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Cassava bagasse - Low cost substrate for thermo-tolerant xylanase production using Bacillus subtilis.

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Abstract: The present study is mainly focused on the production of alkaline tolerant and thermostable xylanase by *Bacillus subtilis* in submerged fermentation. To make process economical, the production of xylanase was carried out using various agricultural wastes such as wheat bran, wheat flour and cassava bagasse as a carbon source. Enzyme production and activity was maximum using cassava bagasse as a carbon source. Then screening of nitrogen source was done according to enzyme activity using cassava bagasse as a carbon source. It was found that yeast extract was the best nitrogen source. Effect of initial pH and temperature on enzyme activity was studied and maximum enzyme activity was obtained at pH 8 and 60^0C. Then effect of substrate concentration on xylanase activity was investigated at 60^0C and pH 8 and Michaelis-Menten parameters K_m and V_m for xylanase were determined to be 1.91g/l and 0.11 g/l min. Screening of metal ions on enzyme activity was carried out to enhance the enzyme activity. Xylanase activity was increased by metal ions such as Mg^{2+}, Ca^{2+}, Fe^{2+}, Zn^{2+} and Mn^{2+} , where as the activity of enzyme was decreased by Cu^{2+} and Hg^{2+} ions.
Keywords: xylanase, enzyme kinetics, metal ions, submerged fermentation.

Introduction

Xylan is a major constituent of hemicelluose and hetero polymer containing D-xylose monomer attached by β-1-4-glycosyl bond; it also has branches of glucuronic acid, arabinose, acetyl residues or mannose[1]. Endo-β-1,4, xylanase is used for depolymerisation of xylan. Xylanase is broadly used in industries for bio bleaching and bio pulping, lignocellulose bioconversion, vegetable processing, fruit processing, food, textile and detergent[2]. In paper and pulp industries, xylanase without cellulase plays a vital role and provides an eco-friendly process than chemical beaching[2].

Xylanase production was investigated in submerged and solid state fermentation by microorganisms such as bacteria, actinomycetes and fungi[3-6]. Bacteria are widely used for xylanase production since less time

requirement for growth and lower risk for contamination. The application of xylanase produced from actinomycetes and fungi have been declined in most industrial processes, since xylanase from fungi and actinomycetes showed poor activity under high temperature and alkaline pH[7-8]. Xylanase have wide range of application, hence its production must be amplified from bacterial sources, because bacterial xylanase are stable and shows good activity at high temperature and pH[9-11]. In order to reduce cost of fermentation process for making the process economically feasible[4], agricultural-based crude substrate such as rice straw, rice bran and wheat bran have been used as a substrate[12, 13].

More amount of lignocellulose wastes are produced through an industrial process like cassava bagasse and utilized for xylanase production through fermentation process[14]. Due to greatest applications, it is essential to produce thermostable xylanase under economical condition. In this paper, cassava bagasse, an agro industrial residue has been selected as a substrate for the production of thermostable xylanase and optimized parameters such as substrate concentration, temperature, and metal ions for better enzyme activity.

Materials and Methods:

Microorganism:

Xylanase producer Bacillus subtilis MTCC 7086 was obtained from Microbial Type Culture Collection (MTCC), Chandigarh, India. This stock culture was introduced in to the growth medium containing beef extract: 1g/l, yeast extract: 2g/l, peptone: 5g/l, NaCl: 5g/l and kept in an incubated shaker at 30^0C and 150 rpm.

Preparation of carbon source

Agricultura wastages such as cracked wheat flour, wheat bran and cassava bagasse were used as a carbon source. Carbon sources were cleaned with water, dried, made into fine powder and then subjected to steam treatment. Then, production medium containing carbon source, yeast extract: 2g/l, peptone: 5g/l, $MgSO_4$:0.5g/l, NaCl: 0.5g/l, $CaCl_2$:0.15g/l was sterilized at 121^0 C and 15 psi. Before sterilization production medium was adjusted to pH 7.5 using 0.1N HCl or NaOH. 5%V/V inoculum was added to the sterilized production medium.

Screening of carbon source and nitrogen source on xylanase production

Carbon sources such as as wheat bran, wheat flour and cassava bagasse were taken for xylanase production. Then nitrogen sources such as yeast extract, beef extract and trypton were screened according to xylanase production.

Enzyme assay

After fermentation, sample was centrifuged at 10000 x g for 10min and temperature below 4°C. The supernatant collected was taken as the crude enzyme solution. Xylanase concentration was estimated by Lowry's method with serum bovine albumin as a standard[15]. Xylanase activity was determined by 1% (W/V) birchwood xylan (4-O-methylglucurono xylan) solution as a substrate[16]. The removal of reducing sugars in 10 min at 50 ˚C, pH 5.3 (0.05M citrate buffer) was estimated as xylose equivalents using the dinitrosalisylic acid method.[17]

Enzyme kinetic studies

Xylan degradation reaction was carried out at various pH conditions (4–9) for understanding the influence of initial pH. Then enzyme-substrate reaction was carried out at different temperatures ranging from 30°C to 90°C at optimum pH. Effect of substrate concentration on enzyme activity was performed at optimum pH and temperature for kinetic parameters evaluation. Screening of metal ions such as Fe^{2+}, Cu^{2+}, Ca^{2+}, Mg^{2+}, Hg^{2+}, Mn^{2+}, and Zn^{+2} on xylanase activity was studied.

Results And Discussion

Screening of carbon source on xylanase activity

Figure 1 shows the influence of carbon source for xylanase activity. Activity was maximum when medium containing cassava bagasse as the substrate. Using wheat bran, very low level of xylanase was achieved. In contrast Yasinok *et al*.[18] and Annamalai *et al*[19] explained maximum production of xylanase from wheat bran. High concentration of xylanase was obtained by wheat bran as a substrate using *Bacillus subtilis*-BS05 [20].

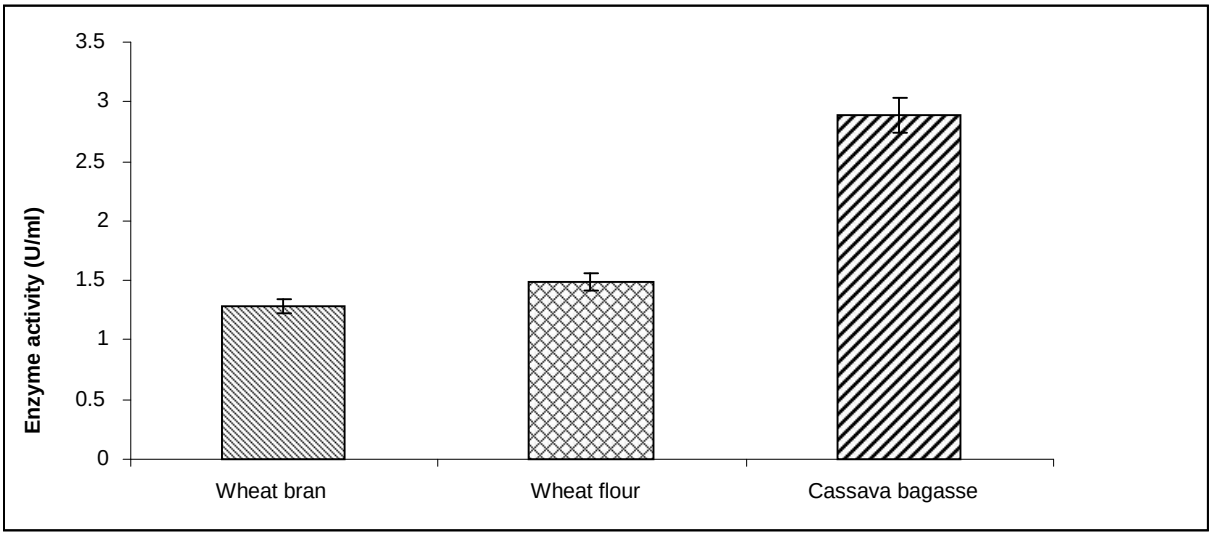

Figure 1: Influence of different carbon sources on xylanase production using *Bacillus subtilis* (±5% error bar)

Screening of nitrogen source on xylanase activity

Figure 2 shows the screening of nitrogen sources such as trypton, beef extract and yeast extract for maximum xylanase production. Maximum xylanase production was supported by yeast extract. Annamalai *et al.*[19] produced xylanase using *Bacillus subtilis* and yeast extract as the nitrogen source. Sharma *et al.*[21] have produced maximum concentration of xylanase using yeast extract. These results match with our findings.

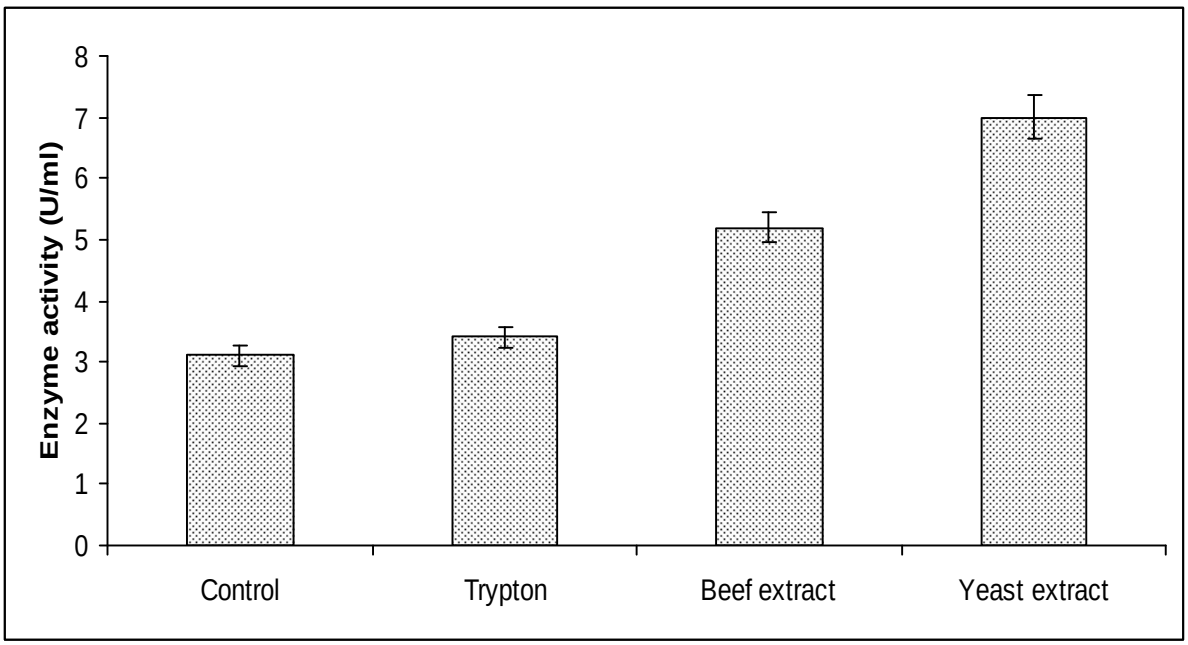

Figure 2: Influence of different nitrogen sources on xylanase production using *Bacillus subtilis* (±5% error bar)

Enzyme kinetics and Characterization studies

Effect of pH on enzyme activity

Enzyme activity was so high at pH 8 and decreased drastically and at pH 4 it was almost zero (Figure 3). Xylanase obtained from *Bacillus sp*. K1 [22]and Bacillus *sp*. C-125 [23]exhibited an optimum pH of 6. Cordeiro*et al.*[24] reported that optimum pH for xylanase activity was 6.5. Optimum pH for xylanase activity from *B. Stearothermophilus* was about 6-7[25]. Some xylanases isolated from *B.subtilus*[26] and *B.agaradhaereus*[27] was more active at pH 5.6. Yasinok*et al.*[18] have reported that the optimum pH as 7.5 for xylanase activity produced from *Bacillus sp.* Few xylanases obtained from *Enterobacter sp.* MTTC 5112 [28]and *Staphylococcus sp.*[29] have an optimum pH of 9.

Effect of temperature on enzyme activity

Optimum temperature for the enzyme activity was found to contain maximum at 60^0C and then decreased (Figure 4). Xylanase activity was so high at 50^0C using *streptomyces sp.*[30]. Xylanase obtained from *B.Pumilus*[18], *B. Stearothermophilus* T-6[25] and *Streptomyces sp.*[31] exhibited an optimum temperature 60^0C. Xylanase isolated from *Bacillus sp.,* was more active at 60 ºC and pH 9[22, 32.] Xylanase produced from *B. Circulans* AB 16 was more stable at 80^0C and less stable at 65 ^{0}C [33].

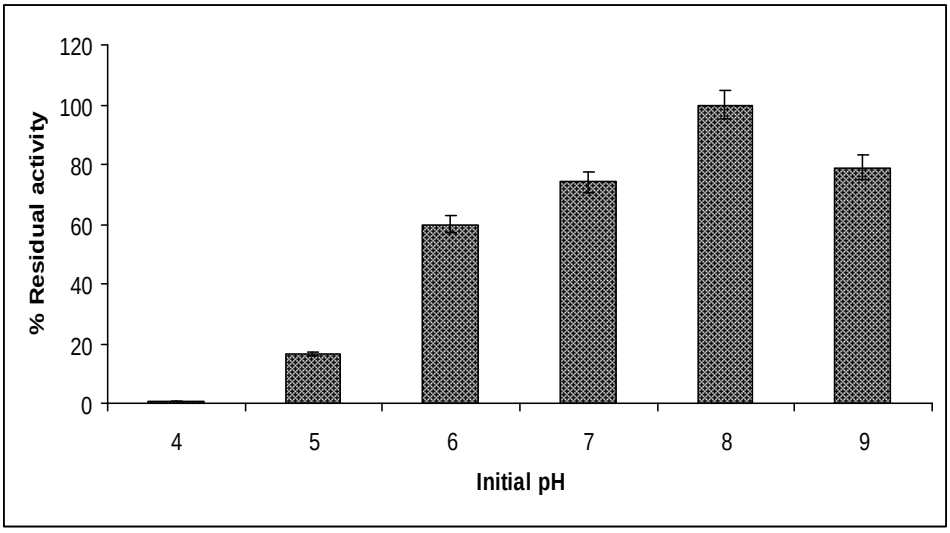

Figure 3: Effect of pH on enzyme activity (±5% error bar)

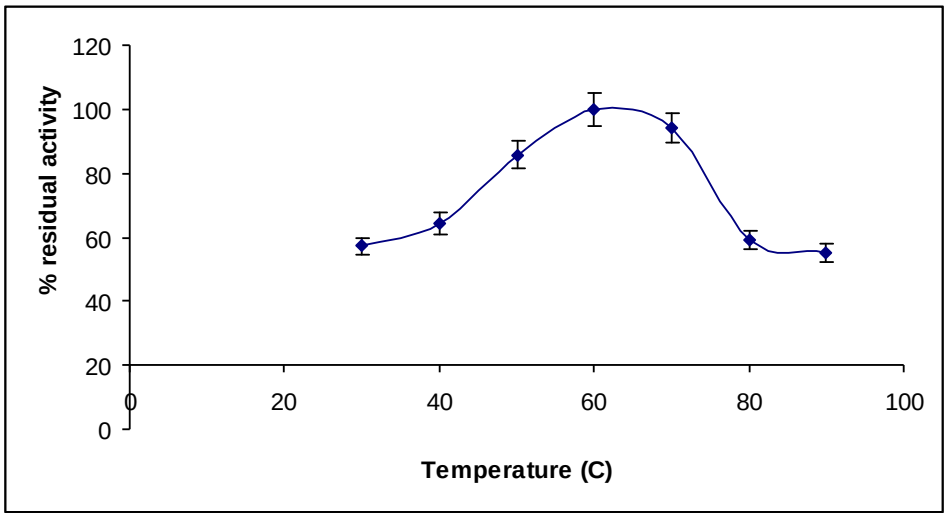

Figure 4: Effect of temperature on enzyme activity (±5% error bar)

Kinetic parameters determinations

Kinetic parameters such as Km and Vm values were determined at 60 °C and pH 8 using different concentrations of birch wood xylan as a substrate. The Km and Vm were found to be 1.81 g/L, and 90 U/ ml (Figure 5). Generally Km value for xylanases obtained from some microbial sources was relatively low (0.025-1.7 mg/ml)[10]. Low affinity of enzyme towards its substrate is indicated by high Km[34].
Monisha *et al.*[35] reported that Km and Vm were found to be 4 mg/ ml and 0.068 × 10^{-4} mM/min mg for partially purified xylanase from *Bacillus pumilus* which was comparatively lesser than the present study. Km and Vm values for xylanase from *Bacillus subtilis* ASH were found to contain 3.33 mg/ml and 100 IU/ml respectively[36].

Effect of metal ions on enzyme activity

Figure 6 shows the screening of metal ions on enzyme activity. Enzyme activity was stimulated by Zn^{+2}, Fe^{+2}, Mg^{+2} and Ca^{+2} whereas Cu^{2+}, Hg^{+2}inhibited xylanase activity. Annamalai *et al.*[19] reported that xylanase activity was increased in the presence of Ca^{+2}, Fe^{+2} and Mg^{+2}, whereas Hg^{+2} inhibited its activity. Thiagarajan *et al.*[37] Hg^{2+} inhibited xylanase activity. Our findings were consistent with their results.

Conclusion

In this report, production of xylanase was investigated by *Bacillus subtilis* using cassava bagasse in submerged fermentation. Maximum production was happened using cassava bagasse which makes the process economically viable. The enzyme was stable and more active in alkaline pH 8 and 60^0C. The alkaline and thermostable xylanase produced from the *Bacillus subtilis* appears to have potential for industrial applications.

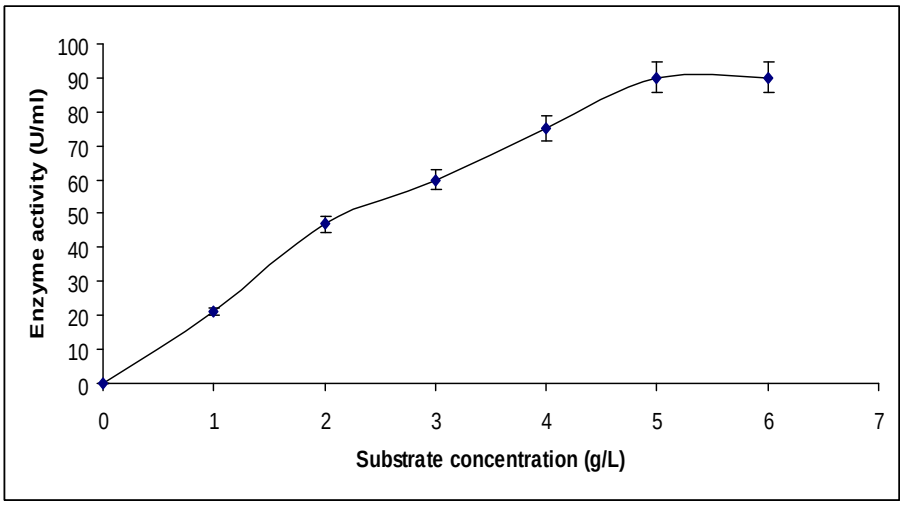

Figure 5: Effect of substrate concentration on xylanase activity (±5% error bar)

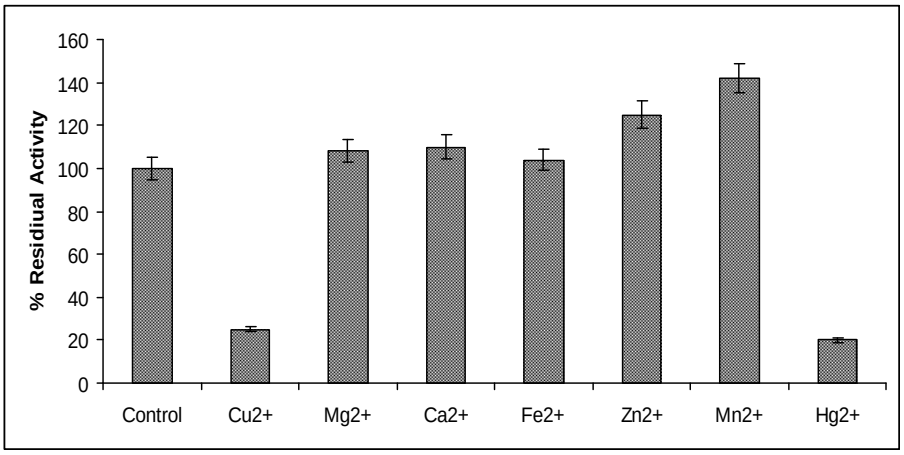

Figure 6: Effect of metal ions on enzyme activity (±5% error bar)

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