



## Amylase, Cellulase and Xylanase production from a novel bacterial isolate *Achromobacter xylosoxidans* isolated from marine environment

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### Abstract

A potentially novel aerobic and multi enzyme (i.e) amylase, cellulase and xylanase producing bacterium designated as *Achromobacter xylosoxidans* was isolated from marine environment. For these enzyme production different pretreated agro waste were used such as rice straw, cone straw, ragi straw, millet straw and sugarcane bagasse. Amylase production of 1095.6 U/ml/min was achieved under the optimized conditions of temperature of 35<sup>0</sup>C, pH 7, NaCl 3% and alkali pretreated substrate. Xylanase production was exhibited under the optimized conditions of temperature 40<sup>0</sup>C, pH 7, NaCl 4% and alkali pretreated substrate. Cellulase production of 64.8 U/ml/min was observed at 40<sup>0</sup>C, pH 7, NaCl 3% and alkali pretreated sugarcane bagasse. Among the pretreated substrates alkali pretreated sugarcane bagasse was the best substrate for these multi enzymes production.

**Keywords:** multi enzyme, *Achromobacter xylosoxidans*, agro waste, sugarcane bagasse.

### Introduction

Microorganisms are the most important sources for enzyme production. Selection of the right organism plays a key role in high yield of desirable enzymes. For production of enzymes for industrial use, isolation and characterization of new promising strains using cheap carbon and nitrogen source is a continuous process. Microorganisms have become increasingly important as producer of industrial enzymes. Due to their biochemical diversity and the enzyme concentrations may be increased by environmental and genetic manipulation, attempts are now being made to replace enzymes, which traditionally have been isolated from complex eukaryotes. Starch degrading amyolytic enzymes are most important in the biotechnology industries with huge application in food, fermentation, textile and paper (Alariya *et al.*, 2013).

The cellulase has three major hydrolases: the endo- -1,4-glucanases (EG I, EG II, EG III, EG IV, and

EG V; EC 3.2.1.4), which hydrolyze the glucosidic bonds randomly in cellulose fiber; the exo- -1,4-glucanases or cellobiohydrolases (CBH I and CBH II; EC 3.2.1.91), which act on the reducing and nonreducing ends of polymers, releasing cellobiose; and the -1,4-glucosidases (BG I and BG II; EC 3.2.1.21), which hydrolyze oligosaccharides and cellobiose into glucose (Chandra, *et al.*, 2010).

Xylanases are group of enzymes mainly consisting of endoxylanase (EC 3.2.1.8) which primarily cleaves -1, 4 linked xylan backbone and -xylosidase (EC 3.2.1.37) which converts xylooligomers to monomeric xylose sub unit (Ritter *et al.*, 2013). In addition to inducing cellulase production, cellulose induces the production of xylanases, which is attributed to the fact that the regulator of cellulase production, ACEII, also affects the regulation of xylanase production.

Though there are about 3000 enzymes known today, only few are industrially exploited. These are mainly extracellular hydrolytic enzymes, which degrade naturally occurring polymers such as starch, proteins, pectins and cellulose. In the production of glucose syrup, the  $\alpha$ -amylase is used in the first step of enzymatic degradation yielding a mixture of glucose and fructose with high fructose content. The amylases can be derived from several sources such as plants, animals and microbes. The microbial amylases meet industrial demands because it is economical when produced in large quantities (Alariya *et al.*, 2013). The application of lignocelluloses for ethanol production from agricultural and industrial wastes has led to extensive studies on amylase, cellulase and xylanase producing fungi and bacteria. The ideal pre-treatment would accomplish reduction in crystallinity, reduction in lignin content and increase in surface area (Prasad and Sethi, 2013).

Present work is focused on a novel multiple enzyme (i.e) amylase, cellulase and xylanase producing marine bacteria *Achromobacter xylosoxidans*. rice straw, sugarcane bagasse, corn straw, ragi straw and millet straw were subjected to evaluate the effect of pretreatment's on the amylase, cellulase and xylanase enzyme activity. This study will help to identify effective pre-treatment and the relative importance of individual pre-treatment on the rate of enzymatic hydrolysis of the lignocellulosic biomass.

## Materials and Methods

### Microorganisms

The bacterial strain *Achromobacter xylosoixdans*, was isolated from Vellar estuary, Portonovo (Lat. 11<sup>o</sup>29' N; Long. 79<sup>o</sup>46'), South East Coast of India.

### Substrate preparation

In an attempt to choose a potential substrate for submerged fermentation which supports enzyme production various agro residues like rice straw, sugarcane bagasse, corn straw, ragi straw and millet straw were treated using alkali and as well as acid.

### Alkali pretreatment

About 50g milled dried rice straw or corn straw or sugarcane bagasse was suspended in 5% NaOH in ratio of 1:10 (w/v) substrate and NaOH. After that the samples were incubated in water bath at 85<sup>o</sup>C for 1 hour (Yoswathana and Phuriphapat, 2010). Finally

pretreated sample was pressed through cheese cloth. The amount of reducing sugar in juice was measured as described by Miller (1959).

### Acid pretreatment

About 50g chopped dried substrates was suspended in acid solution (1% sulfuric acid) in ratio of 1:10 (W/V) substrate and sulfuric acid. The mixtures were autoclaved at 121<sup>o</sup> C for 15 min. (Yoswathana and Phuriphapat, 2010). After that the treated sample was pressed through cheese cloth and the amount of reducing sugar in juice was measured as above.

### Enzyme production

#### Submerged fermentation

Fermentation was performed in 250ml Erlenmeyer flask containing 10 g of substrates pretreated agro waste (alkali and acid treated rice straw, sugarcane bagasse, corn straw, ragi straw and millet straw) inoculated in to Mandel's medium (1976) which is used as mineral medium for this fermentation. The Mandel's medium was prepared with the following composition (g/l); Urea - 0.3; Peptone - 0.75; Yeast extract - 0.25; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> - 1.40; KH<sub>2</sub>PO<sub>4</sub> - 2.0; CaCl<sub>2</sub> - 0.3; MgSO<sub>4</sub>.7H<sub>2</sub>O - 0.3. And trace elements (mg/l) FeSO<sub>4</sub>.7H<sub>2</sub>O - 5; MnSO<sub>4</sub>.4H<sub>2</sub>O - 1.6; ZnSO<sub>4</sub>.7H<sub>2</sub>O - 1.4 and CoCl<sub>2</sub>.6H<sub>2</sub>O - 20.0 (Mandel's *et al.*, 1976). The trace elements were autoclaved separately and added after cooling of other components stabilized. The flask was cooled down at room temperature and a known amount of sterilized trace elements was added. The flasks were then inoculated with 5ml of the fresh bacterial culture and incubated for 2 days at the ambient temperature (37<sup>o</sup>C). Three replicates were used for each treatment.

### Optimization

#### Optimization of culture conditions

The factors such as temperature, pH, salinity, sources of carbon and nitrogen affecting production of amylase, cellulase and xylanase were optimized by varying parameters one at a time. The experiments were conducted in 200mL Erlenmeyer flask containing production medium. After sterilization by autoclaving, the flasks were cooled and inoculated with culture and maintained under various operational conditions separately such as temperatures (25<sup>o</sup>C, 30<sup>o</sup>C, 35<sup>o</sup>C, 40<sup>o</sup>C and 45<sup>o</sup>C), pH (4, 5, 6, 7, 8 and 9), NaCl (1%, 2%, 3%, 4% and 5%), carbon source

(starch, cellulose, xylose, sorbose, fructose, glucose, manitol, sucrose and galactose) and nitrogen source (ammonium nitrate, ammonium sulphate, sodium nitrate, urea, gelatin, casein, yeast extract and peptone). After incubation the cell free extract was assayed in triplicate samples for enzyme activity.

### Optimization of agro waste

To find out the suitability of agro- based waste as substrate for enzyme production. Different untreated and pretreated (acid and alkali) agro waste like rice straw, corn straw, ragi straw, millet straw and sugarcane bagasse were taken in a growth medium (Mandel's mineral medium) under submerged fermentation. The enzyme activity is measured of an incubation period of 24-48 hrs.

### Mass culture for enzyme production

Based on the optimization results for the mass scale cultures 1000ml of amylase production medium (pH 7; Temp  $-35^{\circ}\text{C}$ ; NaCl 3%; and alkali pretreated corn straw) cellulose production medium (pH 8; Temp  $40^{\circ}\text{C}$ ; NaCl 4%; alkali pretreated sugarcane bagasse) and xylanase production medium (pH 7; Temp  $40^{\circ}\text{C}$ ; NaCl 3%; alkali pretreated sugarcane bagasse) were prepared. After sterilization, 1ml of enzyme producing organism *A. xylosoxidans* containing approximately  $10^6$  cells/ml were inoculated and incubated for optimum temperature and optimum incubation period.

### Enzymes assay

#### Amylase activity

An amylase activity was determined as described by Okolo *et al.*, 1995. The reaction mixture consisted of 1.25 ml of 1% starch in 0.1 M acetate buffer; 0.2 ml of 0.1 M acetate buffer (pH 7.0); 0.25 ml of distilled water and 1.0 ml of crude enzyme. After 10 min. of incubation at  $50^{\circ}\text{C}$ , the liberated reducing sugar was estimated by the addition of 1ml of 3, 5 dinitrosalicylic acid (DNS) followed by boiling for 10min (Bernfield *et al.*, 1955) and the OD was measured at 540nm and one unit of the enzyme was defined as the enzyme required to release  $1\mu\text{mol}$  of reducing sugar in one minute under the assay conditions. All enzyme assays were performed in triplicates.

#### Cellulase activity

Carboxymethyl cellulose (CMCase) activity was determined according to the method of Mandels *et*

*al.*, 1976. 0.5 ml of 1% carboxymethyl cellulose (CMC) in 0.1 M citrate buffer pH 5.6 was placed in a test tube and 1.0 ml of culture filtrate was added. The test tube was incubated at  $40^{\circ}\text{C}$  in a water bath with shaker for 30 min. The reaction was terminated by adding 2.0 ml of 3,5 -dinitrosalicylic acid (DNS) reagent to the reaction mixture, boiled for 5 min. (Miller, 1959). The absorbance of the appropriately diluted reaction mixture was read at 540nm using a spectrophotometer. One unit of cellulase was defined as the amount of enzyme that released  $1\mu\text{mol}$  reducing sugar as glucose equivalent per min in the reaction mixture under the specified assay conditions. All enzyme assays were performed in triplicates.

#### Xylanase activity

Xylanase activity was determined according to the method of Bailey *et al.*, 1992. 0.5 ml of 0.8 % w/v of birch wood xylan (0.8g in 100 ml of 0.1M citrate buffer at pH 5.6) was placed in a test tube and 1 ml of enzyme filtrate was added. The reaction mixture was incubated in a water bath with shaker at  $50^{\circ}\text{C}$  for 30 min. and the reaction was terminated by the addition of 2.0ml of 3,5 - dinitrosalicylic acid (DNS) reagent to the reaction mixture heated for 5min at  $80^{\circ}\text{C}$  in a water bath. Absorbance was read at 540 nm using spectrophotometer to determine the concentration of sugar released by the enzyme. One unit (v) of xylanase was defined as the amount of enzyme that released  $1.0\mu\text{mol}$  reducing sugar as xylose equivalent per min in the reaction mixture under the specified assay conditions. All enzyme assays were performed in triplicates.

### Results

The present study was on the production of bacterial enzymes viz. amylase, cellulase and xylanase using cheaper substrates. When the pH was evaluated from 4 to 9, enzyme activity was in the range of pH 4 to pH 6 with the maximum recording amylase at pH 6 (481.1 U/ml/min), cellulase at pH 6 (17.8 U/ml/min) and xylanase at pH 6 (104.5 U/ml/min). Likewise when temperature range of  $25^{\circ}\text{C}$  to  $50^{\circ}\text{C}$  was tested, the enzyme activity was recorded. The xylanase enzyme activity was maximum at  $45^{\circ}\text{C}$  (108.6 U/ml/min) and minimum at  $25^{\circ}\text{C}$  (68.0 U/ml/min). For cellulase enzyme activity was maximum at  $40^{\circ}\text{C}$  (108.6 U/ml/min) and minimum at  $25^{\circ}\text{C}$  (19.0 U/ml/min). Amylase enzyme activity was maximum at  $40^{\circ}\text{C}$  (790 U/ml/min) and minimum at  $25^{\circ}\text{C}$  (320 U/ml/min). Likewise when NaCl concentration range of 1 % to 7% was tested, the enzyme activity was recorded. The

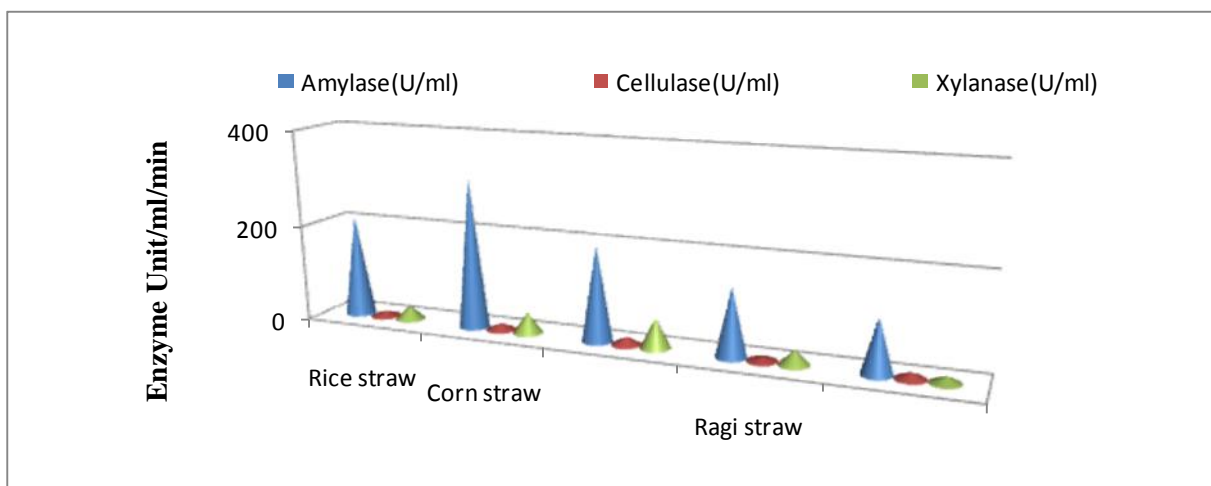
xylanase and amylase enzyme activity was maximum at 3% (107.3 U/ml/min and 489.4 U/ml/min). For cellulase enzyme activity was maximum at 4% (20.11 U/ml/min).

Among nine different carbon source tested starch was found to be best source (499.9 U/ml/min) followed by fructose (474.3 U/ml/min), maltose (432.9 U/ml/min) and glucose (354.3U/ml/min) for amylase activity. For cellulase activity, cellulose was found to be best source (38.5 U/ml/min), compared to other substrates. For xylanase enzyme activity, sucrose was found to be best source (139.1 U/ml/min) observed to other carbon sources. Likewise for amylase activity, gelatin was found to be best nitrogen source (476.8U/ml/min). For cellulase enzyme activity, urea (47.55 U/ml/min) was recorded to be the best nitrogen source followed by

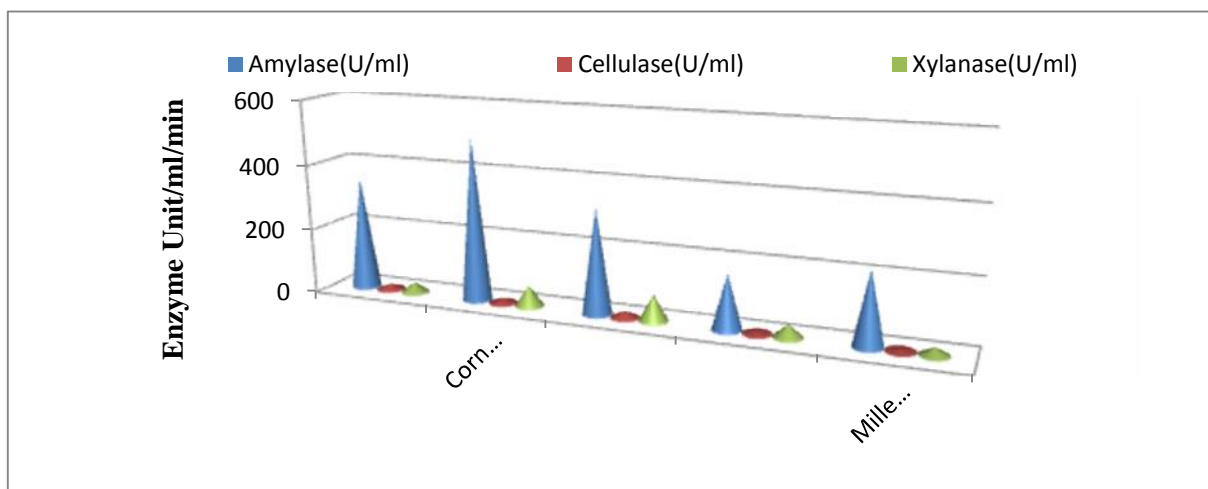
other nitrogen source. For xylanase, sodium nitrate was found to be a best nitrogen source when observed to other nitrogen sources.

Mass scale was done with the respective optimized parameters 1095.6 U/ml/min of amylase, 64.8 U/ml/min of cellulase and 145.54 U/ml/min of xylanase was obtained at 72 hrs.

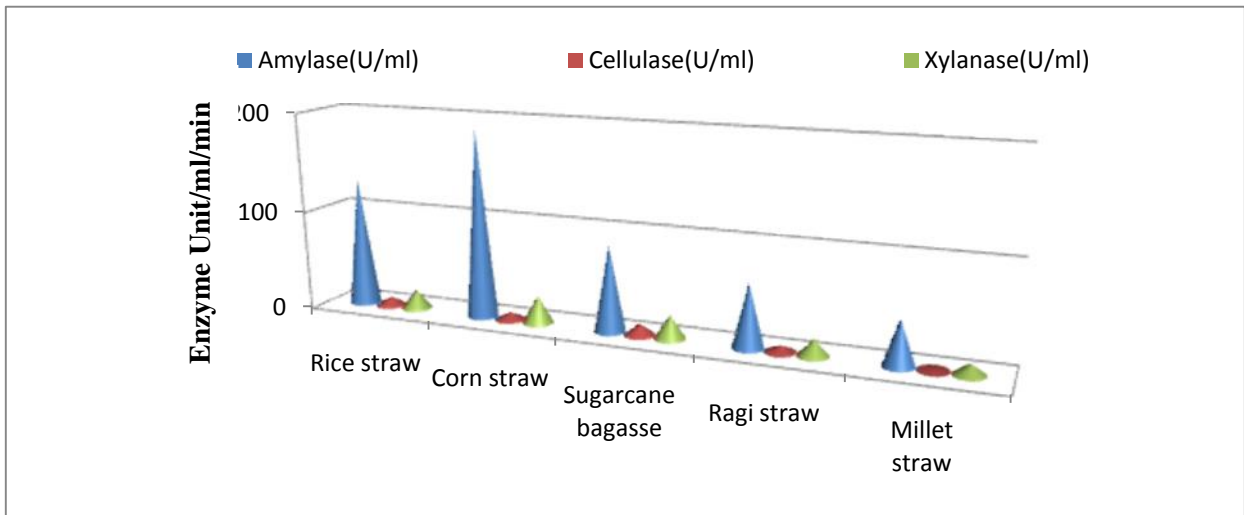
When carbon and nitrogen sources were replaced with 1% of corn straw was found to be the best substrate among substrates tested for amylase enzyme production. For cellulase and xylanase enzyme, sugarcane bagasse was observed as the best source followed by other substrates. Thus the study processed the potential of *A. xylosoxidans* strain to produce three enzymes using cheaper agricultural source.



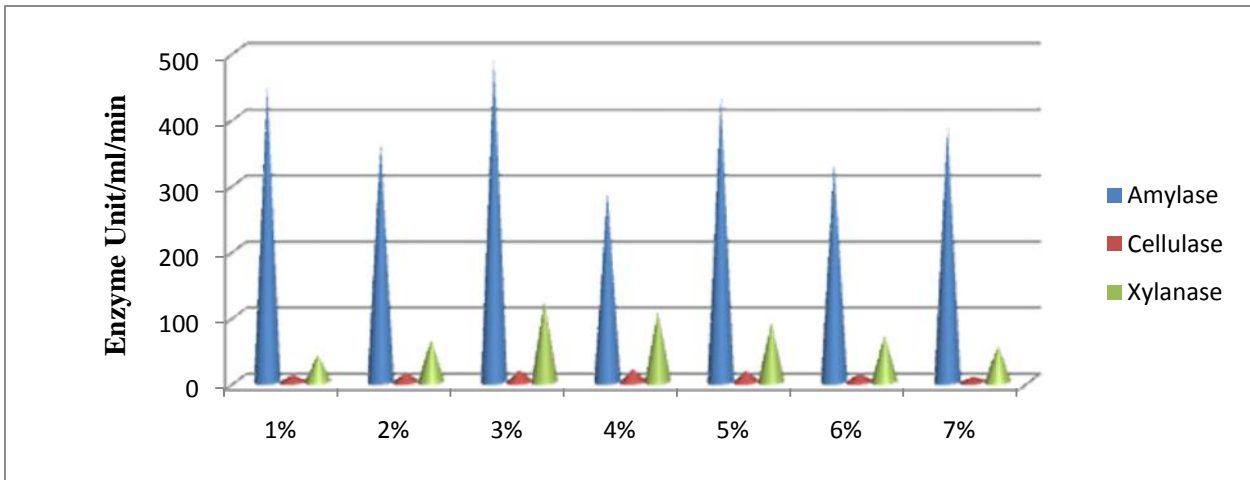
**Effect of Acid treated substrate of *Achromobacter xylosoxidans***



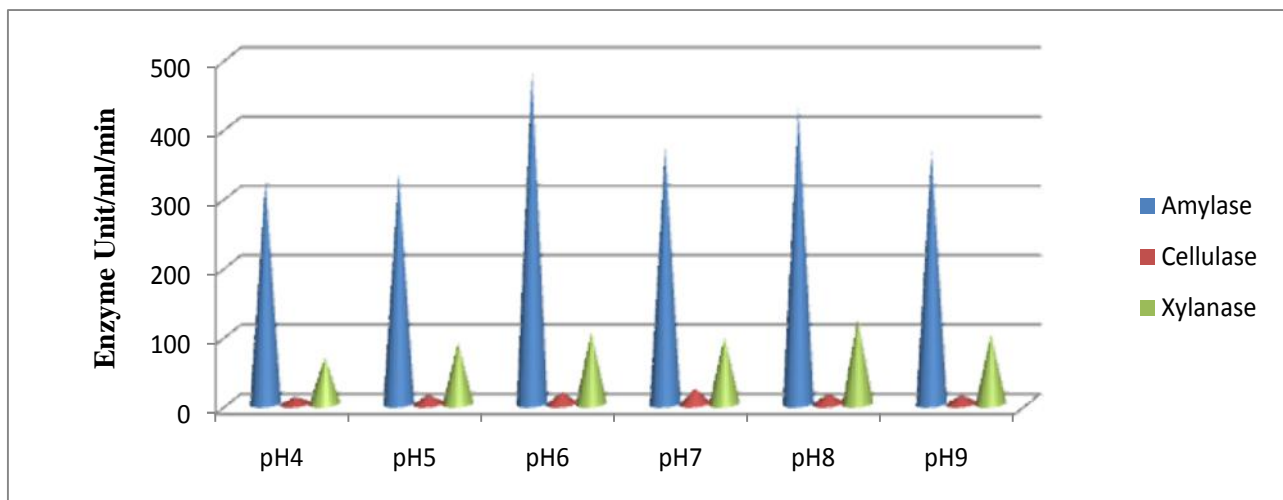
**Effect of Alkali treated substrate of *Achromobacter xylosoxidans***



Effect untreated substrate of *Achromobacter xylosoxidans*

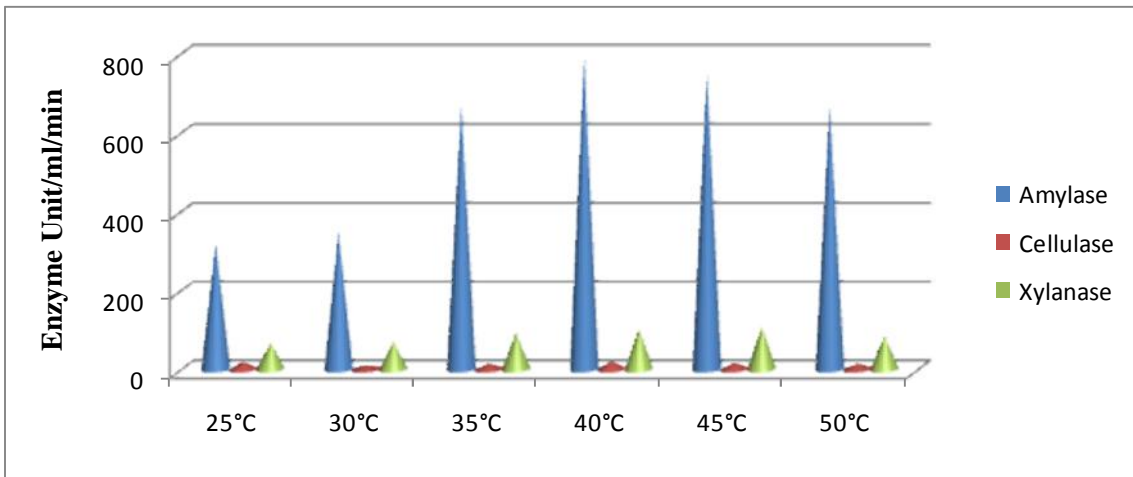


Effect of NaCl concentration of *Achromobacter xylosoxidans*

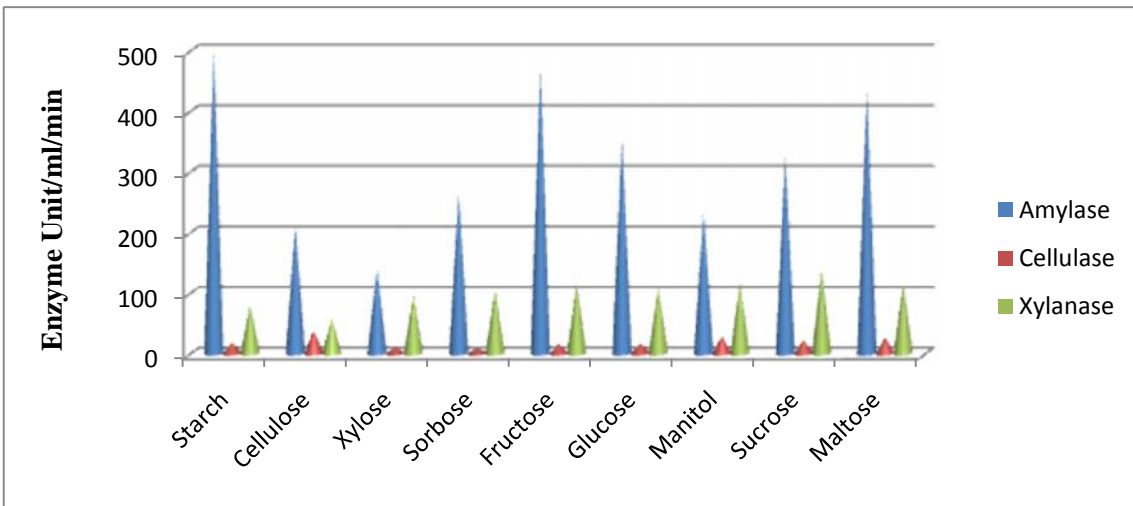


Effect of pH of *Achromobacter xylosoxidans*

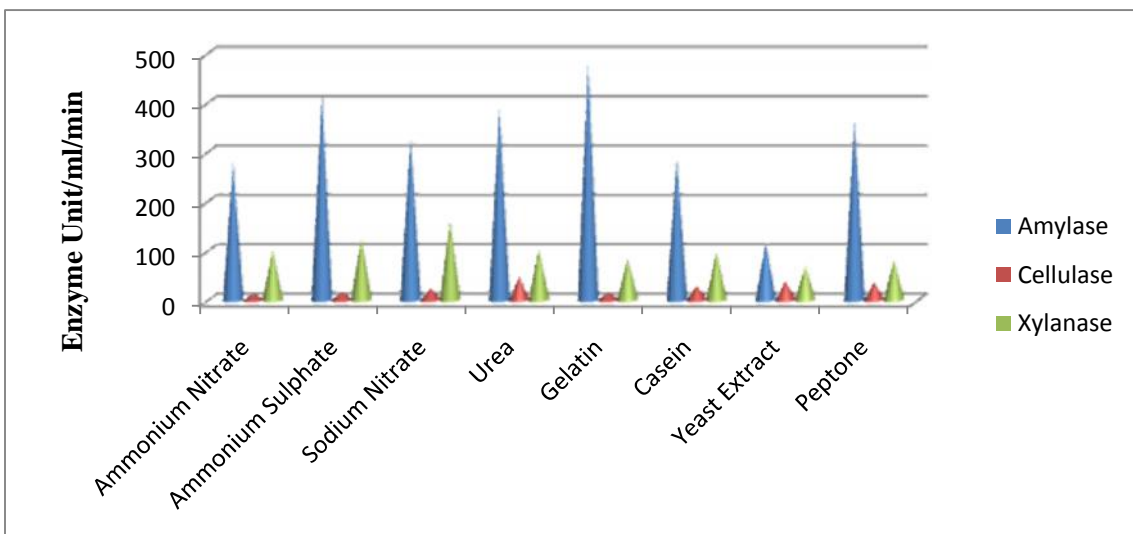




Effect of temperature of *Achromobacter xylooxidans*



Effect of Carbon source of *Achromobacter xylooxidans*



Effect of Nitrogen sources of *Achromobacter xylooxidans*

## Discussion

The present study evaluated the effect of various substrates for enzyme production was examined with 10 g of each rice straw, corn straw, ragi straw, millet straw, and sugarcane baggase in 250 ml Erlenmayer flasks with Mandels's mineral salt solution. Mass cultivation was carried out at 35<sup>0</sup>C, pH 7, NaCl 3% and using alkali treated rice straw as substrate at 1095.6 U/ml/min was found activity was observed amylase. Cellulase production was carried out at pH 7; temp 45<sup>0</sup>C; NaCl 4% and alkali pretreated sugarcane bagasse as substrate at which was the observed 64.8 U/m/min. Xylanase enzyme production was carried out at pH 7; temp 40<sup>0</sup>C; NaCl 3% and alkali pretreated sugarcane bagasse and the enzyme activity was found to be 45.54 U/ml/min.

The high level of xylanase production on sugarcane bagasse suggested that xylan might be present in this substrates. Similarly, among the lignocellulosic substrates tested, wheat bran gave maximum yield of xylanase by *Cellulosimicrobium* sp. as among substrates tested viz. gram bran, rice husk, rice bran, wood dust and apple pomace (Rajashri and Anandrao, 2012). The best substrate for xylanase production was wheat bran due to its nutritional content and large surface area (Babu and Satyanarayana, 1995), where as wheat bran contained xylan and protein, which were served as carbon and nitrogen sources for microorganisms, respectively (Thiago and Kellaway, 1982). Amylase also found to have maximum production by *Clostridium thermosulforegens* using wheat bran (Mrudula *et al.*, 2011). Wheat bran characterized by its better air circulation, loose particle binding and efficient penetration by mycelia and cheaper, therefore it showed a better prospect economically in fermentation processes (Unakal *et al.*, 2012). Therefore, physicochemical parameters of SmF using different substrate for optimization production of enzymes and saccharification content of *A.xyosoidanas* was performed in the present study.

The usual temperature maintained in SmF systems is in the range of 25-32<sup>0</sup>C, depending on the growth kinetics of microorganism employed for fermentation purposes (Lonsane *et al.*, 1985). In the present study, the maximum production of amylase was found at 40<sup>0</sup>C (720 U/ml/min), where as cellulase (21.3 U/ml/min) and xylanase (108 U/ml/min), by *A. xylosoidanas* was achieved at 35<sup>0</sup>C, and 45<sup>0</sup>C. In an other study, the optimum temperature recorded for maximum growth and -amylase production by *B. subtilis* was 35<sup>0</sup>C (Unakal *et al.*, 2012).

The initial pH of fermentation medium has a significant effect on bacterial growth and enzyme production (Murad and Saleem, 2001). In this attempt to study the effect of pH on enzyme production and saccharification using wheat bran by *A. xylosoidanas*, the production medium having pH's ranged from 4 to 7 showed a maximum production of cellulase (22.8 U/ml/min), xylanase (104.5 U/ml/min), and amylase (481.1 U/ml/min) at pH 7, 6 and 5, respectively.

The influence of carbon sources such as starch, cellulose, xylose, sorbose, fructose, glucose, manitol, maltose, sucrose and galactose at 1% were tested for the production of cellulase, xylanase and amylase by *A. xylosoidanas*. An ascending order of cellulase production was noted with as the prime source cellulose followed by manitol, maltose, sucrose, starch, glucose, fructose, xylose, and sorbose. Likewise sucrose was the most preferable source for xylanase production followed by manitol, xylose, cellulose, fructose, maltose, glucose, sorbose and starch. The same carbon supplements except starch caused repressive effect on amylase production by *A. xylosoidanas*. Supplementation of carbon sources increased -amylase production by *B. cereus* during SSF using wheat bran (Singh *et al.*, 2010). *B. thermooleovorans* was reported to prefer starch, glucose, lactose, maltose and maltodextrins as carbon sources for -amylase secretion (Arunava *et al.*, 1993; Narang and Satyanarayana, 2001). In contrast, carbon sources such as glucose, maltose and starch did not enhance -amylase production by thermophilic *B. coagulans* in solid-state fermentation using wheat bran (Babu and Satyanarayana, 1995). Xylanase production by *Bacillus* sp. AR-009 grown on wheat bran was repressed upon addition of lactose, glucose and sucrose (Gessesse and Mamo, 1999).

Gelatin was the potential nitrogen source for amylase production followed by ammonium sulphate (476.8 U/ml/min.). Whereas for xylanase production sodium nitrate (158.8 U/ml/min) was the best source and for cellulase production it was urea (328.0 U/ml/min) that resulted in maximum production. Hashemi *et al.*, 2010 reported that ammonium nitrate was the best inducer for -amylase secretion followed by yeast extract 'by *Bacillus* sp. On the other hand, the limitation or starvation of nitrogen during the fermentation resulted in the limited growth of *B. subtilis* and the enhancement of -amylase production (Mirminachi *et al.*, 2000). For *Bacillus* sp. AR-009, yeast extracts increased xylanase production (Gessesse and Mamo, 1999).

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