillus fumigatus*. The maximum production of endoglucanase (CMCase; 6.97 U/ml/min and FPase; 6.82 U/ml/min) was recorded by using M2 medium. Total protein concentration was 0.46 mg/ml in the M2 medium. All other media gave less enzyme productivity compared with M2 medium.

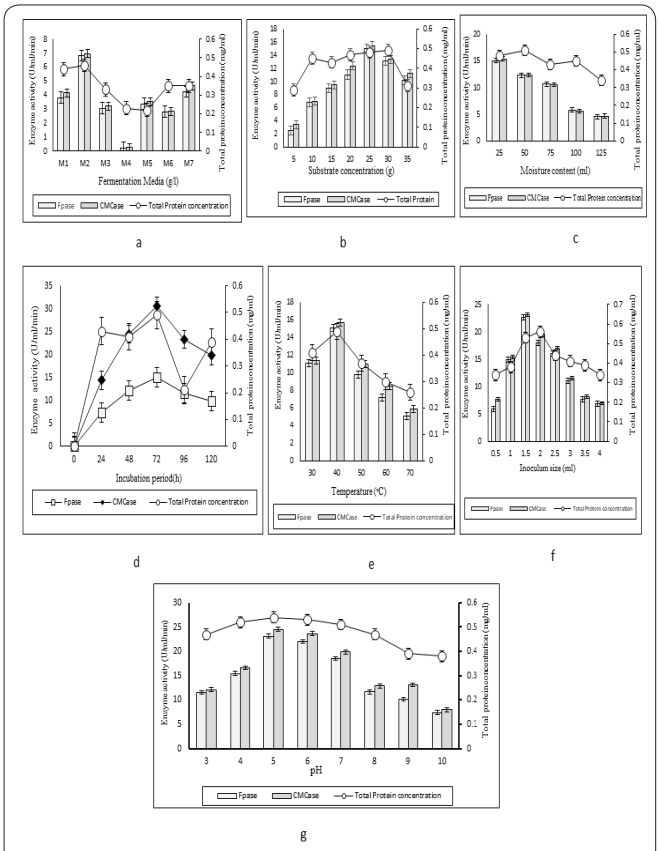

Figure1. Influence of different Physical parameters on the production of endoglucanase production (a) Fermentation media (b) substrate concentration (c) Moisture content (d) Incubation period (e) Incubation temperature (f) inoculum size (g) pH.

Influence of wheat bran concentration on endoglucanase production

The impact of wheat bran concentration on endoglucanase production was evaluated (Fig 1b). The substrate concentration was tested in the range of 5 - 35g. The maximal CMCase (15.41 U/ml/min) and FPase (15.00 U/ml/min) productivity was noted in 25g of wheat bran

Influence of moisture content on endoglucanase production

The influence of moisture content from the range of 25 - 125ml was noted (Fig1 c). The highest production of both CMCase (15.40 U/ml/min) and FPase (15.01 U/ml/min) was obtained at 25ml of moisture content. The lowest enzyme production was recorded at 125ml.

Impact of incubation period on endoglucanase production

The effect of incubation period on endoglucanase production was recorded. The best enzyme production of both CMCase (15.42 U/ml/min) and FPase (15.10 U/ml/min) was observed at 72 hours of incubation time (Fig 1d). Below or above this optimum time period, a sequential decrease in the values of enzyme activities was noted.

Impact of incubation temperature on endoglucanase production

The effect of varying incubation temperature (30-70^{o} C) on the productivity of endoglucanase was evaluated (Fig 1e). The maximum enzyme productivity of both CMCase and FPase was noted at 40 ^{o} C.

Effect of inoculum size on endoglucanase production

By varying the inoculum size, from 0.5ml - 4ml, change in enzyme production was noted (Fig 1f). Maximum enzyme productivity of CMCase and FPase was found at 1.5ml of inoculum size. Further increase or decrease in the size of inoculum resulted in reduction of CMCase and FPase production.

Impact of pH on endoglucanase production

The variation in enzymatic productivity of both CMCase and FPase by a sequential change in pH from 3 – 10 was noted. Maximum endoglucanase productivity was obtained at acidic condition of pH5 (Fig 1g). Any variation in an optimal pH drastically affects the enzyme production.

Impact of varying carbon sources on endoglucanase production

The variation in enzyme activity of CMCase and FPase was evaluated by using different carbon sources at the concentration of 1% (Fig2a). As compared to other carbon sources, glucose gave the highest production of CMCase (29.97 U/ml/min) and FPase (28.23 U/ml/min).

Impact of varying concentration of glucose on endoglucanase production

The impact of different glucose concentration (0.5-3%) on endoglucanase production was tested (Fig 2b). Optimal CMCase (30U/ml/min) and FPase (28.24 U/ml/min) productivity was recorded with the addition of 1% glucose. Lowest productivity was found at 3% of glucose.

Evaluation of additional nitrogen sources for endoglucanase production

The addition of different nitrogen sources at the concentration of 1% resulted in variation in enzyme production. The optimal CMCase and FPase production were obtained by using peptone as a nitrogen source

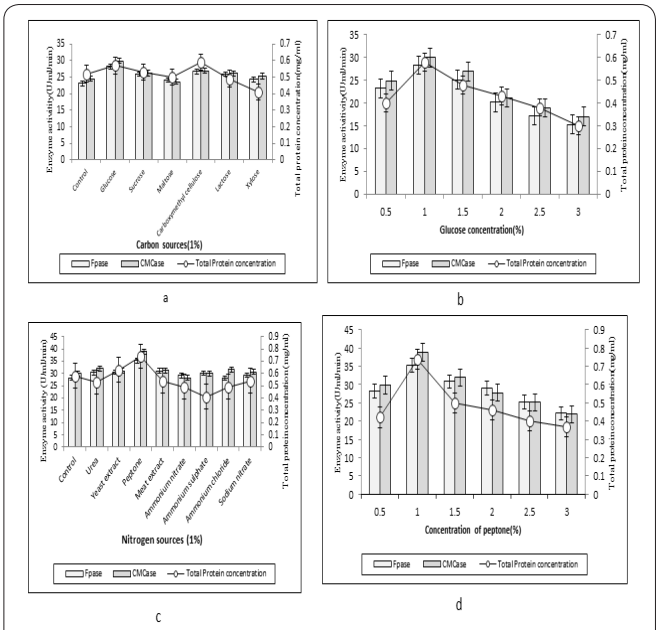

Figure 2. Influence of different nutritional factors on the production of endoglucanase production (a) Carbon sources (b) glucose concentration (c) Nitrogen sources (d) concentration of peptone.

(Fig 2c). However, other nitrogen sources such as yeast extract, ammonium chloride and urea gave less enzyme production as compared to peptone.

Influence of peptone concentration on endoglucanase production

A change in CMCase and FPase activity was recorded by increasing the percentage of peptone from 0.5% to 3%. Highest enzyme activity values of CMCase (38.83 U/ml/min) and FPase (35.22 U/ml/min) were noted at 1% of peptone (Fig 2d).

Discussion

The choice of appropriate fungal strain is a fundamental step for the hyper production of endoglucanase. It includes the different steps like isolation, identification and screening. In current research different thermophilic fungal strains were isolated and screened both qualitatively and quantitatively. The strain showing greater capability of endoglucanase production was identified as *A.fumigatus* BBT2.

Fermentation media is one of the significant optimization factors in endoglucanase production. The selection of simple, economical, easily available and cost effective media is necessary for biosynthesis of endoglucanase. Seven different media were evaluated. The highest productivity was recorded in M_2 medium. Perhaps the capability of wheat bran for the maximal enzyme productivity was depends upon its chemical properties because it forms an appropriate fraction of carbohydrates, fiber, fats and proteins which are required for the growth of fungi as well as endoglucanase production. The reduction in enzyme productivity in contrast to M_2 was either due to the scarcity of certain constituents in the media that were obligatory for the growth of fungi as well as enzyme production or probably because of repressor substances which were contemporary in the constituent of medium (23). The substrate concentration has a direct impact on enzyme productivity, therefore it is important to find the optimum substrate concentration. The substrate concentration in the range of 5-35g was screened. Both CMCase and Fpase showed maximal activity in the presence of 25g wheat. The appreciably decreased in enzyme activity was noted below or above this level. The reason of low enzyme yield at smaller concentration levels may be due to insufficient supply of minerals and nutrients required for proper growth above 25g substrate concentration low yield was reported it was probably due to poor supply of oxygen (24).

Moisture contents play a key role in the growth of fungi as well as production of enzymes. In current research influence of varying moisture contents (1:1-1:5) on the productivity of enzyme was evaluated. The moisture content in 1:1 ratio was found to be optimum. Any upsurge or decline in moisture contents caused decrease in CMcase and Fpase production. Probably the Steric hindrance and interference in oxygen supply are two major reasons of low enzyme yield at a high level of moisture contents. While at the low moisture content decrease in CMcase and Fpase activity was due to a reduction in substrate solubility increased water tension and less degree of swelling (25-26). Our findings were in accordance to Bansal *et al.* (2012) who reported 1:1 ratio of water content was optimum for endoglucanase production. In the present study impact of varying time of incubation (24-120h) was investigated. The optimal endoglucanase production was noted at 72hours. Beyond this level reduction in CMcase and Fpase activity was noted. The reason of lesser activity below 72 hours may be due to insufficient time of growth. Above 72 hours of incubation, the decrease in enzyme production was probably due to the reason, that the selected strain was able to give maximum enzyme production at exponential phase and after the completion of it, enzyme activity was fallen. The presence of byproducts after 72 hours of optimum period may be the reason of low enzyme yield, as they could hinder the fungal growth (27).

Suitable temperature for fungal growth is also one of the important factor which affects enzyme production. In present investigation impact of different temperature (30^{o}C -70^{o} C) was evaluated. Optimal CMcase and Fpase activity was noted at 40^{o}C. The decline in enzyme activity above the optimal temperature was perhaps due to reduction in moisture content of medium due to evaporation of water at high temperature (28). Our results were in accordance with Hoa and Hung[29] who mentioned 40^{o}C was optimal temperature for the growth of thermophilic fungi and enzyme production.

The size of inoculum has paramount significance for the production of endoglucanase. The 0.5- 4ml of inoculum size tested for endoglucanase production. Appreciable increase in enzyme production was recorded at 1.5ml. A decline in enzyme productivity was observed above or below this concentration. Likely the reason was that overgrowth of fungi creates anaerobic conditions and exhaustion of nutrients which reduce metabolic activity of fungi and in turn enzyme production (3). Under the optimal level reduction in the enzyme activity was more likely to be the insufficient availability of fungal growth, which ultimately affects the enzyme productivity (31, 32).

Optimization of pH was carried out in the range from 3 - 10. Paramount increase in the enzyme production was resulted by at pH 5. Our results were in agreement with Maurya *et al*. (33). Below or above the optimal level fall in the production of enzymes was recorded. This is imputable to the reason that enzymes work more effectively over a small range of pH and is highly sensitive to slight alteration in pH value (34). Selection of appropriate carbon and nitrogen sources has paramount significance in the production of enzyme. In present study different carbon sources (glucose, maltose, sucrose, CMC, lactose and xylose) were screened. An appreciable rise in enzyme production was observed in the presence of glucose. Furthermore, the concentration of glucose was also tested from 0.5- 3%. Our results were in accordance with Mandels and Reese (35) wherein, glucose at 1% concentration level gave higher yield in contrast to other carbon sources. Enzyme activity was decreased below and above this level due to insufficient amount of glucose and catabolic repression (33). Different nitrogen sources (urea, yeast extract, peptone, meat extract and ammonium chloride, ammonium sulphate, ammonium nitrate and sodium nitrate) were also screened. Peptone at1% concentration gave optimal

production. Our results are in agreement to Deswal *et al.* (36) who stated 1% pepton was best for endoglucanase production, in contrast to other nitrogen sources.

The utilization of agricultural by products in fermentation medium reduces the cost and makes the process economical. The *A. fumigatus* BBT2 has promising potential of producing valuable products like EG by using the agriculture by products. The optimization of vital factors enhances the production of endoglucanase.

Interest conflict

Authors declared there is no conflict of interest associated with this work**.**

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