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Optimization of process parameters for xylanase production by *Bacillus* sp. in submerged fermentation

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ABSTRACT

In this study an attempt was made to optimize the cultural and nutritional conditions for xylanase production by Bacillus species in submerge fermentation process. Whole fermentation process was carried out in 250 ml Erlenmeyer flask with agitation speed of 140 rpm. *Bacillus subtilis* exhibit maximum xylanase production at initial medium pH of 8, substrate concentration of 2% with inoculum size of 2% at 35 °C for 48 h of fermentation period. Further supplementation of sucrose, $(NH_4)_2SO_4$ and peptone as a carbon and nitrogen sources favored enzyme production. The other strain *Bacillus megaterium* showed its peak xylanase production at initial medium pH of 8, inoculum size of 1.5% with substrate concentration of 1.5% at incubation temperature of 40 °C for 72 h of fermentation period. The best carbon and nitrogen sources are xylose, KNO_3 and malt extract. Both strains can also utilize molasses at 0.5% concentration for xylanase production can grow in medium containing 0.2% NaCl (*B. subtilis* BS04) and 0.8% NaCl (*B. megaterium* BM07) respectively. The optimum temperature of xylanase was 50 °C and pH was 5 and 5.5 by *B. subtilis* BS04 and *B. megaterium* BM07 respectively.

1. Introduction

Xylanase (E.C 3.2.1.8) is the enzyme which degrades β-1, 4 xylan by cleaving β-1, 4 glycosidic linkages thus forming usable products such as xylose, xylobiose like xylo-oligosaccharides (Bernier, Desrochers, Jurasek, & Paice, 1983; Chakrit, Khin, & Khanok, 2006). In annual plants and hardwoods, xylan is the most abundant non-cellulosic polysaccharide which accounts for 20–35% of the total dry weight in biomass (Bernier et al., 1983; Elegir, Szakacs, & Jeffries, 1994). Xylanases can be produced by bacteria and fungi in both liquid culture and solid culture. The production of microbial xylanases is preferred over plant and animal sources, because of their availability, structural stability and easy genetic manipulation (Bilgrami & Pandy, 1992). Mostly bacteria especially from the genus *Bacillus* are world widely used for the production of extracellular hemicellulases (Coughlan & Hazlewood, 1993; Srinivasan & Meenakshi, 1999).

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Mostly the production of xylanases has been studied in submerged liquid culture but there are few reports concerning the xylanase production in solid state fermentation using lignocellulosic wastes (Couri, Terzi, Pinto, Freitas, & Costa, 2000; de Souza, de Souza, & Peralta, 2001; Kalogeris, Christakopoulos, Kekos, & MacRis, 1998). Submerged fermentation process is mostly preferable because of more nutrients availability, sufficient oxygen supply and less time required for the fermentation than other fermentation techniques (Gomes, Gomes, & Stiener, 1994; Gouda, 2000; Hoq, Hempel, & Deckwer, 1994; Veluz, Taksuo, Hiroshi, & Yusaku, 1999). Today, xylanases production is highly important due to its industrial exploitation like, pulp and paper industry, baking industry, clarification of juices and also in liquification of fruits and vegetables (Gupta & Kar, 2009). In this study we reported here the xylanase production from *Bacillus subtilis* BS04 and *Bacillus megaterium* BM07 in submerged fermentation. To our knowledge this is the first report on xylanase production by *B. megaterium* in submerged fermentation.

2. Materials and methods

2.1. Procurement of substrates

Sugarcane bagasse was procured from local market of Lahore, city and used as substrate for xylanase enzyme production in submerged fermentation.

2.2. Bacterial strain

Two bacterial strains *B. subtilis* BS04 and *B. megaterium* BM07 were locally isolated from soil. The strain was maintained on nutrient agar (Oxoid) slants and store at 4 °C.

2.3. Cultivation of bacterial cells

Twenty five milliliter of nutrient broth (Oxoid) was sterilized in each two 250 ml Erlenmeyer flask at 121 °C for 15 min. After sterilization, the media was inoculated with a loopful of 24 h old strains of *B. subtilis* BS04 and *B. megaterium* BM07 incubated at 37 °C for 24 h with the agitation speed of 140 rpm. Vegetative cells were used as a source of inoculum throughout the study.

2.4. Fermentation technique

Twenty five milliliter of fermentation media (g/l: Sucrose 20, K_2HPO_4 0.5, NaCl 0.2, $MgSO_4.7H_2O$ 0.16, Yeast extract 0.5.) with 2% substrate (sugarcane bagasse) were sterilized in each 250 ml Erlenmeyer flask at 121 °C for 15 min. After sterilization, the media was inoculated with 2% solution containing vegetative cells of 24 h old *B. subtilis* BS04 and *B. megaterium* BM07 in each flask and incubated at 37 °C for 48 h of fermentation period with the agitation speed of 140 rpm.

2.5. Preparation of enzyme

After the termination of fermentation period, the fermented broth was filtered through muslin cloth and finally by centrifugation at 4 °C, 8000 × g for 10 min to remove the bacterial cells and unwanted particles. The clear filtrate obtained after centrifugation was used as a source of crude enzyme.

2.6. Assay of xylanase enzyme

Xylanase enzyme in the culture filtrate was estimated as reported earlier (Irfan, Nadeem, Syed, & Baig, 2012). Reaction mixture containing 0.5 ml of appropriately diluted culture filtrate with 0.5 ml of 1% birchwood xylan (Sigma) solution prepared in citrate buffer (0.05 M, pH5.0) was incubated for 15 min at 50 °C. After incubation the reaction was stopped by the addition of 1.75 ml of 3, 5 dinitrosalicylic acid and heated for 10 min in boiling water bath. After cooling the reducing sugars liberated were measured by spectrophotometrically at 550 nm and expressed as xylose equivalent. Xylose was taken as standard. One unit enzyme activity was defined as the amount of enzyme required to produce 1 μmole reducing sugar as a xylose equivalent per minute under standard assay conditions. Units were calculated by using following formulae.

$$\text{Xylanase activity (IU)} = \frac{\text{Reducing sugars (mg/ml)} \times 1000}{\text{Incubation time (15 min)} \times 150}$$

2.7. Optimization of cultural and nutritional conditions for xylanase production

Different cultural conditions like time course of fermentation (24–120 h), initial medium pH (4–10), incubation temperature (25–50 °C), inoculum size (0.5–3%), substrate concentration (0.5–3.0%) and various nutritional conditions such as additional carbon sources (glucose, sucrose, fructose, CMC, arabinose & xylose), nitrogen sources (KNO_3, $NaNO_3$, $(NH_4)_2SO_4$,NH_4Cl, ammonium citrate, peptone, yeast extract, tryptone, Malt extract & urea) molasses supplementation (0.5–3.0%) and NaCl concentration (0.2–1.0%) were optimized for enhanced production of xylanase by two tested strains of *B. subtilis* BS04 and *B. megaterium* BM07 in submerged fermentation process.

2.8. Effect of pH on activity of xylanase

The optimum pH for the enzyme was determined by incubating crude enzyme with substrate (1% xylan) prepared in appropriate buffers; 0.05 M citrate buffer (pH 3.0–6.0), 0.05 M sodium phosphate buffer (pH 6.0–8.0), 0.05 M Tris–HCl (pH 8.0–9.0) and 0.05 M glycine-NaOH (pH 9.0–11.0). Enzyme and substrate was incubated for 30 min at 50 °C. After incubation the reaction was stopped by the addition of DNS reagent and absorbance was measured at 550 nm.

2.9. Effect of temperature on activity of xylanase

The effect of temperature on activity of Xylanase was determined by incubating crude enzyme mixture in 1% xylan in 0.05 M citrate buffer, pH 4.8. at temperatures between 40 and 90 °C with regular interval of 5 °C. Enzyme activity was