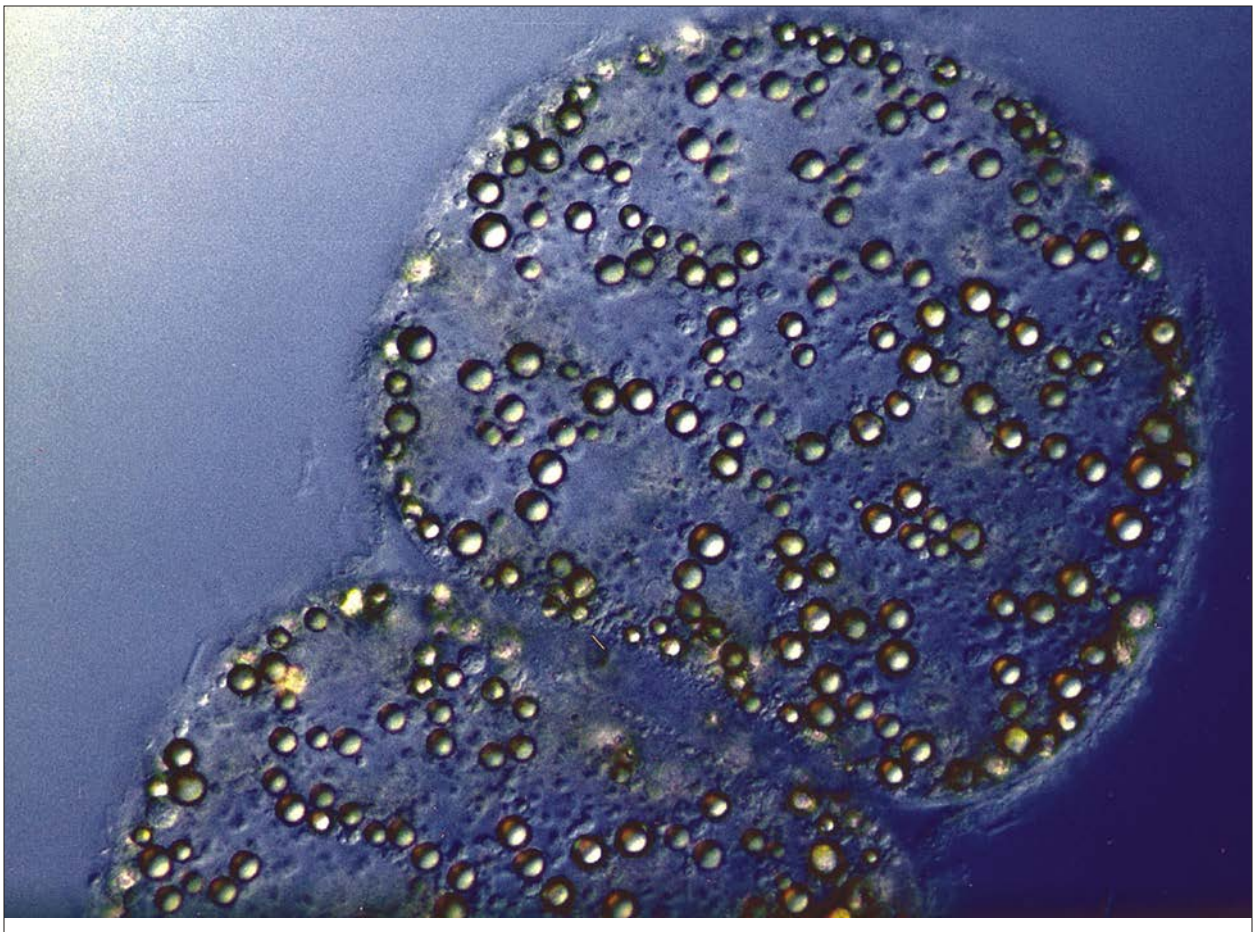

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Exploitation of Agro-Industrial Wastes as Substrates for Cellulase Production by *Bacillus licheniformis* MTCC 429

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ABSTRACT

The aim of the present investigation was to exploit the cheap agro-waste, sugarcane bagasse as a substrate for cellulase enzyme production using *Bacillus licheniformis* MTCC 429. The cellulase producing ability of *B. licheniformis* MTCC 429 was assessed using CMC agar plates. The factors affecting cellulase production were optimized by varying the parameters of incubation period, temperature and pH. The maximum cellulase enzyme production was achieved when the production medium pH was maintained at pH 7.0, temperature of 35°C with an incubation time of 48 h. Among the different concentration of sugarcane bagasse hydrolysate tested, CMC medium amended with 5% seemed to exhibit maximum cellulase productivity. Further, the partially purified enzyme was tested for its pH and temperature stability. The properties presented by *B. licheniformis* MTCC 429 suggest that the strain showed enhanced production of lignocellulose-degrading cellulase enzyme under the optimized conditions. The temperature and pH stability of the enzyme suggested its potential for industrial applications involving elevated conditions.

Key words: *Bacillus licheniformis*, sugarcane bagasse, cellulase enzyme

INTRODUCTION

Cellulose is the major constituent of the plant cell wall responsible for their structural framework. Although, we exploited enormous natural cellulosic sources, there are still surplus quantities of cellulosic sources that are not exploited more efficiently (Hao *et al*., 2006). With this context, it is necessary to develop an economically profitable process for the production of value added products from the unexploited cellulosic wastes. Cellulase, the enzyme hydrolyzing cellulose, plays a major role in global enzyme market. The production of cellulase by various microorganisms has been reported earlier (Shamala and Sreekantiah, 1986; Muniswaran and Charyulu, 1994).

Cellulosic wastes originate from both agricultural and industrial activities. The crop residue, crop-processing wastes and sawdusts come under agricultural cellulosic wastes. Previously, these wastes were viewed valueless and considered for disposal only. These waste materials can be exploited as raw materials for the production of potential value added products as suggested in this present investigation. The exploitation of cellulosic wastes as substrates possesses significant environmental, industrial and commercial implications. The agro-industrial waste has been previously exploited for the production of α-amylase using *Bacillus subtilis* CBTK 106 (Krishna and Chandrasekaran, 1996).

Among the various microorganisms reported for enzyme production, *Bacillus* species are considered as the microbial workhorses widely explored for multiple enzyme production. It is

estimated that 50% of global enzyme market depends on *Bacillus* spp. (Patagundi *et al*., 2014). There are several reasons to consider *Bacillus* spp. for the purpose including high growth rate, capacity to adapt to industrial processes and also can be augmented for increased enzyme production. Many researchers have exploited the extracellular enzyme production ability of various *Bacillus* spp. using submerged or solid state fermentation (Apun *et al*., 2000; Lee *et al*., 2008; Abdel-Mawgoud *et al*., 2008; Santhi, 2014). On this context, the present study was aimed to exploit *B. licheniformis* MTCC 429 for cellulase production ability using sugarcane bagasse as medium substrates.

MATERIALS AND METHODS

Microorganism: The bacterial strain, *Bacillus licheniformis* MTCC 429 procured from MTCC, IMTECH, Chandigarh was used for the present study. The culture was maintained on nutrient agar slants at 4°C until further use.

Cellulolytic activity of *B. licheniformis*: A loopful of *B. licheniformis* culture suspension was transferred into carboxymethyl cellulose (CMC) agar plates and incubated for 48 h. At the end of incubation, the CMC agar plates was flooded with 1% congo red and allowed to stand for 15 min. The plates were then counter stained with 1M NaCl and observed for the development of clearance zone.

Optimization studies: To determine the optimum incubation time for *B. licheniformis* for its maximum cellulolytic activity, 100 mL of sterile CMC broth containing (g L^{-1}) carboxymethylcellulose (0.5 g L^{-1}), $NaNO_3$ (0.1 g L^{-1}), K_2HPO_4 (0.1 g L^{-1}), $MgSO_4$ (0.05 g L^{-1}) and yeast extract (0.05 g L^{-1}) was prepared and inoculated with 1% (v/v) of *B. licheniformis* inoculum suspension (Kasana *et al*., 2008). The flasks were incubated in shaker (120 rpm) at 37°C. About 10 mL of culture medium was withdrawn from the production medium every 6 h upto 96 h duration. To study the optimum pH for the maximum cellulase production, sterile CMC broth with pH ranging from 5.5-8.5 with a gradation of 0.5 were prepared; inoculated with *B. licheniformis* and incubated for 48 h. Similarly, for the effect of incubation temperature on cellulolytic activity, different flasks with production medium was inoculated with *B. licheniformis* and incubated at temperatures ranging from 25-55°C with a gradation of 5ºC for 48 h. At the end of incubation time, the cellulolytic activity was assayed using the cell free supernatant which was obtained by centrifuging the broth at 8000 rpm for 20 min at 4°C.

Assay for cellulase activity: The cellulase activity in the culture supernatant was assayed directly by dinitrosalicyclic acid (DNS) method (Miller, 1959). About 0.5 mL of culture supernatant was added to 0.5 mL of 1% (w/v) CMC solution prepared in 10 mM sodium phosphate buffer and the reaction mixture was incubated at 70°C for 10 min. The DNS reagent was prepared by mixing the sodium potassium tartrate solution (45 g in 75 mL distilled. H_2O) and DNS solution (1.5 g in 30 mL of 2M NaOH) and making up the volume to 150 mL. After incubation, 1 mL of DNS reagent was added to the reaction mixture and was boiled for 5 min. The reaction mixture was cooled to room temperature and the absorbance was read spectrophotometrically at 540 nm. One unit (U) of cellulase activity represents the amount of enzyme required to release 1 μmol of glucose per minute under the assay conditions.

Cellulase production using sugarcane bagasse: Sugarcane bagasse was collected from a local juice market, oven dried, ground and sieved to obtain fine powder. The substrate was pretreated chemically by submerging the powder in 2% NaOH overnight at 37°C. The concentrated hydrolysate formed was detoxified using hot distilled water, wash along with calcium oxide till the pH reached 5.5. The preparation was further incubated at 30°C for 1 h at 200 rpm. At the end of the incubation period, the hydrolysate was filtered under vacuum and autoclaved. The sterilized hydrolysate was cooled and used for further studies (Ashfaque *et al.,* 2014). Different concentration (1-10%, w/v) of sterilized hydrolysate was amended aseptically in sterile CMC broth medium and the contents were mixed. The medium was then inoculated with *B. licheniformis* (1% inoculum) and incubated for 48 h at 35°C. At the end of the incubation, the cell free supernatant was assayed for cellulase activity.

Purification and characterization of cellulase: The cell free supernatant from the production medium amended with sugarcane bagasse hydrolysate was collected and precipitated with ammonium sulphate at 70% saturation. Then, it was dialyzed overnight against 50 mM acetate buffer (pH 5.0) and the resultant product was further subjected to ion exchange chromatography with DEAE Sephadex column (A-50). The phosphate buffer (50 mM, pH 7.0) was used as running buffer and 50 mM phosphate buffer with 1M NaCl (pH 7.0) was used as elution buffer for purification process (Bai *et al*., 2012). The fraction with maximum cellulase activity was used for stability studies.

Effect of pH and temperature on cellulase activity and stability: The partially purified enzyme obtained was further tested for its activity and stability at different pH and temperature. To study the cellulase activity at different pH, assay reaction mixture with different pH was prepared using buffers such as 0.1 M sodium acetate (pH 5-5.5), citrate phosphate (pH 6.0-6.5), sodium phosphate (pH 7.0-7.5) and 0.1 M Tris-HCl buffer (pH 8.0-9.0) and the enzyme activity estimated after 30 min. The stability of the enzyme was studied by pre-incubating the enzyme in different buffers at different pH for 30 min at 37°C. Similarly, the effect of temperature on enzyme activity was determined by incubating the reaction mixture at different temperatures (30-90°C) for 30 min and activity was assayed (Ladeira *et al*., 2015). The temperature stability was determined by measuring the residual activity of the enzyme after incubating the enzyme at various temperatures.

RESULTS

The development of clear zones around the bacterial colony indicated the cellulolytic ability of *B. licheniformis* MTCC 429. During the incubation time course study, maximum cellulolytic activity of 0.34 U mL^{-1} was obtained after 48 h of fermentation (Fig. 1), further increase in the process time results in low yield of enzyme. As a result, 48 h was chosen as an optimum incubation time for further studies. Similarly, the maximum cellulase production was attained in medium with an initial pH of 7.0 (Fig. 2). The temperature is also a factor impinging the enzyme production by bacteria and maximum enzyme production was recorded at 35°C (Fig. 3). Different concentrations of sugarcane bagasse hydrolysate amended in the CMC medium was investigated for better cellulase production. Based on the results obtained, 5% (w/v) of sugarcane bagasse hydrolysate yielded maximum cellulolytic activity of *B. licheniformis* MTCC 429. However, further increase in

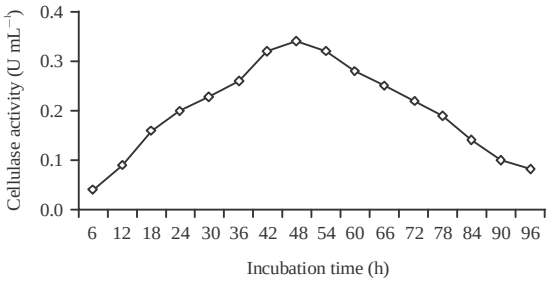

Fig. 1: Effect of incubation time on cellulase production by *B. licheniformis* MTCC 429

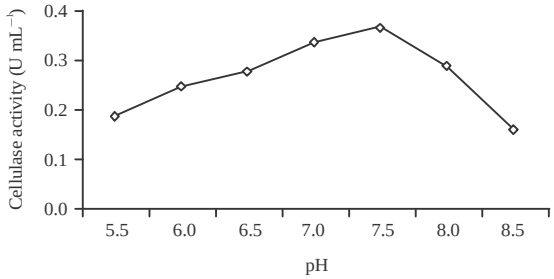

Fig. 2: Effect of pH on cellulase production by *B. licheniformis* MTCC 429

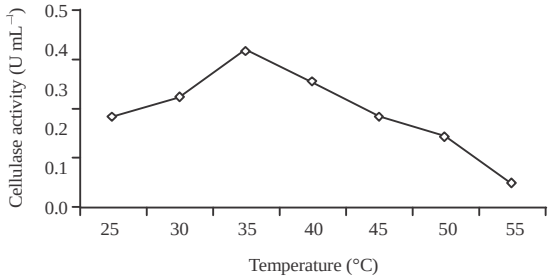

Fig. 3: Effect of temperature on cellulase production by *B. licheniformis* MTCC 429

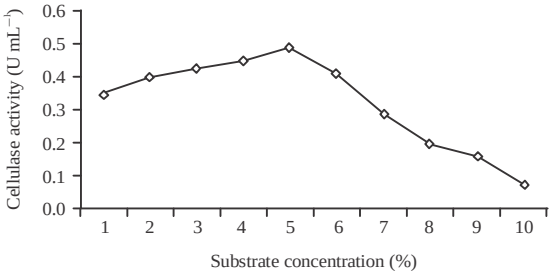

Fig. 4: Effect of concentration of sugarcane bagasse hydrolysate on cellulase productivity

the sugarcane bagasse hydrolysate concentration (more than 5%) resulted in decrease in cellulase production (Fig. 4). These results evidenced the suitability of using sugarcane bagasse as substrate for the production of cellulase by *B. licheniformis* MTCC 429.

The two cellulose active fractions were pooled, concentrated and subjected to characterization studies. When tested for the effect of pH, the optimum pH for the cellulolytic activity of partially

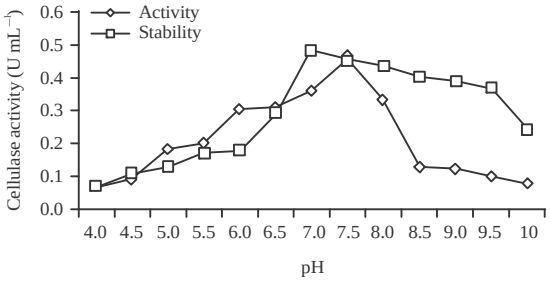

Fig. 5: Effect of pH on activity and stability of purified cellulase enzyme

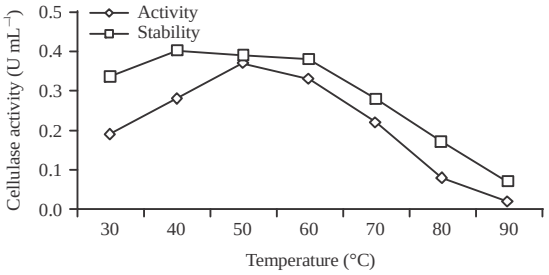

Fig. 6: Effect of temperature on activity and stability of purified cellulase enzyme

purified cellulase was 7.5. While testing the pH stability, the cellulase enzyme was found to be stable at a pH range between 7.0 and 9.0 (Fig. 5). The effect of temperature on the activity of partially purified cellulase from *Bacillus licheniformis* MTCC 429 was tested. The cellulolytic activity was increased with temperature upto 50°C and beyond that reduction in cellulolytic activity was observed. The optimum temperature of cellulase enzyme activity was found at 50°C. The role of the temperature in the stability of cellulase enzyme was also analyzed. The cellulase enzyme remained stable up to 60°C and lost its activity thereafter (Fig. 6).

DISCUSSION

Incubation time was found to be having an important role in the cellulase production. Behera *et al.* (2014) have also reported similar trend in cellulase production using *Bacillus* spp. The decrease in cellulolytic activity after 48 h might be due to either depletion of nutrients in the medium. The alteration in pH due to fermentation might also be the reason for decrease in cellulase activity which causes the denaturation of cellulase enzyme (Dave *et al*., 2015).

Among all the process parameters considered during enzyme production, pH seems to be of major interest because the metabolic activities of the bacteria were sensitive to even slight changes in pH (Juhasz *et al.*, 2004). There exists a strong relationship between the pH of the medium and enzyme production with considerable variation in cellulase production with even slight changes in the production medium. Similar results were obtained with *Bacillus* spp. which shown pH 7.5 as optimum for maximum cellulase productivity (Kim *et al*., 2005). In the present study, maximum yield of enzyme was observed at 35°C, which is in line with those of cellulase production using the *B. subtilis* (Shabeb *et al*., 2010). Several available reports revealed that the maximum cellulase production was achieved at 35-40ºC by *Bacillus* spp. (Ahmad *et al*., 2004; Nakasaki and Adachi, 2003) and *Pseudomonas flourescens* (Bakare *et al*., 2005).

Among the various lignocellulosic wastes tested, sugar cane baggase was found to be a better substrate for the extracellulase production by *B. licheniformis* MTCC 429. Waseem *et al.* (2014)

have also screened various agro-wastes and found cotton stalk as better substrate for cellulase production by *Bacillus* spp. isolated from soil samples. The incorporation of soyabean, banana fruit stalk and wheat bran favoured maximum cellulase productivity by *B. subtilis* and *B. pumilus* (Poorna and Prema, 2007). It is very essential to determine the ability of the enzyme to withstand different conditions; therefore stability of proteins against different factors is very important before applying to any industries. In the present study, optimum pH and temperature for the cellulolytic activity of partially purified cellulase was found at 7.5 and 50°C, respectively. The endoglucanase produced by alkaliphilic *Bacillus* spp. JAM-KU 023, shown to have maximum cellulolytic activity at the optimal pH range from 7-9.5 (Dave *et al*., 2015). Since, most of the industrial process involves elevated temperatures, thermostable enzymes stable at elevated temperatures are mostly preferred (Rastogi *et al*., 2010). The thermostable cellulase enzyme produced by *B. licheniformis* DR, isolated from hot spring showed optimum cellulase yield at 50°C and the enzyme was found to be stable up to 75°C for 30 min (Irfan *et al*., 2012).

CONCLUSION

The *B. licheniformis* MTCC 429 produced significant amount of cellulase under optimum conditions using sugarcane bagasse. The pH and temperature stabilities of the purified enzyme indicated their potential applications in various industries. The results of the present study indicated the suitability of cheap and easily available sugarcane bagasse as substrate for the production of cellulase in submerged fermentation. The usage of such cheap substrates minimizes the production cost of the enzymes. The accumulation of such waste poses a serious environmental problem and hence, maximum utilization of such agro-waste can also contribute to their efficient solid-waste management.

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