OPTIMIZATION OF PROCESS PARAMETERS FOR CELLULASE PRODUCTION BY NOVEL THERMOTOLERANT YEAST

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The goal of the present study was to investigate production of cellulase in low cost medium by thermotolerant yeast. After screening, an efficient yeast isolate having capability of C_1 (exo-gluconase) and Cx (endo-gluconase) production was isolated and designated as strain R-1. Maximum enzyme production was achieved at 50 °C, pH 5.5 in the medium containing bagasse powder 4% (w/v), and ammonium sulphate 0.1% (w/v) after 72 hours of incubation. The composition containing bagasse powder, 4% (w/v); ammonium sulphate, 0.5 % (w/v); and glucose, 0.5% (w/v) achieved better production after complete medium optimization. The yeast isolate was able to tolerate wide ranges of temperature, pH, and substrate concentration for higher enzyme production. The isolated yeast was able to produce C_1 (exo-gluconase) and Cx (endo-gluconase) enzymes in appropriate concentrations on a crude cellulosic substrate. Therefore, yeast may be used to power alcohol production.

Keywords: Cellulases; Exo-gluconase; Endo-gluconase; Bagasse; Yeast

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INTRODUCTION

Microbial diversity from various habitats such as soil, river water, hypersaline lakes, and insects offers vast opportunities for exploration, as these habitats are the source of useful biomolecules which are a great relevance to the biotechnology industry, and include enzymes, fatty acids, pigments, antibiotics, *etc*. (Butinar *et al*. 2005; Slavikova and Vadkertiova 2003). Moreover, the development of biotechnology has raised much interest in using cellulase producing microorganisms to convert lignocellulosic biomass from agro-industrial wastes to glucose that can be used in applications such as production of bioethanol (Amita *et al*. 2006). Furthermore, cellulase could be used in waste water treatment, the food industry, the pulp and paper industry, textiles and laundry, and in animal feed (Enari 1983; Suchita and Ramesh 2006).

Cellulases degrade cellulose by the synergistic action of three enzyme activities: endoglucanase, which is also called as carboxymethyl cellulase (CMCase) (endo-1,4-β-Dglucanase, EG, EC 3.2.1.4); exoglucanse (also called as cellobiohydrolase) (exo-1,4- β -D-glucanase, CBH, EC 3.2.1.91); and β-glucosidase (1,4-β-D-glucosidase, BG, EC 3.2.1.21) (Li *et al*. 2006; Gao *et al*. 2008). Researchers have a strong interest in cellulases because of their applications in several industries (Jamil *et al*. 2005; Ahmed *et al*. 2005). In recent years, interest in cellulases has increased due to their application in the production of bioenergy and bio-fuel (Ahmed and Vermette 2008; Zhou *et al*. 2008).

A number of microorganisms' *viz*. bacteria, fungi, actinomycetes, and yeasts are capable of producing extracellular cellulase enzyme (Kirk *et al*. 2002). Many fungal

strains secrete higher amounts of cellulases than bacterial ones. Cellulases from *Trichoderma* and *Aspergillus* species have been investigated in detail over the past few decades (Fang *et al.* 2008; Tao *et al*. 2010). But due to their long growth cycle, such fungi have a huge spore formation, and this limits their performance in terms of cellulase production and its safe utilization. Yeasts such as *Pichia stipitis*, *Candida shehatae*, and *Pachysolan tannophilus* have the ability to use both C5 and C6 sugars (Agbogbo and Coward-Kelly 2008).

Industrial applications of these enzyme systems require high temperature tolerant microorganisms, because during fermentation of cellulose, the temperature can increase up to 8 °C from the initial commercial level at production. The substrate concentration, pH range change, and microaerophilic environment create problems during fermentation (Singh *et al.* 2009; Zhang *et al.* 2009).

Bagasse is abundantly produced by the sugar industry as a byproduct, and it is also used for fuel. Bio-conversion of sugarcane bagasse into ethanol seems to be the most plausible solution to the increasing demands of energy and the current saving of foreign exchange (Xiros and Christakopoulos 2009).

The aim of the present study was to isolate a thermotolerant cellulase producing yeast *Candida* sp. with bagasse as a main substrate. So, in this study, bagasse was used as a substrate to investigate optimum fermentation conditions for the production of cellulase from *Candida* sp.

MATERIALS AND METHODS

Substrate

Bagasse was obtained from bagasse storage ground of K. M. Sugar Mills, Faizabad U.P. India, air dried and stored in an oven at 65 °C to a constant weight. Substrate was ground to 2 mm sieve and stored in air tight plastic jars.

Isolation, Screening, and Identification of Cellulase Producing Yeast

Thermotolerant cellulolytic yeasts were isolated from soil or the bagasse storage ground of K. M. Sugar Mills, Faizabad U.P. India, in the months of May to June 2010-2011, where the maximum day temperature during the summer is 40 to 42 °C. Soil samples were collected in sterile polyethylene bags by a random sampling method. The 1 g collected soil samples were suspended in 9 mL sterile distilled water and kept on a magnetic stirrer for 1 to 2 minutes. The samples were serially diluted up to 10^{-2} to 10^{-4}, and 0.1 mL mixture was spread on cellulose agar plates by surface overlay and the sandwiched agar plate method for aerobic and facultative anaerobic thermotolerant yeasts. Cellulose agar medium consisted of 2% (w/v), carboxymethyl cellulose (CMC) and 0.1% (w/v) ammonium sulphate with an initial pH of 5.5 at 40 °C for 48 hours of incubation. The plates were stained with Congo red, followed by destaining with 1 M NaCl solution. Colonies having a clear halo zone were further selected for biochemical studies (Ghose 1987). The best isolate was identified as the genus *Candida* on the basis of morphological characteristics using the standard identification manual (Kurtzmam and Fell 1998).

Enzyme Production

The culture was grown in a 150 mL Erlenmeyer flask that contained 50 mL of basal medium containing 4.0% bagasse and 0.5% ammonium sulphate. The pH of the medium was adjusted to 5.0 prior to sterilization. The flask was inoculated and incubated at 40 ± 2 ºC under stationary condition for 72 days. The crude enzyme was filtered and centrifuged at 10000 rpm for 10 min and carried out enzyme assay.

Enzyme Assays

Cellulase activity was measured by the Nelson-Somogyi method (Nelson 1944; Somogyi 1952). The medium contained bagasse powder 4 % (w/v) and ammonium sulphate 0.1 % (w/v). Cx (beta 1-4 exogluconase) activity was determined by a reaction mixture (1 mL) containing 0.1 mL of culture supernatant, 0.5 mL of 1 mol/L substrate solution, and 0.4 mL of distilled water. The reaction mixtures were incubated for 10 minutes at 40 °C. The reaction was stopped by adding 1 mL of alkaline copper tartrate solution and boiling for 10 min. Then after cooling, 1 mL of arsenomolybdate solution was added for color stabilization. Thereafter activities were measured at 620 nm against a reagent blank by a spectrophotometer. C_1 (beta 1-4 endogluconase) activity was measured by using an absorbent cotton assay (Mandels and Weber 1969). It was measured by incubating 1.0 mL of the enzyme with the addition of 4.0 mL of distilled water preparation with 0.25 g absorbent cotton at pH 6.0, 50˚C for 24 hrs. The reducing sugar was estimated by the NS (Nelson-Somogyi; Mandels and Weber 1969) method as discussed above for Cx activity. After that the activities were measured at 620 nm against a reagent blank by a spectrophotometer. One unit of enzyme activity was defined as 1 µg of reducing end group (glucose) released per min at 40 °C.

Inoculum Preparation

Mother culture was prepared by inoculating one full loop of 24 h grown culture on CMC agar plate in 50 mL of CMC broth and incubating at 40 °C for 24 h to achieve active exponential phase containing of 50×10^8 cfu/mL. A suitable amount (0.5%, v/v) of this cell suspension was used to inoculate the test flasks.

Biomass Determination

Yeast cells in broth were harvested by centrifugation (10000 rpm for 10 min at 4 °C), washed with distilled water, and dried in an oven at 80 °C until reaching a constant weight. The biomass was reported in the form of dry cell mass (g/L).

Determination of Protein Concentration

Quantitative estimation of protein content was done by the method of Lowry *et al.* (1951) using Bovine Serum Albumin (BSA) as the standard and expressed as mg/mL.

Optimization of Physico-Chemical and Nutritional Parameters for Cellulase Production

The various process parameters influencing cellulase production were optimized individually and independently of the others. Therefore, the optimized conditions were subsequently used in all the experiments in sequential order. For optimization, the CMC medium was inoculated and incubated at different temperature *viz.* 25, 30, 35, 40, 45, 50, 55, and 60 °C under the standard assay conditions. The samples were withdrawn at every

8 h interval up to 48 h to study the effect of incubation period. The influence of pH on the enzyme activity was determined by measuring the enzyme activity at varying pH values ranging from 4.0 to 8.0 at 40 ºC using different suitable buffers, 1 mol/L sodium acetate (pH 4.0, 4.5, 5.0, and 5.5) and 1mol/L sodium phosphate (pH 6.0, 6.5, 7.0, 7.5, and 8.0), respectively. The growth medium was supplemented with different carbon sources *viz.* fructose, galactic, glucose, lactose, mannitol, soluble starch, sorbitol, sucrose, and xylose (at the level of 1%, w/v). Different organic nitrogen sources (1 % w/v beef extract, gelatin, malt extract, peptone, and yeast extract) and inorganic nitrogen sources (1 % w/v sodium nitrate, ammonium nitrate, ammonium chloride, potassium nitrate, ammonium sulphate, and urea) (at the level of 1%, w/v) were also used for enzyme production. Thereafter, optimized carbon and nitrogen sources were further optimized at different concentrations (0.1 to 0.8%, w/v).

Statistical Analysis

Each experiment was performed thrice in triplicates, and mean standard deviation for each experimental result was calculated using the Microsoft Excel.

RESULTS

Isolation, Screening, and Identification of Yeast

A total of 25 yeast isolates were isolated from the bagasse storage ground's soil of the K. M. Sugar Mill. Out of these, only 10 isolates of yeast showed a maximum clear zone on the cellulose-containing medium after staining with Congo red. One isolate, R-1, showed maximum clear zone diameter and was identified as genus *Candida*. This was further optimized for enzyme production at different physico-chemical and nutritional parameters.

Effect of Temperature on Cellulase Activity

The yeast isolate R-1 was examined for cellulase enzyme (Cx and C_1) production at different temperatures from 30 °C to 60 °C. Yeast showed better enzyme production from 40 °C to 50°C, but 50 °C was found to be the most effective temperature for optimum enzyme production. Yeast isolate showed a maximum Cx 44.84 and C_1 46.15 Unit/mL enzyme production with 0.25 g/100mL biomass production and 0.3 mg/mL protein concentration at 50 °C. Above and below this temperature, the enzyme production was less (Fig. 1). From our results, it was concluded that the yeast strain could be able to tolerate wide range of temperatures for higher cellulase production.

Effect of pH on Cellulase Activity

The effect of pH on the crude cellulase enzyme production by yeast isolate R-1 was examined at different pH values from pH 4.0 to pH 8.0 (Fig. 2). The yeast strain showed a wide range of pH tolerance (pH 5.0 to 6.5) capacity for cellulase enzyme production, but maximum enzyme production was achieved at pH 5.5. The yeast isolate R-1 showed maximum Cx 44.84 and C_1 46.15 Unit/mL enzyme production with 0.29 g/100mL biomass production and 0.34 mg/mL protein concentration at pH 5.5. Above and below this pH, the enzyme production was lower.

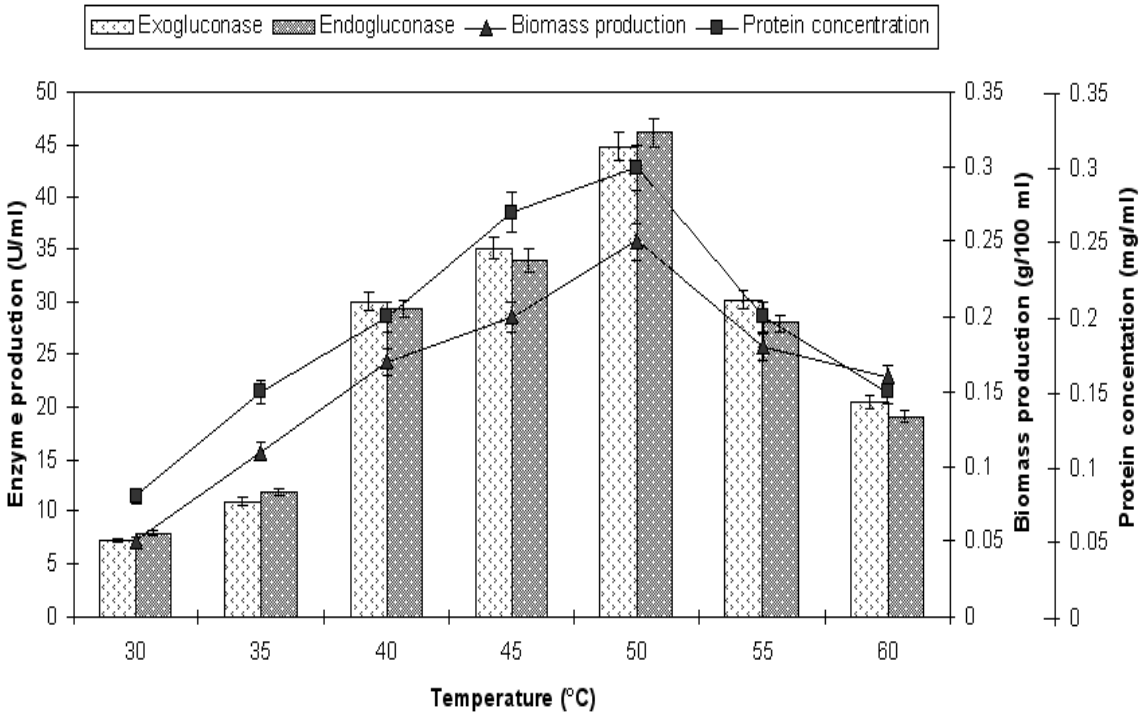

Fig. 1. Effect of temperature on cellulase production; The test flasks were inoculated and incubated at different temperatures for 72 hours under static conditions. Error bars presented are mean values of ± standard deviation of duplicates of three independent experiments.

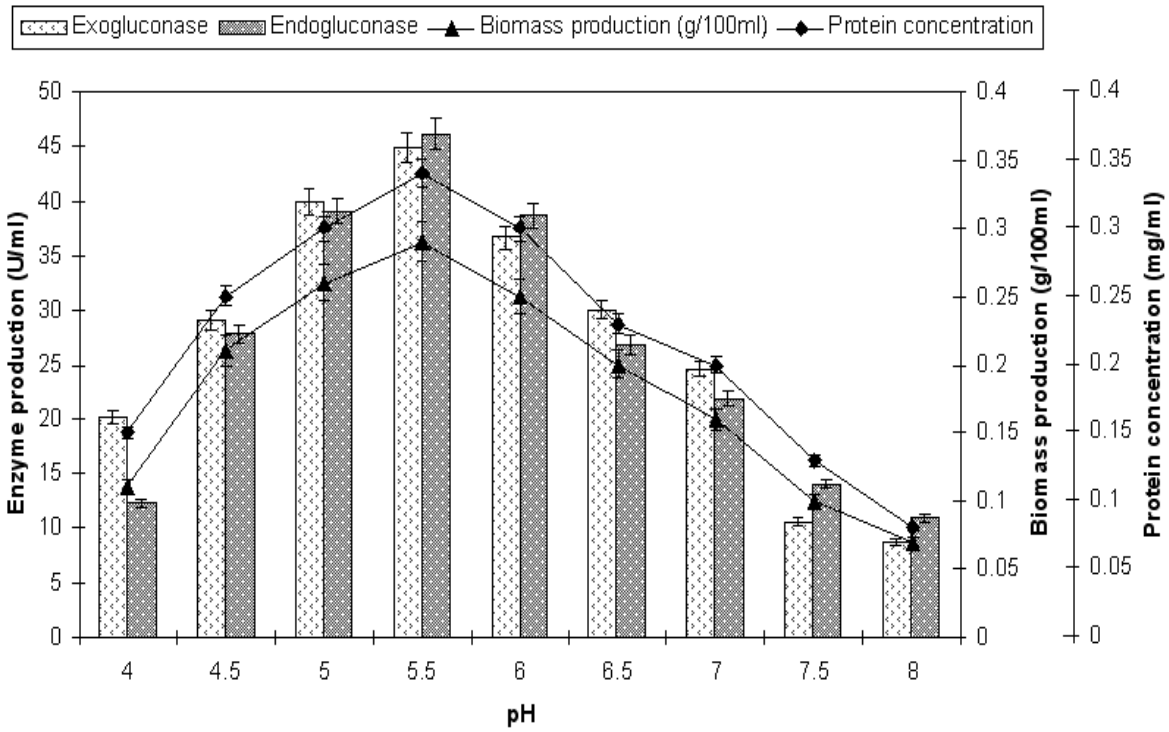

Fig. 2. Effect of pH on cellulase production; The test flasks were inoculated and incubated at 50 ºC at different pH for 72 hours under static conditions. Error bars presented are mean values of ± standard deviation of duplicates of three independent experiments.

Effect of Carbon Sources on Cellulase Activity

Addition of different carbon sources had both positive and negative effects on cellulase production (Fig. 3). Maximum cellulase production was obtained with glucose (Cx 345.4 and C_1 355.5 Unit/mL) followed by sucrose, fructose, and xylose. Starch, galactose, sorbitol, mannitol, and lactose had negative effects on cellulase production.

Different concentrations of glucose (0.1 to 0.8 %, w/v) were also studied on cellulase production. It was observed that 0.5 % (w/v) gave better results in cellulase production (Cx 575.9 and C_1 605.5 U/ml) as well as biomass production (Fig. 4). When the concentration of glucose was increased further, cellulase activity gradually decreased.

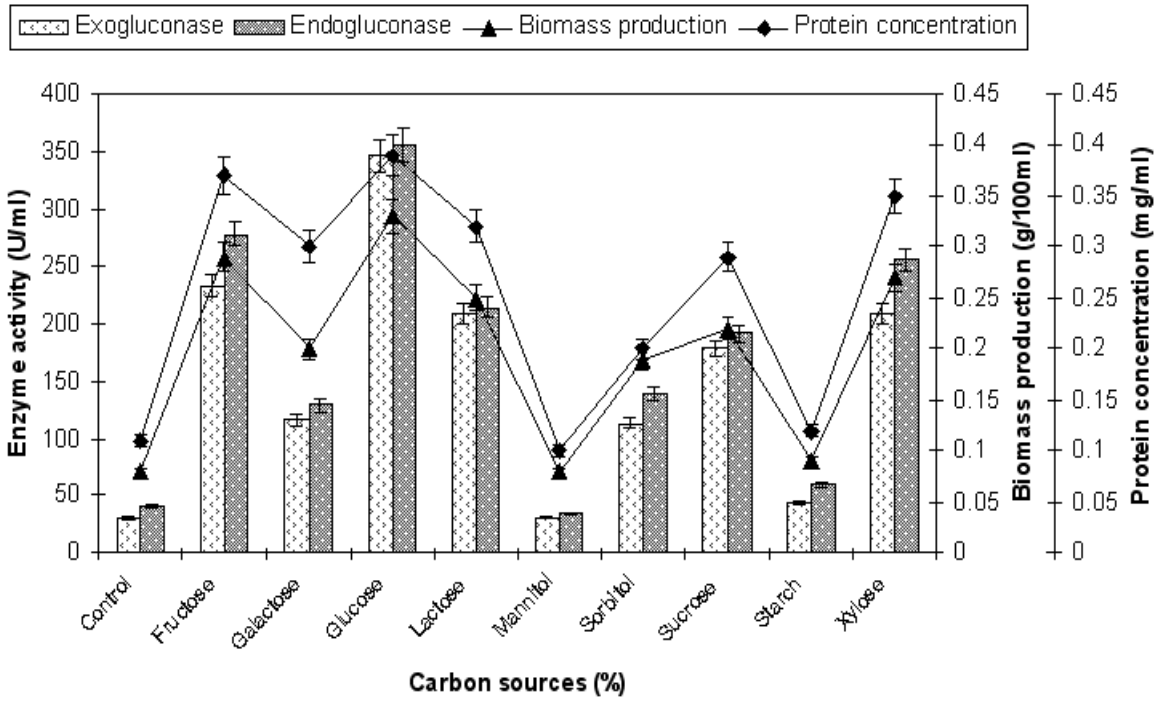

Fig. 3. Effect of different carbon sources on cellulase production; The control flask does not contain any carbon sources. Test flasks were amended with different sugar in the medium (1.0 % w/v) and were inoculated and incubated at 50 °C for 72 hours at pH 5.5. Error bars presented are mean values of ± standard deviation of duplicates of three independent experiments.

Effect of Organic Nitrogen Sources on Cellulase Activity

The influence of different organic nitrogen source was also optimized for better cellulase production. It was observed from Fig. 5 that all the organic nitrogen sources showed decreased cellulase production, except that malt extract exhibited Cx 610.9 and C_1 675.5 U/mL cellulase production.

When different concentrations (0.1 to 0.8 %, w/v) of malt extract were added to the medium, it did not exhibit a significant increase in cellulase production. Figure 6 indicates that maximum cellulase production (Cx 698.9 and C_1 796.4 U/mL) was achieved with 0.41 g/100 mL biomass production and 0.5 mg/mL protein concentration at 0.6% concentration of malt extract.

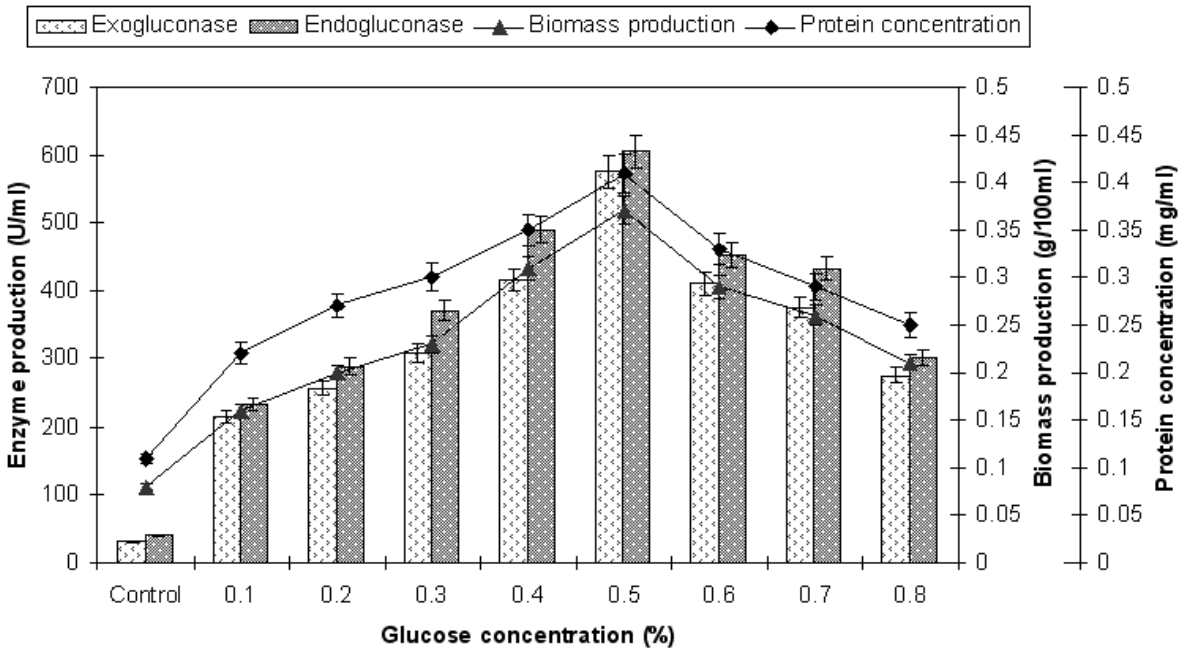

Fig. 4. Effect of different concentration of glucose on cellulase production; the control flask does not contain glucose. Test flasks were supplemented with a different concentration of glucose (0.1 to 0.8 % w/v) in the medium which was inoculated and incubated at 50 °C for 72 hours at pH 5.5. Error bars presented are mean values of ± standard deviation of duplicates of three independent experiments.

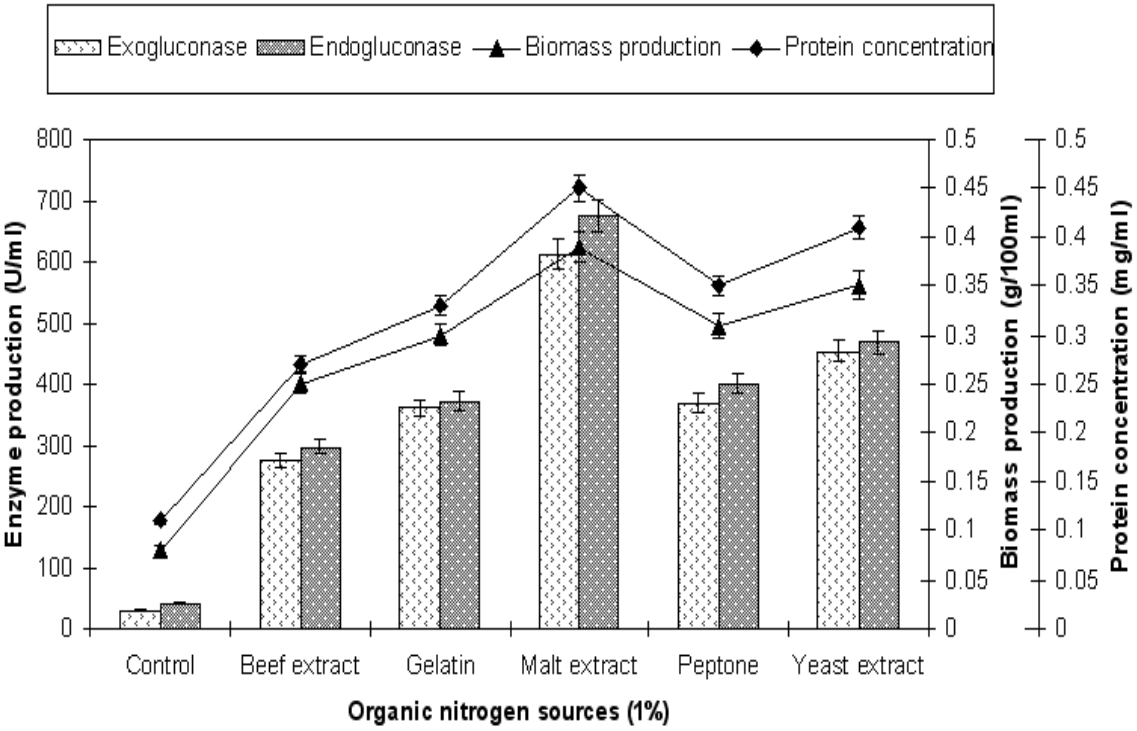

Fig. 5. Effect of different organic nitrogen sources on cellulase production; the control flask did not contain any organic nitrogen source. Test flasks were supplemented with different organic nitrogen source in the medium (1.0 % w/v) and were inoculated and incubated at 50 °C for 72 hours at pH 5.5. Error bars presented are mean values of ± standard deviation of duplicates of three independent experiments.

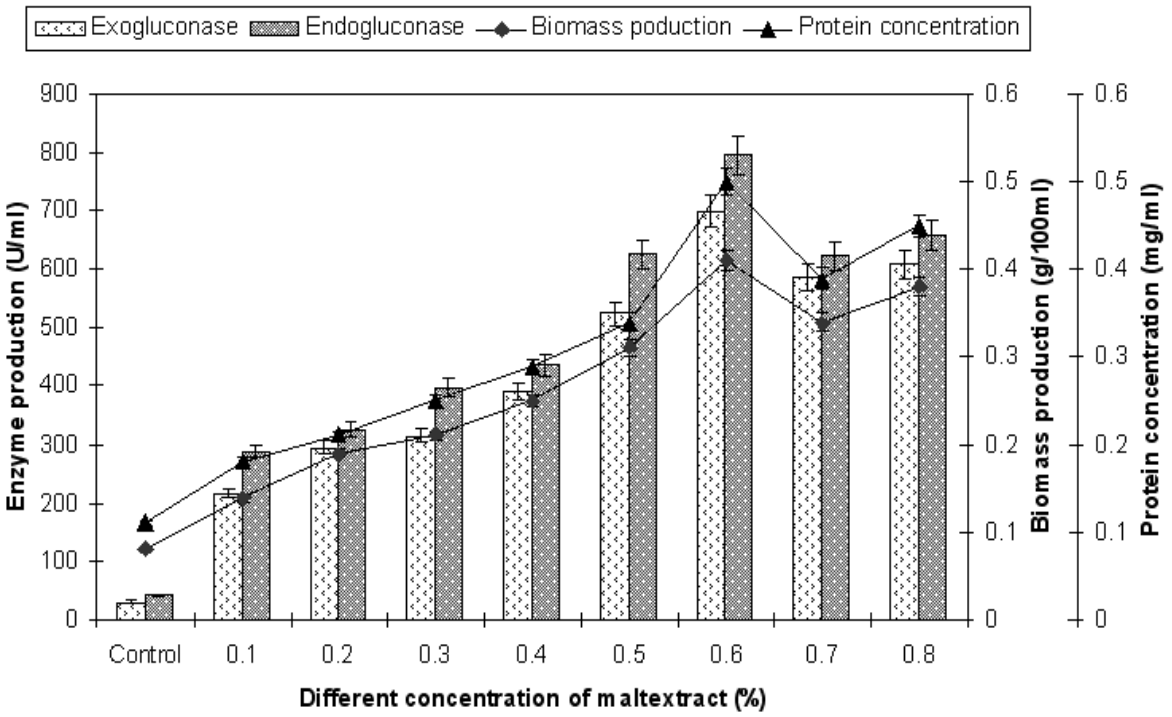

Fig. 6. Effect of different concentration of malt extract on cellulase production; the control flask does not contain malt extract. Test flasks amended with different concentration of malt extract (0.1 to 0.8 % w/v) in the medium which were inoculated and incubated at 50 °C for 72 hours at pH 5.5. Error bars presented are mean values of ± standard deviation of duplicates of three independent experiments.

Effect of Inorganic Nitrogen Sources on Cellulase Activity

Figure 7 indicates that cellulase productions by yeast isolate R-1 also were affected by different inorganic nitrogen sources; however, while maximum cellulase production (Cx 798.8 and C_1 867.6 U/mL) was achieved in the presence of ammonium sulphate, other nitrogen sources showed inhibitory effects on cellulase production.

When different concentrations (0.1 to 0.8 %, w/v) of ammonium sulphate were added to the medium, significant increases in cellulase production were observed, and maximum cellulase yield (Cx 907.5 and C_1 995.8 U/mL) was observed at a concentration of 0.5% (Fig. 8).

DISCUSSION

Nature provides a rich reservoir of different types of microorganisms. Most of them are beneficial for human welfare. Various microbial metabolites are *viz.* enzymes, antibiotics, organic acids, vitamins, amino acids, single cell proteins polysaccharides, *etc*. Microorganisms have been isolated from the natural ecosystem and developed for industrial use with or without strain improvement.

The main aim of this investigation was to isolate a potential cellulase producing strain from the natural ecosystem.

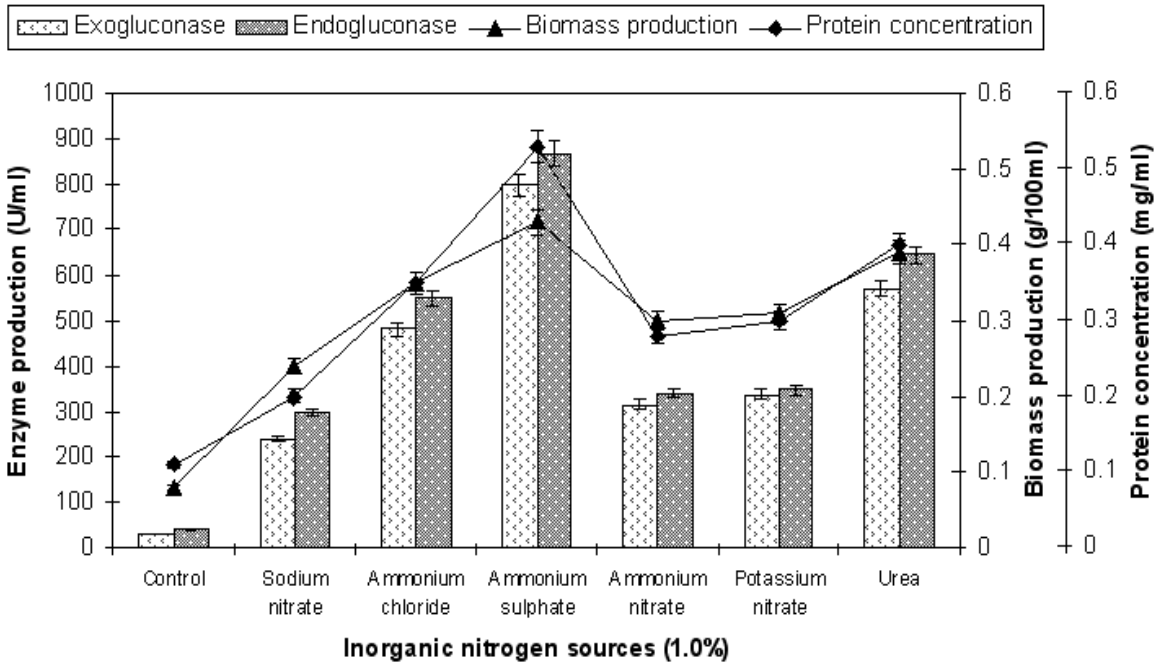

Fig. 7. Effect of different inorganic nitrogen sources on cellulase production; the control flask does not contain any inorganic nitrogen sources. Test flasks amended with different inorganic nitrogen source in the medium (1.0 % w/v) which inoculated and incubated at 50 °C for 72 hours at pH 5.5. Error bars presented are mean values of ± standard deviation of duplicates of three independent experiments.

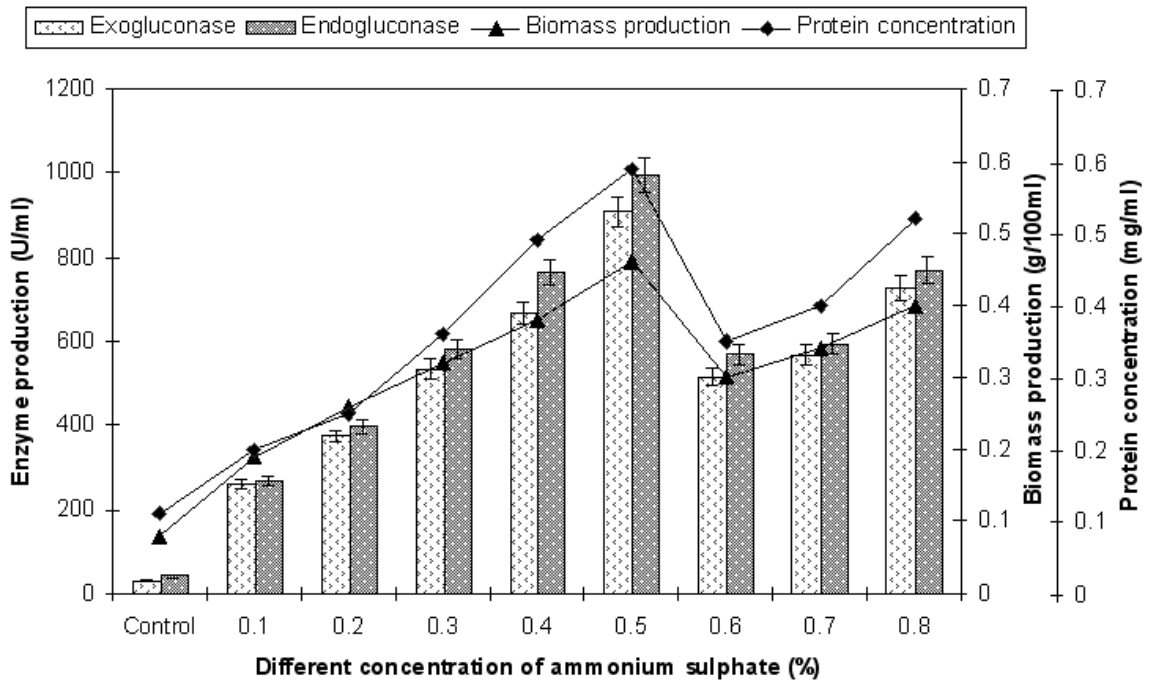

Fig. 8. Effect of different concentrations of ammonium sulphate on cellulase production; the control flask does not contain ammonium sulphate. Test flasks were supplemented with different concentrations of ammonium sulphate (0.1 to 0.8 % w/v) in the medium, which were inoculated and incubated at 50 °C for 72 hours at pH 5.5. Error bars presented are mean values of ± standard deviation of duplicates of three independent experiments.

Cellulases are the key enzyme complex for the saccharification of cellulosic biomass. For an efficient saccharification, all three enzymes *viz.* C_1, Cx, and β glucosidase are required by a single thermotolerant microorganism in order to reduce the fermentation cost (Durrani *et al.* 2002; Lin and Tanaka 2006).

In the present investigation, an efficient, thermotolerant yeast was isolated, which has wide adaptability at varying temperatures, pH values, and also requires microaerophilic conditions for higher production of C_1 and Cx cellulases. For ethanol production, saccharification of cellulose, and simultaneously the production of glucose, can easily be fermented to ethanol by suitable *Saccharomyces* spp. for power alcohol. In this regard, there are few reports on the use of such yeast, which has good capability to produce C_1 and Cx enzyme for cellulose hydrolysis.

Temperature is one of the critical factors that profoundly influence the production of end product. The temperature for cellulase production of *Candida* was optimized. The production of cellulases by *Candida* in fermentation medium at different temperatures (30 to 60 °C) was carried out. Maximum C_1 and C_X activities were achieved at 50 °C (Fig. 1). Further increase in temperature resulted in a decrease in cellulase production. Similarly, Jang and Chen (2003) reported higher cellulase production at 50 °C by *Streptomyces* T3-1. In contrast, Pourramezan *et al.* (2009) reported optimum temperature for higher cellulase production was 30 °C and the lowest yield was achieved at 45 °C. Alam *et al.* (2004) also reported high cellulase production with maximum growth at 35 and 40 °C by the yeast *Streptomyces omiyaensis.*

The influence of pH on enzyme production was found to be an important parameter (Odeniyi *et al.* 2009). Our strain showed maximum enzyme production at pH 5.5. Moreover, reduction in enzyme production resulted above and below this pH value. The instability of enzymes at very low or very high pH values is due to the fact that they are proteins that are generally denatured at extreme pH values (Steiner *et al.* 1994). Haltrich *et al.* (1996) also reported that high acidic and high basic pH values adversely affected enzyme production, but a medium with acidic pH 5 was ideal for enzyme of fungal cultures. The cellulase enzyme has been reported to have a broad range of pH activity (pH 4 to 7) with optimal results at pH 5.0, which is close to the optimum pH value of most *Bacillus* cellulases (Fukumori *et al.* 1985). It has been found that cellulases from *T. reesei* work better in a more acidic pH (4.5 to 5.0), and that a maximum production of cellulase occurs at pH 4.5 (Yang *et al.* 2004; Gomes *et al.* 2006). It is well established that production of enzymes at different pH values depends upon the nature of strain, especially in the variation of metabolic activities, architecture of the microbial cell, and specific genetic diversity.

Cellulase production by *Candida* sp. was significantly influenced by the type of carbon source present in the basal medium. Glucose was the most effective as a sole carbon source for cellulase enzyme production. Increased enzyme activities for C_1 (355.5 U/mL) and Cx (345.4 U/mL) were obtained in culture medium containing 1.0% of glucose. Cellulase production increased with increases in initial glucose concentration from 0.1 to 0.5% while further increases in glucose concentration slightly reduced the yield. Mandels and Reese (1957) also reported that maximum yields of cellulase were obtained on 1% different carbon substrate using *T. viride.* Gautam *et al.* (2010) also reported maximum cellulase production from glucose at 1% concentration by *Trichoderma viride.*

To evaluate the effect of nitrogen source on cellulase formation, different organic and inorganic nitrogen sources were supplemented in the basal medium. Data revealed that supplementation with organic and inorganic nitrogen sources stimulated the cellulase production. The maximum enzyme production was obtained with malt extract (1.0%) in the case of organic nitrogen sources, which brought about an improvement in cellulase components, including C_1 and Cx. But in the case of inorganic nitrogen sources, ammonium sulphate gave maximum cellulase production as compared to malt extract *Candida* sp. It was reported that good cellulase yield can be obtained with ammonium compound as the nitrogen source. Ammonium compounds are reported to be the most favorable nitrogen compounds for protein and enzyme synthesis (Rajoka 2004). Though the addition of organic nitrogen sources such as beef extract and peptone resulted in increased growth and enzyme production, it has been reported that they are not an effective replacement for inorganic nitrogen sources because of their higher cost (Sun *et al.* 1999).

In the present study, a strain of yeast has been isolated, which has the capability to produce very high quantity of cellulases at wider physico-chemical and nutritional levels. Hence, this isolate may have the potential to be used at an industrial level. Moreover, several other efficient strains are under trial for higher saccharification of lignocellulosic biomass by mix culture, with capability of producing xylanases and cellulases.

CONCLUSIONS

The present work showed that cellulase production by *Candida* sp. was increased by culturing this strain in bagasse medium. It was found that bagasse and ammonium sulphate can be used to generate cellulases by yeast without costly pretreatment or nutrient supplementation. Medium optimization greatly enhanced the cellulolytic enzyme yield. Bagasse (4% w/v) showed excellent potential as a substrate for cellulases production. 0.5% glucose and 0.1% ammonium sulphate gave higher cellulases production at pH 5.5, 50 °C, and 72 h of incubation. The present research indicated that *Candida* sp. effectively produced cellulases and could be utilized for industrial production of cellulase. The possibility of using locally available substrates such as bagasse for cellulases production was promising.

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