Optimization of Culture Conditions for the Production of Extracellular Cellulases via Solid State Fermentation

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Authors' contributions

This work was carried out in collaboration between all authors. Author THA designed the study, all authors performed the statistical analysis, wrote the protocol, author DHIE wrote the manuscript, managed the analyses of the study and the literature searches. All authors read and approved the final manuscript

Original Research Article

ABSTRACT

Aim: The aims of the present study were to screen different filamentous fungi for extracellular cellulases production and to optimize solid-state fermentation medium and culture conditions to enhance cellulases production.
Study Design: Using agro-industrial waste as raw material for the production of cellulases by a hyper cellulase producing fungus and evaluating the influence of various parameters to design a suitable SSF process for cellulase production.
Place and Duration of Study: Department of Microbial Chemistry, Genetic Engineering and Biotechnology Division, National Research Centre (NRC), Cairo, Egypt, between January 2013 and October 2013.
Methodology: Different filamentous fungi were grown and maintained on potato dextrose agar slants at 28ºC for 7 days. The spores were washed down by distilled water. Then, 2.0 ml aliquots were used to inoculate 250 ml Erlenmeyer flasks, containing rice straw as the only carbon source. The inoculated flasks were incubated for 5 days at 28ºC. The enzymes were extracted by mixing homogenously the fermented substrate with 50 ml citrate phosphate buffer (0.1 M, pH 5.0) and agitated (150 rpm) for 1 hr. Pooled extracts were centrifuged at 5000 rpm for 15min and the clear supernatant was used as a source of extracellular enzyme.
Results: *Aspergillus oryzae* NRRL 3484 exhibited relatively higher cellulases production.

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The optimum incubation period, temperature, and initial moisture level were reported on the 7th day, at 28°C, and 70%, respectively. Peptone proved to be the suitable nitrogen source followed by yeast extract, while pH 5.0 was ideal for cellulases production.

Conclusion: Using ligninolytic fungi, including their enzymes, may be one potential alternative to provide a more practical and environmental-friendly approach for enhancing the nutritive value of rice straw. Moreover, the application of ligninolytic fungi or their enzymes combined with chemical pre-treatments to rice straw may be an alternative way to shorten the period of the incubation times and (or) decrease the amount of chemicals, effecting some synergy.

1. INTRODUCTION

Biotechnology offers an interesting alternative for the manufacture of value added natural products by using microorganisms and enzymes [1]. Natural high molecular weight matter such as lignocellulosic materials undergo enzymatic hydrolyzation by extracellular enzymes to low molecular weight substances which could then be transferred to the cell through the cell membrane. This key biochemical process makes change of the composition of organic matter and biological availability [2]. There are several reports describing use of agro industrial residues for the production of cellulase such as wheat straw, wheat bran and rice straw as substrates [3-4]. The management of such residues effectively and economically must be given utmost priority in the country is ensuring not only in reducing the detrimental impact of such waste to the environment, but most importantly in the transformation of such residue into useful raw materials for production added value commodities of industrially commercial potentials [5]. Several physical and chemical treatments have been used to improve the degradability and voluntary intake of rice straw; however, using these treatments is still restricted in terms of safety concerns, high costs and potentially negative environmental consequences. Using ligninolytic fungi, including their enzymes, provide a more practical and environmental-friendly approach for enhancing the nutritive value of rice straw. Moreover, the application of ligninolytic fungi or their enzymes combined with chemical pre-treatments to rice straw shorten the period of the incubation times and (or) decrease the amount of chemicals, effecting some synergy [6-7].

Plant cell walls are the most abundant renewable source of fermentable sugars on earth [8-9] and are the major reservoir of fixed carbon in nature [10-11]. The major components of plant cell walls are cellulose, hemi cellulose and lignin, with cellulose being the most abundant component [12]. Plant biomass comprises on average 23% lignin, 40% cellulose and 33% hemi cellulose by dry weight [13]. Annually, 830 Gt of renewable plant biomass is formed consisting mainly of cellulose and hemicelluloses. Plant biomass is an alternative natural source for chemical and feed stocks with a replacement cycle short enough to meet the demand of the world fuel market [14]. In recent years, much work has been carried out towards efficient utilization of agro-industrial residues to produce enzymes of commercial importance by microorganism [15]. Enzymatic hydrolysis of cellulose, the most abundant renewable resources on the earth, offers an attractive alternative for the generation of sugars which can serve as the raw material for the production of various products of commercial interest such as bio-ethanol. The most promising technology for the conversion of the lingo cellulosic biomass to fuel ethanol is based on the enzymatic breakdown of cellulose using cellulase enzymes [16].

Researchers have strong interests in cellulases because of their applications in industries of starch processing, animal feed production, grain alcohol fermentation, malting and brewing, extraction of fruit and vegetable juices, pulp, paper and textile industries [17-18]. Moreover, there are growing markets for produced cellulases in the field of detergent industry and saccharification of agriculture wastes for bioethanol technology [19-20]. The cellulase complex consists of three major enzyme components, an endo-1,4-β-glucanase [Carboxymethyl cellulase (EC 3.2.1.4)], a 1,4-β-cellobiohydrolase [Exoglucanase (EC 3.2.1.91) and a 1,4-β-glucosidase [Cellobiase (EC 3.2.1.21)], which act synergistically during the conversion of cellulose to glucose [21-23].

Cellulases are widely distributed in all living kingdoms; they occur ubiquitously in mammals, plants and microbial kingdoms including bacteria, yeasts, and fungi [24]. Although a large number of microorganisms are capable of degrading cellulose, only a few of these produce significant quantities of cell free enzymes capable of completely hydrolyzing crystalline cellulose *In vitro*. The production of cellulase has been reported from a wide variety of bacteria [25] and fungi [26]. However, filamentous fungi are preferred for commercial enzyme production, because the level of the enzymes produced by these cultures is higher than those obtained from yeast and bacteria [27]. Therefore, much work has been directed to find suitable cellulase-producing fungi through strain selection and development. *Trichoderma reesei* produces very high titers of cellulase system with very little β-glucosidase, which is a practical disadvantage, as β-glucosidase plays an important role in the hydrolysis of cellulose by converting cellobiose to glucose [28]. Otherwise, *Aspergillus* species are the well known efficient producer of β-glucosidase compared with *Trichoderma* sp. [29-30].

The cost of production and low yield of cellulases are the major problems for industrial applications. It has been reported that solid state fermentation (SSF) technique as an attractive process to produce cellulases economically is mainly due to its superior productivity, simple technique; low capital investment, low energy requirement and less water output [31-33], better product recovery and lack of foam build up and reported to be most appropriate process for developing countries [34]. Production of cellulases by fungi in SSF using agricultural wastes has been reported by Fawzi [35], Abo-State et al. [18] and Mrudula and Murugammal [5]. Filamentous fungi are the most commonly used microorganisms in SSF because they are able to grow on solid materials with low water contents. Thus, the objective of this study was to investigate high level production of extracellular cellulases through different filamentous fungi and optimizing some parameters in solid-state fermentation medium to enhance cellulase system production.

2. MATERIALS AND METHODS

2.1 Microorganism

Thirteen filamentous fungi belong to the genera *Aspergillus*, *Trichoderma* and *Scopulariopsis*, obtained from different sources were screened for their abilities of utilizing rice straw as main carbon source for cellulases production. The source of each culture is listed as follows:

- NRRL: Northern Regional Research Laboratory, United States Department of Agriculture. Peoria, Illinois, USA.
- ASU: Local isolated strains: Faculty of Science, Ain Shams University, Department of Microbiology, Cairo, Egypt.

The stock cultures were maintained routinely on potato dextrose agar (PDA) slants containing (g/l): Potato, 300; dextrose, 20.0; agar; 20.0; distilled water, 1000 ml, adjusted at pH 6.0. The freshly grown slants at 28°C subsequently used for further work or stored at 4°C. The slants were subcultured routinely every 4-5 weeks interval.

2.2 Substrate

Rice straws (RS) were collected after harvesting from the local rice fields, Kalubia governorate, Egypt. The air-dried straws were cut into 1 cm, dried at 80°C for 24 hrs in air-circulation oven, then ground to uniform size (No. 6 meshes) in an electric grinder, finally packed and stored in plastic bags at room temperature for later use.

2.3. Pretreatment of Substrate

Different treatments carried out for delignification of RS before being used for production of the cellulolytic enzyme system:

2.3.1 Alkali pretreatment

5% (w/v) slurry of native RS in 160 ml of 1% NaOH aqueous solution was kept on a gyratory shaker (150 rpm) for 24 h at ambient temperature.

2.3.2 Alkali/steam pretreatment

5% (w/v) slurry of substrate in 160 ml 1% NaOH was pressure cooked at 121°C for 1 h.

2.3.3 Alkali/microwave pretreatment

The microwave/alkali pretreatment was carried out as follows: 5% (w/v) slurry of RS was suspended in 160 ml of 1% NaOH aqueous solution in a 500 ml beaker and positioned at the centre of a rotating circular glass plate in the microwave oven at 700 W for 15 min.

2.3.4 Steam pretreatment

5% (w/v) slurry of substrate in 160 ml distilled water was pressure cooked at 121°C for 1h.

2.3.5 Hydrochloric acid pretreatment

5% (w/v) slurry of substrate were soaked in 160 ml 1% HCl and kept on a gyratory shaker for 24 hrs at 30°C with 150 rpm oscillation min^{-1}.

2.3.6 Sulfuric acid pretreatment

5% (w/v) slurry of substrate were soaked in 160 ml 1% H_2SO_4 and kept on a gyratory shaker for 24 hrs at 30ºC with 150 rpm oscillation min^{-1}.

After acid and alkali pretreatments, treated RS were collected by filtration and extensively washed with distilled water. The pH was adjusted to approximately 5.5. Steam-pretreated rice straws were washed once. All treated substrates were dried overnight at 45°C in a forced-draft oven until constant weight.

2.4 Chemicals and Buffers

p-Nitrophenyl-β-D-glucopyranoside (*p*-NPG) was purchased from Sigma Chemicals Company, St. Louis, Mo, U.S.A; carboxy-methylcellulose (CMC) from Mallinckrodt Inc., Paris, Kentucky; 3,5-dinitrosalicylic acid (DNS) from Oxford Laboratory, Mumbai; Folin-Ciocalteu phenol reagent from Gomhoria Company for chemical and clinical supplies, Cairo, Egypt. Buffers were prepared according to the method presented by Gomori [36]. All other chemicals were also of the best analytical grade and of high purity.

2.5 Screening Medium for Cellulase Production

Different filamentous fungi were grown on PDA agar slants for 7 days at $28^{\circ}C$. After incubation, conidia were scraped and 5.0 ml of sterile distilled water was added to each slant and spores were extracted by hand-shaking. Then, 2.0 ml aliquots were used to inoculate 250 ml Erlenmeyer flasks, each containing (g/l): Rice straw, 200; $NaNO_3$, 2.0; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.5 and KCl, 0.5, adjusted at pH 5.0 before autoclaving ($121^{\circ}C$ for 20 min). Thereafter, the inoculated flasks were incubated for 5 days at 28ºC under static condition.

2.6 Enzyme Extraction

The enzyme was extracted by mixing homogenously the entire fermented substrate for each flask with 50 ml buffered solution (0.1 M citrate phosphate buffer; pH 5.0) and agitated on a rotary shaker (150 rpm) at room temperature with a contact time of 1 hr. Dampened cheese cloth was used to filter the extract, pooled extracts were centrifuged at 5000 rpm for 15min and the clear supernatant was used as a source of extracellular enzyme [37]. The clarified filtrates were checked for CMC-ase, FP-ase and β-glucosidase activities.

2.7 Enzyme Activity

2.7.1 Cellulases assay

Cellulase [filter paper activity (FP-ase) and carboxymethylcellulase (CMC-ase)] activities were assayed according to the method described by Bai et al. [38]. The amount of reducing sugars released was determined by dinitrosalicylic acid (DNS) method against boiled enzyme as a control and D-glucose as a standard. One unit of enzyme activity (FP-ase and CMC-ase) is defined as the amount of enzyme which releases 1 µmole of reducing sugars per min with glucose as standard, under the assay condition described above. The values of enzymatic activities were expressed as U/g-ds.

2.7.2 β- Glucosidase assay

According to Jatinder et al. [39]; β-glucosidase activity was determined photometrically by measuring the increase in absorbance at 420 nm, after 30 min incubation of 0.1 ml of properly diluted enzyme with 5 mM *p*-nitrophenyl-β-D-glucopyranoside (*p*-NPG) at 50°C, using standard curve of *p*-nitrophenol. The reaction was terminated by adding 2.0 ml of 1 M sodium carbonate solution (Na_2CO_3). One unit of β-glucosidase is defined as the amount of enzyme which releases one µmole of *p*-nitrophenol per min under culture conditions. The unit of β-glucosidase was expressed as U/g-ds.

2.8 Protein Determination

Protein content was determined according to Lowry et al. [40] method by measuring optical density of developed color at 660 nm. The µg of protein was estimated using µg standard of bovine serum albumin (BSA).

2.9 Statistical Analysis

Statistical analysis was carried out according to the method described by Kenney and Keeping [41].

2.10 Optimization of Cellulases Production

Optimization of various physicochemical parameters and media components required for maximum enzymes production by the selected fungal strain was evaluated in 250-ml Erlenmeyer flasks, and the medium described above was taken as the basal medium. The parameters studied included initial pH values of the medium (4.0, 5.0, 6.0, 7.0, 8.0 and 9.0), optimal duration for the enzyme production (during the fermentation, the flasks were taken at regular intervals of 24 h), incubation temperatures (23, 28, 35 and 40°C). In addition, the effects of different nitrogen sources ($NaNO_3$, NH_4Cl, $NH_4H_2PO_4$, L-asparagine, L-glutamine, peptone and yeast extract, at 0.33 g nitrogen/L as nitrogen base) and moisture level on cellulase production were also examined by varying the RS to moisture ratio (w/v) within the range of 40 to 90% using the previously mentioned medium. In addition, the effect of using different media for enzymes production was also evaluated. All the experiments were carried out in triplicate and the mean values were taken.

3. RESULTS AND DISCUSSION

3.1 Screening of Different Filamentous Fungi for Cellulase Production

The capability of different filamentous fungal strains on cellulase production on rice straw is shown in Fig. 1, from which it was clear that *Aspergillus oryzae* NRRL 3484 gave the highest cellulase production (371 and 344 U/g ds for FP-ase and CMC-ase, respectively) followed by *A. oryzae* NRRL 447 (359 and 336 U/g ds for FP-ase and CMC-ase, respectively). These two fungal strains produce the three hydrolytic enzymes of cellulase system, namely, exo-(1,4)-β-D-glucanase, endo-(1,4)-β-D-glucanase and β-glucosidase in appreciable amounts. The presence of β-glucosidase besides the other two components will trigger the degradative reaction of cellulose towards the formation of glucose as an end-product [42]. Jahromi et al. [43] reported that total cellulase activity of *A. terreus* was indicated by the activity of filter paper activity, carboxymethylcellulase and β-glucosidase. In this concern, Woodward [44] reported that in most studied cellulolytic fungus, absence of appreciable levels of β-glucosidase is one of the limitations of *Trichoderma* cellulases for enzymic conversion of lignocelluloses.
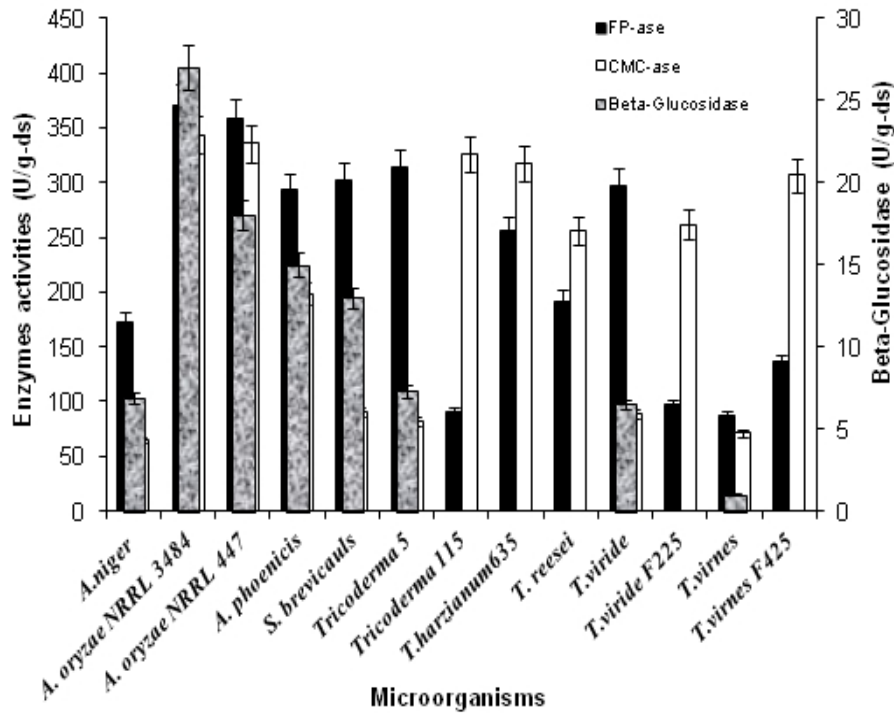
Fig.1 Screening of different filamentous fungi for extracellular cellulases production using RS under SSF

A capacity to degrade cellulose is a character distributed among a wide variety of aerobic, facultative aerobic, anaerobic bacteria and fungi. It has been reported in the recent studies that higher levels of cellulases were obtained with *Aspergillus* and *Trichoderma* sps [45-46]. However, *Aspergillus* species are known to be the most efficient producer of β-glucosidase compared with *Trichoderma* sp. [29,47-48]. Kang et al. [49] have reported the 129 units of CMC-ase activity using *Aspergillus niger* KK2 and RS as substrate in solid state fermentation. Moreover, the results obtained in the present study revealed that the other fungal strains gave low activity especially *Trichoderma virnes* which gave the lowest cellulase activity (87 and 71 U/g ds for FP-ase and CMC-ase, respectively). Therefore, *Aspergillus oryzae* 3484 was chosen for further studies on RS.

3.2 Effect of Rice Straw Pretreatment on Cellulase Productivity

The chemical composition of rice straw varies between varieties and growing seasons, with higher nitrogen and cellulose contents in early-season rice compared to others [50]. Rice straw consists predominantly of cell walls, comprised of cellulose, hemicellulose, and lignin. To break down these components cellulase, hemicellulase and ligninase are required [51]. Different pretreatments of lingo cellulosic materials have been reported in the literature to make the substrates more conducive to SSF [52]. In the present report, different pretreatments were applied to finely grinded rice straw. It was found that the pretreatment of rice straw by NaOH under steam pressure can get rid of about 97% of lignin which represents an excellent delignification level that enables fungal cultures to grow and utilize rice straw as a sole carbon source producing the required cellulase system. It is noteworthy

that the RS treated by NaOH/steam pressure had higher cellulose, lower lignin and hemicelluloses (Data not shown). The cellulose increase came from the solubilization of other components in the NaOH aqueous solution. Moreover, steam pressure enhanced the solubilization of hemicellulose in the NaOH, which led to the close contact between lignin and the NaOH solution and thus enhanced the solubilization of lignin [53]. In most cases, it was found that 1% NaOH treated substrates were much more efficient compared to that treated by 4% NaOH. This is due to the strong effect of alkali treatment which dissolves the lignin seal instead of removing it. The dissolved lignin has been regarded as an effective inhibitor of cellulases [54]. The alkali/steam pressure treatment of RS leads to a change in physical nature of lignin, increase of the available surface area, increase in pore sizes and partial depolymerization of hemicelluloses, which enhance the accessibility and availability of the substrate as reported by Haltrich et al. [55] and Brijwani and Vadlani [56]. The results obtained in the current study also showed that other treatment techniques were not effective in delignification of RS. Therefore, alkali/steam pressure technique was selected for subsequent enzymatic hydrolysis of RS.

The effect of rice straw pretreatment on cellulases production was shown in Fig. 2, from which it is clear that alkali/steam pretreated RS was the most efficient pretreatment for cellulases production (27.3±0.6, 369.1±4.3 and 341.7±4.2 U/g ds for β-glucosidase, FP-ase and CMC-ase, respectively). However, neither of NaOH nor steaming under pressure was efficient for cellulase production. Acids pretreatment failed to produce efficient amounts of enzymes yields.
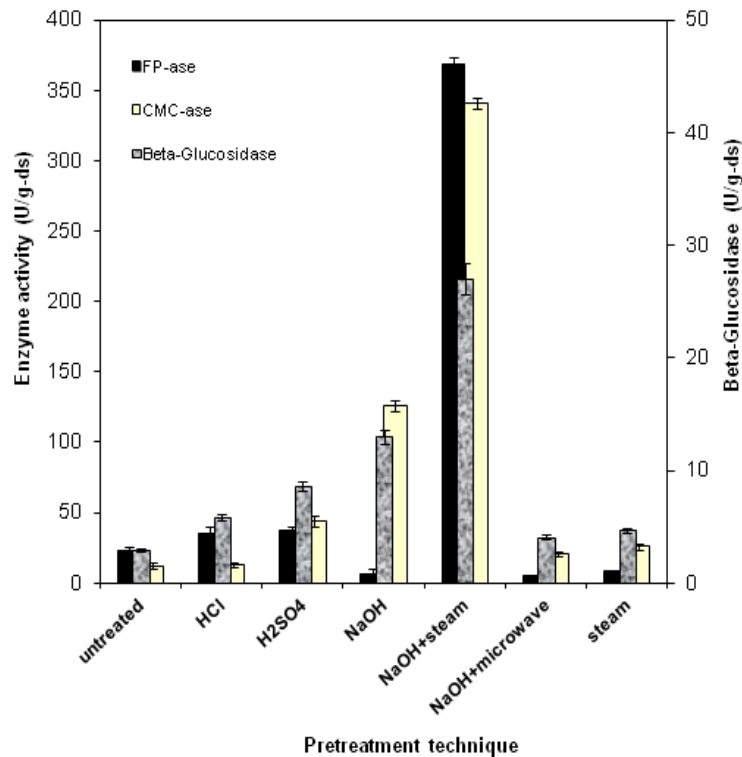

Fig. 2. Effect of pretreatment of RS on cellulases production by *A. oryzae* 3484

3.3 Optimal Conditions for SSF

3.3.1 Effect of different moistening media on cellulase production

Rice straw was moistened using four different liquid media aiming to enhance the cellulase enzymes yields. The results obtained showed that the highest cellulases production (41.4±3.1, 525.3±5.6 and 463.3±4.8 U/g ds for β-glucosidase, FP-ase and CMC-ase, respectively) was reported with medium III [48] which enhance an increase of 42% in enzymes production compared with the basal medium IV [57] and medium II [58]. On the other hand, medium I [43] gave the lowest cellulase system productivity (Data not shown). Thus, medium III was chosen for further studies.

3.3.2 Effect of different incubation periods on cellulases production

With regard to the different incubation periods in this current investigation, the enzyme production rates showed growth relatedness as the incubation period progressed, and optimum cellulase production from *A. oryzae* 3484 grown on RS (566 and 529 U/g ds for FP-ase and CMC-ase, respectively) had been achieved on the 7th day (Fig. 3). The results also showed that there no significant activity could be detected in filtrates after 24 - 48 h incubation. It seemed likely that the cell machinery of the organism during this period is directed towards active vegetative growth and mycelium proliferation. This result is in relation with the findings of Milala et al. [59] for cellulase production by *A. candidus*. In this concern, Badhan et al. [60] reported the highest FP-ase and CMC-ase that represent total cellulase activity on the 8th and 10th days of fermentation (410.76 and 480.48 U/g ds, respectively) and thereafter, the activity declined. In addition, the highest β-glucosidase production was investigated from 6 and 8 days fermentations (16.37 and 15.97 U/g ds, respectively).
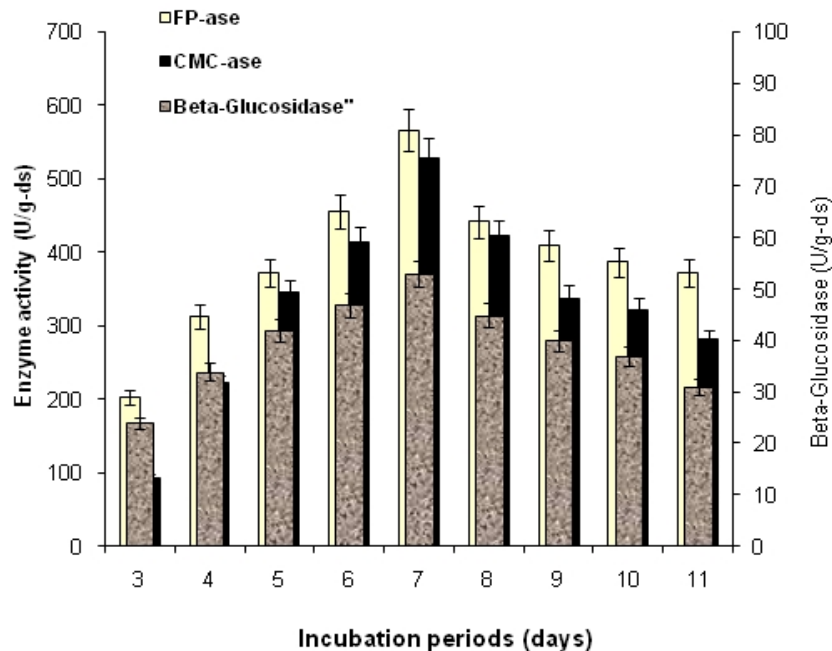

Fig. 3. Effect of different incubation periods on cellulases production by
***A. oryzae* 3484**

On the other hand, Kang et al. [49] found that the highest cellulase activity was obtained after 5-6 days of fermentation by *A. niger* grown on rice straw. Vu et al. [20] reported the highest production of cellulase *Aspergillus* sp. SU14 (28.31 U/g ds) was observed after 3 days of fermentation. Moreover, our study showed that further increase in the incubation time more than 7 days reduced the enzyme production. It might be due to the depletion of macro and micronutrients in the fermentation medium with the lapse in time, which stressed the fungal physiology resulting in the inactivation of secreting machinery of the enzymes [61], or may be due to the denaturation of enzymes, resulting from variation in pH during fermentation as reported by Krishna [62], or the cumulative effect of cellobiose [63].

3.3.3 Effect of initial pH values on cellulases 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 stability in the medium [64]. The optimal pH varies with different microorganisms and enzymes. Thus, Beldman et al. [65] reported that *Aspergillus* species grow and metabolize well in acidic pH medium between pH 3.0 – 5.0. Our study investigate that maximum cellulase production from *A. oryzae* was reported when the pH of the medium was adjusted to 5.0 as shown in Fig. 4.
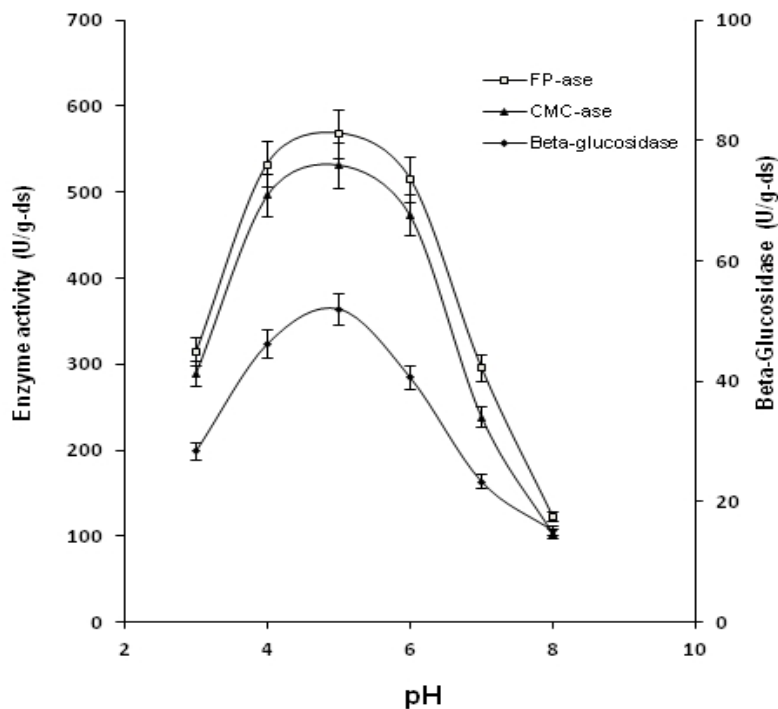

Fig. 4. Effect of initial pH of medium on cellulases production by *A. oryzae* 3484

Moreover, there was a drastic decrease in cellulase activity when the pH of the medium is either increased or decreased from 5.0. Similar observation was reported for cellulase production by *A. terreus* QTC 828 by Ali et al. [66] and *Trichoderma reesei* by Doppelbauer et al. [67], whereas pH 7.0 was reported by Krishna [62] for the production of bacterial

cellulases by using banana wastes in SSF. The bacterium, *Cornybacterium lipophiloflavum* produces maximum cellulase activity (0.80 U/ml) when the pH of the medium was adjusted to 7.0 as investigated by Sakthivel et al. [68]. Pamment et al. [69] found 6.0 as the optimum pH for CMC-ase production by *Chaetomium cellulolyticum*. While Vu et al. [20] reported the highest production of cellulase from *Aspergillus* sp. SU14-M15 at a pH of 3.5.

3.3.4 Effect of incubation temperature on cellulases 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 cellulase production decreasing at higher temperature (Data not shown). The results in the present study confirm the findings of Devanathan et al. [70] and Kathiresan and Manivannan [71] for *Aspergillus niger* and *Penicillium fellutanum*, respectively. On the other hand, Asquieri and Park [72] found that the optimum temperature for production of CMC-ase from thermostable *Aspergillus* sp. was 37ºC, whereas the maximum cellulase production was observed at 40ºC for *A. terreus* QTC 828 and *A. niger* Z10 grown in SSF [66]. About 81% of cellulase production was observed at 35ºC. Whereas, about 53 and 64% of cellulase production was recorded when the organism was grown at 40 and 20ºC, respectively.

Sherief et al. [48] found that the optimum temperatures for cellulase production by *Aspergillus fumigatus* were between 35-45ºC. While, Jahromi et al. [43] reported optimum cellulase production from *Aspergillus terreus* ATCC 74135 at 25°C under solid state fermentation. 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 [73]. In general the temperature maintained in SSF system is in the range of 25 to 35ºC and depends on the growth kinetics of the microorganism employed rather than on the enzyme produced [74].

3.3.5 Effect of initial moisture level of the medium on cellulase production

Moisture content is a critical factor for cell growth and enzyme production under SSF, which determines the outcome of the process. In SSF, moisture level plays an important role in biosynthesis and secretion of many kinds of enzymes, especially cellulases. Very high moisture content in solid medium results in a reduction in enzyme yield due to steric hindrance of the growth of the producer strain by reduction in porosity (inter-particle spaces) of the solid matrix, thus interfering oxygen transfer, while excessively low moisture levels in solid medium causes reduction in solubility of nutrients of the substrate, low degree of swelling and high water tension which leads to poor microbial growth and poor development [74].

The optimum initial moisture level in the current study was reported at 70% for cellulase production by *Aspergillus oryzae* NRRL 3484 on RS. Lower or higher than 70% both decreased the cellulase production (Data not shown). Lower moisture level gives a lower degree of swelling and higher water tension and then reduces the solubility of nutrients. While, higher moisture level decreases porosity, changes particle structure, promotes development of stickiness, decreases diffusion, lowers oxygen transfer or increases formation of aerial hyphae [75]. Vu et al. [20] reported the highest cellulase production (76.6 U/g) by *Aspergillus* sp. SU14-M15 at 50% moisture content. Moreover they found that moisture contents less than 40% or greater than 55% were not suitable for high cellulase production. Maximum enzyme production from *Aspergillus niger* was obtained at coir waste

to distilled water ratio of 1:2 and any further increase or decrease in the ratio resulted in a reduction in cellulase production [5].

3.3.6 Effect of supplementation of rice straw with different nitrogen sources on cellulase production

Different organic and inorganic nitrogen sources were tried to improve cellulase production. As shown in Fig. 5, all nitrogen sources tested enhanced cellulase production to different levels when compared to control. Among the different nitrogen sources used the highest cellulase activity was observed in medium amended with peptone (68.6, 601.7 and 567.2 U/g-ds, for β-glucosidase, FP-ase and CMC-ase, respectively) which was closely followed by yeast extract (Fig. 5).
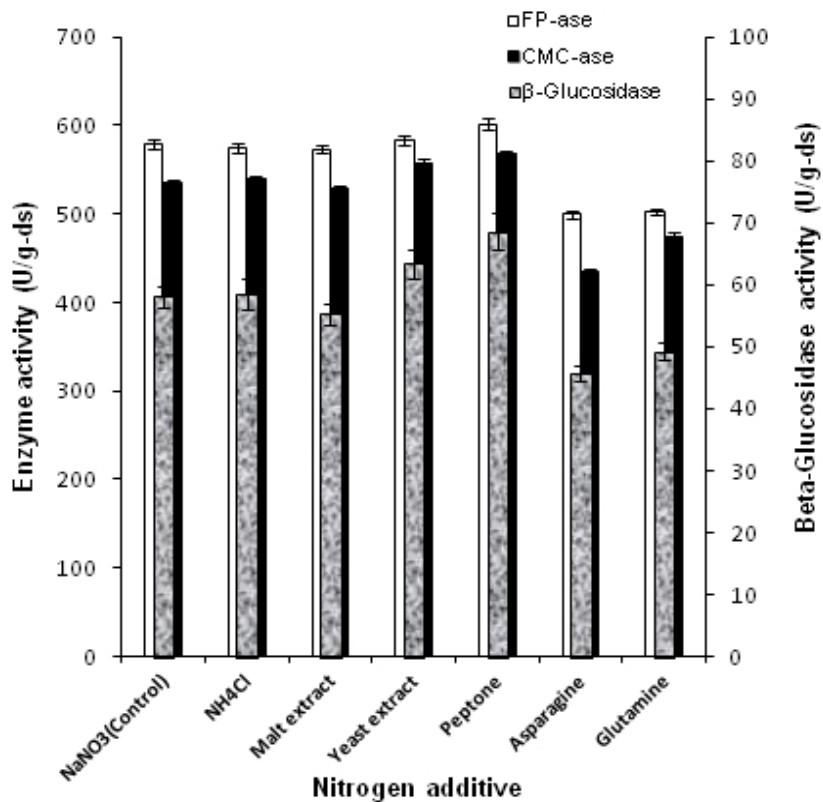

Fig. 5. Effect of different nitrogen additives on cellulases production by
***A. oryzae* 3484**

Organic nitrogen showed superiority over inorganic nitrogen sources for the production of enzymes [76]. It might be due to the deficiency of nitrogen sources in natural RS. These results are in agreement with the reports of Kathiresan and Manivannan [71] and Devanathan et al. [70] for production of cellulase by *Penicillium fellutanum* and *Aspergillus niger*, respectively. Enari et al. [77] reported that good cellulase production by fungi can be obtained with peptone as the organic nitrogen source in SSF.

4. CONCLUSION

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 oryzae* NRRL 3484 over the other tested fungal cultures for production of extracellular cellulases on rice straw via SSF. Alkali-steam pretreated RS was the most efficient pretreatment for cellulases production. The optimum incubation period, temperature, initial pH of medium and moisture level were reported on the 7th day, at 28°C, 5.0 and 70%, respectively. In addition, the supplement of peptone favored the enzyme formation markedly. More investigations are needed for production of cellulases which imported for use in Egypt at a high cost.

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COMPETING INTERESTS

Authors have declared no competing interests.

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