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Optimization and Partial Purification of Xylanase from *Bacillus subtilis*

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Abstract: Enzymes play crucial roles in producing the valuable commodities in our day to day routine life and also producing fuel for automobiles. Enzymes are also important in reducing both energy consumption and combating environmental pollution. Enzymes are a type of protein in all biological entities and perform as biological catalysts. Xylanase enzyme is of great importance commercially because of its high stability at elevated temperatures and in alkaline conditions. The xylanase producing eight *Bacillus subtilis* were isolated and identified by various standard bacteriological methods from Paddy field soil sample, Sathanur village, Tiruvannamalai district. Among eight *Bacillus subtilis* isolates *viz*., (BS1, BS2. BS3. BS4. BS5. BS6. BS7 and to BS8), *Bacillus subtilis* (BS1, BS5 and BS7) isolates shows higher zone of inhibition on selective media plate containing various ingredients including brich wood xylan as substrate. The xylanase activity was assayed at 55°C by determining the concentration of reducing sugars liberated by the activity of the enzyme on its substrate xylan using DNS reagent. The effect of *Bacillus subtilis* isolates (BS1, BS5 and BS7) for the xylanase enzyme production was studied at different pH, temperature, carbon source, nitrogen source and incubation periods. The maximum chitinase production was observed from BS7 at pH 8.0, temperature 50°C, wheat bran as carbon source, beef extract as a nitrogen source at 30 hours of incubation. The xylanase enzyme was partially purified by APS. Purified enzyme exhibited the maximum protease activity in *Bacillus subtilis* (B7) (1.86 U/ml). Among the three isolates BS7 were exhibited maximum xylanase activity.

Key words: Xylanase · *Bacillus subtilis* · Optimization and Partial Purification

INTRODUCTION

Enzymes are substance produced by a living organism which acts as a catalyst to bring about a specific biochemical reaction and convert substrates to specific product [1]. Xylanases are a group of enzymes that depolymerize xylan molecules into xylose units used by microbial populations as a primary carbon source [2]. Xylanases, a group of hydrolytic enzymes, catalyze the hydrolysis of xylan which is genetically single chain glycoproteins, 6-80 kDa and active between 40 to 60°C [3]. The complete enzymatic hydrolysis of xylan into its constituent mono-saccharides requires the synergistic action of a consortium of xylanolytic enzymes. This is due to the fact that xylans from different sources exhibit a significant variation in composition and structure [4].

Xylanase production can be carried out using agricultural waste materials; those are used as substrates which provide carbon and mineral nutrients to the organisms under the controlled conditions [4]. As the price of the substrate plays a crucial role in over all processing cost, cheap substrates such as agro-residues are now a day's becoming choice of many large scale enzyme productions [5]. Xylanase producing microorganisms including various Bacteria *viz*., *Bacillus amyloliquefaciens, Bacillus licheniformis*, *Bacillus subtilis, Streptomyces* spp. and fungi *viz*., *Trametes versicolor*, *Alternaria* sp., *Rhizoctonia solani*, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus brasiliensis Trichoderma atroviride, Fusarium oxysporum, Penicillium digitatum* [6]. The applications of xylanolytic enzymes have increased for the last few

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decades owing to their potential effectiveness in bread making. Starch and non-starch carbohydrate- hydrolyzing enzymes are commonly used in the bread making industry as bread improvers [7]. Enzymes are added to animal ration with the goal of increasing its digestibility, removing anti-nutritional factors, improving nutrient availability, as well as for environmental issues [8]. Production of xylanase from a single microorganism renders its industrial application more feasible and economical [9]. Filamentous fungi have been widely used to produce hydrolytic enzymes for industrial application, including xylanases, whose levels in fungi are generally much higher than those in yeast and bacteria [10].

MATERIALS AND METHODS

Sample Collection: Soil sample was collected from the Sathanur village paddy field land in different areas. The soil samples are taken from 2 to 3 cm depth with the help of sterile spatula and put in sterile plastic bags and it mouth was tied properly and brought to laboratory for further processing.

Isolation of *Bacillus* sp.: One gram of soil sample was serially diluted up to 10^9 dilution and 0.1 ml of serially diluted sample from 10^4, 10^5, 10^6, 10^7, 10^8 was taken and transfers to nutrient agar medium by spread plate technique, the inoculated plates were incubated at room temperature for 48 hours. After the incubation the colonies were observed. To reduce the number of colonies in the inoculation, quadrant streaking is carried out.

Colony Characterization: The *Bacillus* isolates were observed under macroscopically, the colony morphology was noted with respect to colour, size, shape and nature of colony.

Identification of *Bacillus* sp.: The identification of *Bacillus* sp. was done by standard bacteriological methods *viz*., Light microscopy, Motility demonstration, Endospore staining, Hydrolysis of Starch, Hydrolysis of Gelatin, Plating on Selective media and performing Biochemical tests.

Screening of Xylanase Activity: The isolated bacterial cultures were screened for their ability to produce xylanase on selective media containing Magnesium Sulfate 0.5 g/L; Potassium chloride (KCL) 0.5g/L; Potassium Hydrogen Phosphate(K_2HPO_4) 1.0 g/L; Sodium Nitrate ($NaNO_3$) 2.0 g/L; Agar 20.0 g/L; Brich Wood Xylan 10.0 g/L and pH was adjusted to 7.0. The plates were inoculated with bacterial culture and incubated at room temperature for 3 days. After incubation, 1% Congo red solution was added to the plates and incubated for 15 min. The Congo red staining solution in the plate was discarded and washed with 1N NaCl. Formation of clear zone of hydrolysis indicated degradation around the bacterial colonies. Xylanolytic activity of each colony was detected by determining the ratio of diameter of clearance zone (CZ) to the diameter of size of colony (CS). The high zone of clearance was considered for a higher Xylanolytic activity and this bacterial isolates were used for further studies.

Submerged Fermentation: The bacteria were cultured in Erlenmeyer flasks (250 ml) containing 50 ml of medium. The medium composition in (g/L) was NH_4Cl 0.5 g/L; KH_2PO_4 0.5 g/L; KCl 0.75 g/L; $K HPO_2$ 2.5 g/L; $MgSO_4.7H_2O$ 7.0 g/L; Brich wood xylan 10 g/L; NaCl 30 g/L; DH_2O- 1.0 g/L and pH was adjusted to 5.5. Flasks were inoculated with 1ml of one day-old pure bacterial broth culture. Flasks were incubated at 30ºC under static conditions for 3 days. After incubation, the culture fltrate was harvested by filtration using Whatman No.1 filter paper and centrifuged at 10, 000 rpm for 15 min at 4°C. The clear supernatant was used for enzyme assays and stored at 4°C for further uses.

Xylanase Assay: The xylanase enzyme activity was assayed by determining the concentration of reducing sugars liberated by the activity of the enzyme on its substrate xylan using DNS reagent. The reaction mixture was prepared by adding 1.8 ml of 1% birch wood xylan in 50mM Glycine NaOH buffer (pH 9.0) and 0.2 ml of crude enzyme and incubating at 55°C for 10 minutes. The reaction was terminated by adding 3 ml DNS reagent followed by incubation in boiling water for 10 minutes to release the reducing sugars. A control was also set up where crude was added after adding DNS. All the tubes were cooled to room temperature and the absorbance estimated by Colorimeter at 540 nm against blank. All the experiments were set up in triplicates and the results are the mean of the three sets. One unit of xylanase activity was defined as the amount of enzyme required to release 1 µmol of xylose units per minute under the specified assay conditions [11].

Optimization of Cultural Conditions

pH Optimization: The effect of pH values was carried out to determine the optimum pH value for xylanase productivities by *Bacillus subtilis.* The pH was adjusted

at pH – 5, pH – 6, pH – 7, pH – 8 and pH – 9 for the production media using 1 N NaOH or 1 N HCL. Xylanase production using solid state fermentation in different production vessels: enzymes production was studied in 250 ml conical flask.

Temperature Optimization: The effects of temperature values of were carried out to determine the optimum temperature value for xylanase productivities by *Bacillus subtilis*. Xylanase production was studied by incubating the production medium at 30°C, 40°C, 50°C, 60°C and 70°C temperature. Protease production using solid state fermentation in different production vessels: enzyme production was studied in 250 ml conical flask.

Carbon Sources Optimization: The effects of different carbon sources were studied for xylanase production by *Bacillus subtilis*. Four different carbon sources *viz*., Glucose, Maltose, Galactose, Lactose, Fructose and Sucrose were selected for this present study. Xylanase production using solid state fermentation in different production vessels: enzyme production was studied in 250 ml conical flask.

Nitrogen Sources Optimization: The effect of different nitrogen sources was studied for Xylanase production by *Bacillus subtilis*. Four different nitrogen sources *viz*., Peptone, Ammonium chloride, Tryptone, Meat extract, Yeast extract and Ammonium citrate were selected for this present study. The xylanase production by replacing 1% Trisodium citrate in the production medium with 1% and Carbon source. The flasks were incubated at 55°C on shaker for 24 hrs. Xylanase production using solid state fermentation in different production vessels: enzyme production was studied in 250 ml conical flask.

Incubation Optimization: The optimization of incubation time required for xylanase productivities by *Bacillus subtilis* was studied at 12 hrs, 24 hrs, 48 hrs, 72 hrs and 96 hrs. Xylanase production using Solid state fermentation in different production vessels: enzyme production was studied in 250 ml conical flask.

Purification of Xylanase

Ammonium Sulphate Precipitation: The crude enzyme was purified from the culture supernatant by precipitating the proteins using ammonium sulphate precipitation procedure. Initially the needed concentration of $(NH_4)_2SO_4$ ammonium sulfate was obtained by performing precipitation at various Ammonium sulphate concentrations i.e., 30, 40, 50, 60, 70 and 80 % by carried out this procedure at 4 °C for few hours with continuous stirring. After complete dissolution, supernatant was kept at 4°C overnight, for allowing proteins to precipitate. The precipitates were then collected and analyzed for xylanase activity [12].

Partial Purification by Dialysis Method: The culture supernatant fluid was subjected to precipitation with ammonium sulphate to 80 % saturation and stirred for 2 hours. Then the precipitate was allowed to stand for overnight and then collect by centrifugation at 10000 rpm at 4 °C for 30 min. The precipitated pellets were dissolved in phosphate buffer and centrifuged and dialyzed against the same buffer which was then partially purified.

RESULTS AND DISCUSSION

Microbial enzymes in the field of industrial usage have been increasing because of its economical production in respect to biotechnological activities [13]. Xylanases are used as additives in animal feeds for monogastric animals, together with cellulases, pectinases and many other depolymerizing enzymes. Enzyme degradation of arabinoxylans, commonly found as ingredients of feeds, reduces the viscosity of the raw materials thus facilitating better mobility and absorption of other components of the feed and improving nutritional value [14]. The incorporation of xylanase into the rye- or wheat-based diets of broiler chickens resulted in an improvement in weight of chicks and their feed conversion efficiency. Similar improvements can be obtained for pigs fed on a wheat-based diet supplemented with xylanases and phospholipases [15]. The majority of agroindustrial waste is lignocelullosic biomass containing xylan, which is the second most abundant polysaccharide in the world [16]. Xylanase is also used for coffee extraction, extraction of oils and starch from plant origin and for clarification of the fruit juice in combination with pectinases and cellulases, degumming and retting of bast fibres like sun hemp, ramie and jute [17, 18]. A large variety of Xylanases produced by microorganisms become a major group of industrial enzymes that are capable to degrade xylan to renewable fuels and chemicals [19], in addition to their use in food, paper and pulp industries [20, 21].

The xylanase producing bacteria was isolated from paddy field soil, Sathanur Village, Tiruvannamalai district and Xylanase producing *Bacillus subtilis* were isolated and identified based on the standard procedures like serial dilution, spread plate, streak plate methods, cultured in suitable media. Porsuk *et al*. [22]

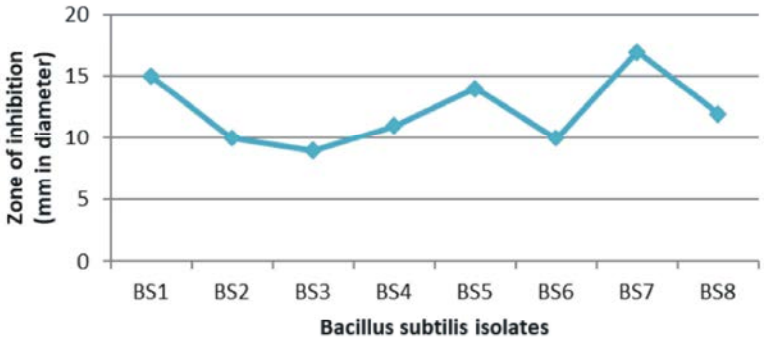

Fig. 1: Screening of *Bacillus subtilis* for its xylanase production

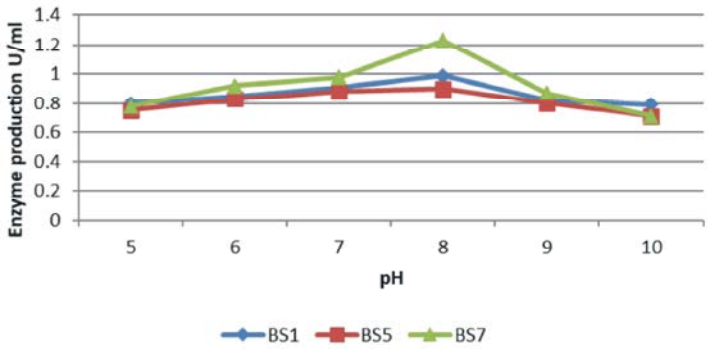

Fig. 2: Different pH on xylanase production by *Bacillus subtilis*

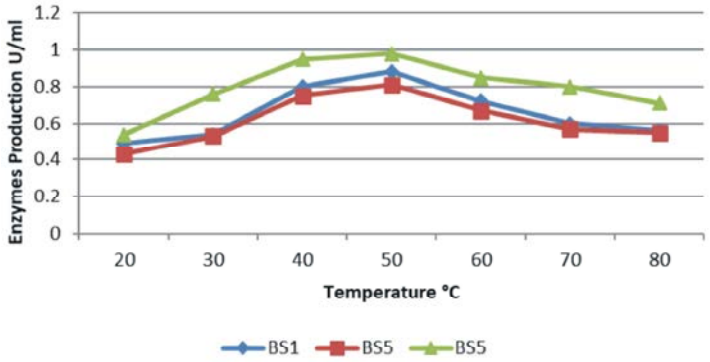

Fig. 3: Different temperature on xylanase production by *Bacillus subtilis*

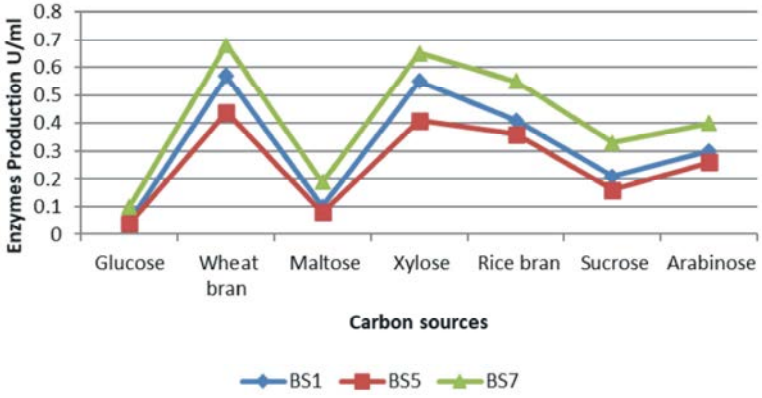

Fig. 4: Different carbon sources on xylanase producing by *Bacillus subtilis*

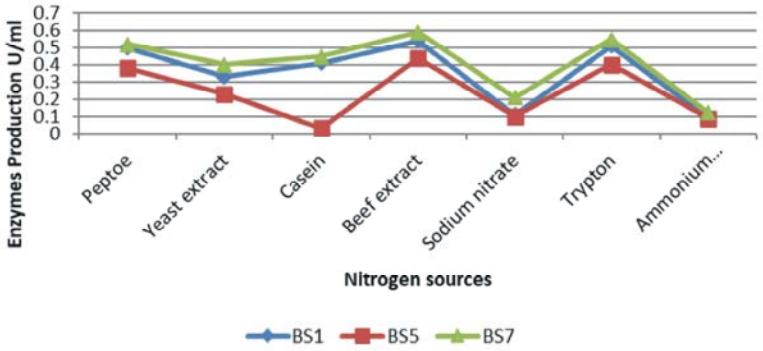

Fig. 5: Different Nitrogen sources on xylanase production by *Bacillus subtilis*

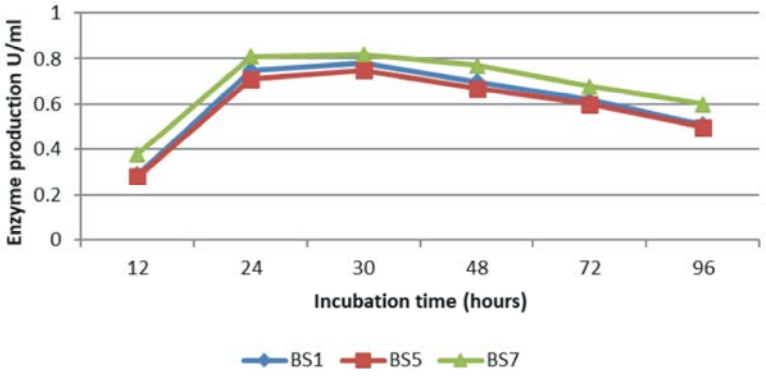

Fig. 6: Different incubation on xylanase production by *Bacillus subtilis*

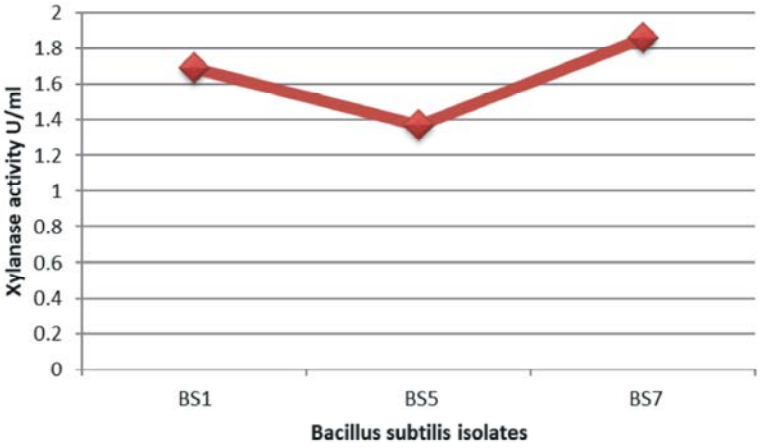

Fig. 7: Purified Enzyme Activity (APS)

studied preliminary morphological characterization showed that the isolate was gram positive, rod shaped, aerobic and endospore former. There are reports on isolation of xylanase producing organisms from soil.

The isolated bacterial cultures were screened for their ability to produce xylanase on selective media plate containing various ingredients including brich wood xylan as substrate and totally eight *Bacillus subtilis* isolates (BS1, BS2, BS3, BS4, BS5, BS6, BS7 and BS8) were tested for their zone of inhibition (mm in diameter) in agar plate and three *Bacillus subtilis* isolates BS1 (15 mm), BS5 (14 mm) and BS7 (17 mm) were recorded higher zone of inhibition and BS1, BS5 and BS7 were selected for the further studies (Figure 1).

Roy and Abedin [23] and Kamble and Jadhav [24] measured the clearing zone of xylanase-producing bacteria (*Bacillus aerophilus, Bacillus subtilis, Bacillus stratophericus* and *Bacillus pumilus*) on xylan agar plate. Teather and Wood [25] found that the plate assay with Congo red is one of the important methods to assess the

xylanolytic activity of the microflora and it is depends on the formation of complex between the dye Congo red and polysaccharide (xylan) the Congo red (reactive dye). The xylanase activity was assayed at 55°C by determining the concentration of reducing sugars liberated by the activity of the enzyme on its substrate xylan using DNS reagent. The three *Bacillus subtilis* isolates BS1, BS5 and BS7 were assayed for xylanase activity by absorbance at 540 nm in colorimeter. The maximum xylanase activity was recorded in *Bacillus subtilis* BS7 (1.87 U/ml). The effect of isolated *Bacillus subtilis* isolates (BS1, BS5 and BS7) for the xylanase enzyme production were studied at different pH *viz*., pH – 5, pH – 6, pH – 7, pH – 8, pH – 9 and pH – 10 (Fig. 2), Among the three isolates tested, *Bacillus subtilis* BS7 recorded maximum xylanase activity at pH 8 (1.23 U/ml). The minimum xylanase activity was observed at the *Bacillus subtilis* BS1 and BS5 at pH 8 (0.99 U/ml, 0.90 U/ml).

The effect of isolated *Bacillus subtilis* isolates (BS1, BS5 and BS7) for the Xylanase enzyme production were determined at different temperatures *viz*., 20°C, 30°C, 40°C, 50°C, 60°C, 70°C and 80°C (Figure 3). The maximum protease activity of *Bacillus subtilis* (BS7) was observed at the temperature 50°C (0.98 U/ml). The minimum xylanase activity was observed at *Bacillus subtilis* (BS1 and BS5) at the temperature 50°C (0.88 U/ml, 0.81 U/ml).

Sharma and Bajaj [26] noticed that xylanase production was seen at different pH may be an indication that the organism may have the ability to induce multiple xylanases at different pH. Xylanases produced by bacteria and actinomycetes (*Bacillus* sp*., Pseudomonas* sp. and *Streptomyces* sp.) are effective in a broader pH range of 5 to 9, with the optimum temperature for Xylanase activity between 35°C to 60°C [27].

The effect of isolated *Bacillus subtilis* isolate (BS1, BS5 and BS7) for the xylanase enzyme production were evaluated on the presence of different carbon sources *viz*., Glucose, Wheat bran, Maltose, Xylose, Rice bran, Sucrose and Arabinose (Figure 4). The highest protease activity was recorded at *Bacillus subtilis* BS7 in the presence of Wheat bran (0.68 U/ml). The lowest protease activity was recorded at *Bacillus subtilis* BS1 and least activity was observed at *Bacillus subtilis* BS5.

The isolated *Bacillus subtilis* isolates (BS1, BS5 and BS7) were estimated for its xylanase enzyme production against the different nitrogen sources *viz*., Peptone, yeast extract, casein, beef extract, sodium nitrate, tryptone and ammonium nitrate (Figure 5). Maximum xylanase production was exhibited at *Bacillus subtilis* BS7 in the presence of Beef extract as nitrogen source (0.59 U/ml) followed by tryptone (0.55 U/ml), peptone (0.52 U/ml), casein (0.45 U/ml), yeast extract (0.40 U/ml), sodium nitrate (0.21 U/ml) and Ammonium nitrate (0.12 U/ml). The minimum Xylanase activity was observed in *Bacillus subtilis* BS1 and BS5 at beef extract (0.54 U/ml, 0.44 U/ml).

Wang, *et al*. [28] noticed that peptone, like yeast extract is a complex organic nitrogen source which might be stimulating growth by releasing NH_4^+ and improving the expression of nitrogen assimilating enzymes. The effect of isolated *Bacillus subtilis* isolates (BS1, BS5 and BS7) for the xylanase enzyme production were studied at different incubation hours *viz*., 12, 24, 30, 48, 72 and 96 (Fig. 6). The maximum xylanase activity was exhibited in *Bacillus subtilis* BS7 at 30 hrs of incubation (0.82 U/ml) followed by 24 hrs (0.81 U/ml), 48 hrs (0.77 U/ml), 72 hrs (0.68 U/ml), 96 hrs (0.60 U/ml) and 12 hrs (0.38 U/ml). The minimum xylanase activity was observed in *Bacillus subtilis* BS1 and BS5 at 30 hrs of incubation (0.78 U/ml, 0.75 U/ml).

The xylanase enzyme was partially purified by APS and the Purified enzyme exhibited the maximum protease activity in *Bacillus subtilis* (B7) (1.86 U/ml) followed by *Bacillus subtilis* (B1) (1.69 U/ml) and *Bacillus subtilis* (B1) (1.37 U/ml) (Figure 7).

Feniksova *et al*. [29] also reported optimum incubation period of 72 hrs for maximum xylanase production by *Bacillus subtilis* in solid state as well as surface culture conditions. Xylanase produced by *Paenibacillus* sp. N1 was growth associated, reaching to maximum after 72 hrs at exponential peak and enzyme production remained more or less the same up to 96 hrs. Maximum production of xylanase is observed by Wahyuntri, *et al*. [30] in a culture incubated at 50°C, pH 7.0 at 72 hrs by *Bacillus* sp. AQ-1.

CONCLUSION

The requirement for large scale enzyme production in industry is low cost, simple cultivation and minimal amount of downstream processing. These goals can be achieved with a production system in which enzyme is effectively secreted into the cultivation medium. Microbial xylanases have great potential for industrial applications. Xylanase enzyme should be promoted in the food processing and pulp and paper industry to replace the (harsh) chemical used during the processing. Xylanase enzyme in combination with some other enzyme can provide better results for sustainable industrial processes. The isolated bacterial isolate *Bacillus subtilis* showed best result in the brich wood xylan as substrate and maximum activity at 55 °C by its substrate xylan using DNS reagent. Maximum production showed at pH 8,

temperature 50°C, wheat bran as carbon source, beef extract as nitrogen source at 30 hours of incubation. The present study investigates the xylanase enzyme has to be produced at more amount and it to be applied for various applications.

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