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Xylanase production using fruit waste as cost effective carbon source from thermo-tolerant *Bacillus megaterium*

Rafid Abdul Wahid Abdul Kareem[1], Imrana Khushk[2], Muhammad Aqeel Bhutto[2], Qureshi Abdul Sattar[2] and Ayyaz Ahmed[3]*

[1]Department of Biochemical Engineering, Alkhwarizmi College of Engineering, University of Baghdad, Baghdad, Iraq.
[2]Institute of Biotechnology and Genetic Engineering, University of Sindh, Jamshoro, Pakistan.
[3]Department of Chemical Engineering, University of Gujrat,Gujrat, Pakistan.

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Thermotolerant *Bacillus megaterium* BRL-0101 was used to produce xylanase in batch fermentations using various fruit waste as cost effective substrates. A mineral medium based on mango peel (100 g/L) as the carbon source and yeast extract + peptone + $NaNO_3$ (10 g/L) as the nitrogen source proved to be the most effective. A 48-h batch fermentation in this medium with a starting pH of 8.0 produced a xylanase titer of ~2,876 U/mL at the optimal fermentation temperature of 50°C. The optimal temperature, initial pH, the carbon source and its concentration, and the nitrogen sources, size of inoculum, inducer and its concentration, were identified after evaluation of multiple nutrient sources and fermentation conditions.

Key words: Xylanase, *Bacillus megaterium*, fermentation, mango peel.

INTRODUCTION

The cost of enzyme production and low enzyme yields are the major challenges in industrial applications of enzymes. Lignocellulosic biomass is an essentially inexhaustible and renewable carbon source, that consists of cellulose, hemicelluloses and lignin (Harris and Debolt, 2010; Kumar et al., 2008). Xylan is second most abundant polysaccharide after cellulose, the major hemicellulose and accounts for 20-35% of total dry weight in plant biomass. Xylanases (EC. 3.2.1.8) show excellent potential due to their wide industrial applications in pulp bleaching, oligosaccharides production (Kuhad et al., 2010; Ninave et al., 2006; Sanghi et al., 2009), the modification of cereal-based food stuffs, improving the digestibility of animal feed stocks, fruit softening and clarifying juices, texture improvement of bakery products, textile industry, (Nagar et al., 2010; Dhiman et al., 2009; Polizeli et al., 2005), pharmaceutical and chemical applications. Furthermore, xylanases in combination to β-xylosidase are utilized for complete saccharification of lignocellulosic biomass for ethanol production (Rojas et al., 2011; Cavka et al., 2011; Lopez et al., 2011; Abdeshahian et al., 2010; Rajoka and Riaz, 2005; Tengerdy and Szakacs, 2003). Xylanases have an annual worldwide market of about US $ 200 million and the widespread use of xylanase in commercialized industrial applications requires extensive studies to

*Corresponding author. E-mail: ayyaz.ahmead@uog.edu.pk.

optimize their production (Mullai et al., 2010).

Microorganisms are the most common source of industrial enzymes due to their broad biochemical diversity and feasibility of large scale production. Xylanases have been reported mainly from bacteria (Anuradha et al., 2007; Dhiman et al., 2008), fungi (Nair et al., 2008), actinomycetes (Techapun et al., 2002; Ninawe et al., 2006) and yeast (Passoth and Hahn-Hägerdal, 2000). Although several thermophilic micro-organisms have been isolated and exploited for enzymes production and characterization, there is still a need for novel strains capable of producing enhanced levels of enzyme in an economically feasible culture system.

The fermentation performance of microorganism is significantly affected by culture conditions and medium constituents such as: carbon source, nitrogen source, inducer, inoculum size, pH of the media, incubation temperature and agitation rate (Kuhad et al., 1993). For the commercial realization and economic viability of xylanase production, it is necessary to optimize cultural conditions of microorganisms so that higher enzyme production could be obtained. The objectives of present work were to exploit *Bacillus megaterium* for hyper xylanase production from low cost carbon source such as fruit waste and to optimize the fermentation profile of xylanase producing strain.

MATERIALS AND METHODS

Microorganisms

The bacterial strains were collected from Department of Microbiology, University of Baghdad, Baghdad, Iraq and the micro-organisms were grown under laboratory conditions. *Escherichia coli, Bacillus subtilis, Bacillus megaterium* and *Bacillus cereus* capable of xylanase production were used in this study. The microorganisms were maintained on nutrient agar medium containing glucose 20 g/L, peptone 10 g/L and agar 20 g/L (Qureshi et al., 2012).

Fermentation medium

It was composed of (g/L) glucose 20, peptone 10, magnesium sulphate 2, ammonium nitrate 1 and sodium dihydrogen phosphate 2. 50 ml of culture medium was taken in a 250 ml Erlenmeyer flask with an initial pH maintained at 6.0. Flasks were cotton plugged and autoclaved at 1.5 kg/cm^2 for 20 min. 5.0 ml of *Bacillus megaterium* seed culture was inoculated in each flask. The flasks were incubated at 37± 2°C. The samples were collected with regular interval for analyzing growth (OD), residual sugars and xylanase activity. 5 g of fruit wastes were treated with 95 ml (1.0%) diluted H_2SO_4 and kept in autoclave at 115°C for 1 h. After 1 h supernatant was separated and used as source of fermentable sugars for microbial growth and enzyme production.

Optimization of fermentation condition

The parameters that strongly influence the xylanase production such as: incubation time; carbon and nitrogen sources, inducer, pH and temperature were optimized in the present study. Time course of fermentation (12-84 h), 5.0% different carbon sources (orange peel, banana peel mango peel, apple pulp, and oilcake were hydrolyzed with 1.0% H_2SO_4) instead of pure glucose, several organic and inorganic compounds separately and in combination (tryptone, ammonium chloride, potassium nitrate, sodium nitrate, yeast extract and corn steep liquor) as nitrogen source in place of peptone, initial pH (4.5-10) and fermentation temperature (30 to 65°C) were optimized in terms of maximum xylanase production.

Assay of xylanase activity

Xylanase activity was determined by mixing 0.5 ml sample (broth) with 0.5 ml of oat to xylan (Fluka, Germany) (1% w/v) in 50 mM citrate buffer (pH 5.3) at 60°C for 15 min (Bailey et al., 1992). Xylose standard curve was used to calculate the xylanase activity. In the assay, the release of reducing sugars was measured using the dinitrosalicylic acid reagent method (Miller, 1959).

One international unit of enzyme activity was defined as the amount of enzyme, releasing 1 mol of reducing group per minute per mille.

RESULTS

In order to attain maximum xylanase production, fermentation parameters such as nutritional (carbon and nitrogen source and inducer) and physiological (incubation time, size of inoculum, pH, temperature) were optimized. The xylanase production by *B. megaterium* BRL-0101 was highest (2876 IU/mL) under the optimized conditions, that is, peptone 0.25%, yeast extract 0.50%, sodium nitrate 0.25%, mango peel 10.0%, pH 8.0, temperature 50°C, incubation time 48 h, agitation rate 150 rpm and using 10.0% (v/v) inoculum. Figure 1 shows the xylanase production from different bacterial species such as *B. subtilis, E. coli, B. cereus* and *B. megaterium*. *B. megaterium* produced better xylanase titer among the tested organisms. The effect of different fruit waste such as banana peel, mango peel, orange peel and apple peel and oilcake as carbon source were observed on enzyme production. The *B. megaterium* BRL-0101 showed 167 IU/mL xylanase activity with 10% mango peel as carbon source. The enzyme titer in the presence of other carbon sources such as; banana peel, mango peel, apple peel and oilcake was much lower as compared to mango peel (Figure 2). Xylanase production was found to vary with change in the concentration of mango peel as sole carbon source. The enzyme activity was measured in the presence of 1-10.0% (v/v) mango peel. Xylanase production was found to be highest with 10.0% (w/v) mango peel for *B. megaterium* BRL-0101 (Figure 3).

Figure 4 shows the time profile of xylanase production, bacterial growth and residual sugars. The highest xylanase activity of *B. megaterium* BRL-0101 (532 IU/mL) was observed after 48 h of incubation but decreased thereafter (Figure 4). The xylanase production show strong correlation with growth, the maximum growth was observed at 24 h and enzyme titer after 48 h. Effect of inoculum size (1-10%) on xylanase activity of *B. megaterium* was tested. Xylanase production increased

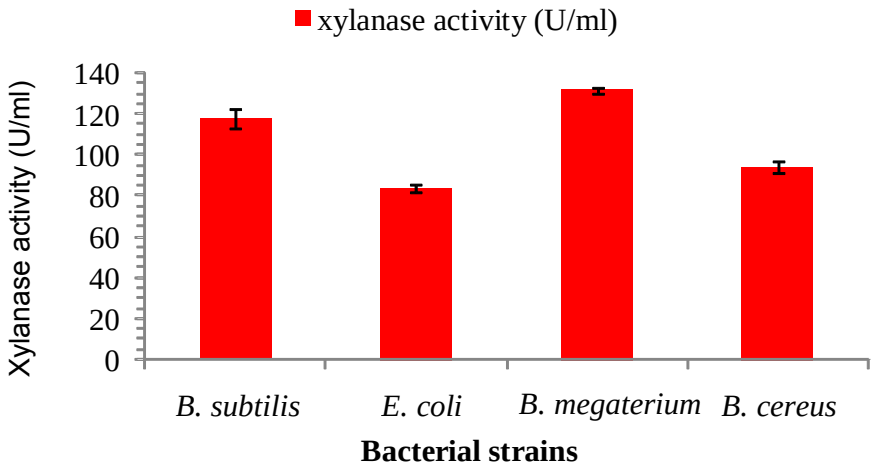

Figure 1. Xylanase production from different bacterial species using glucose as carbon source after 24 h incubation at 37°C, initial pH 6.0.

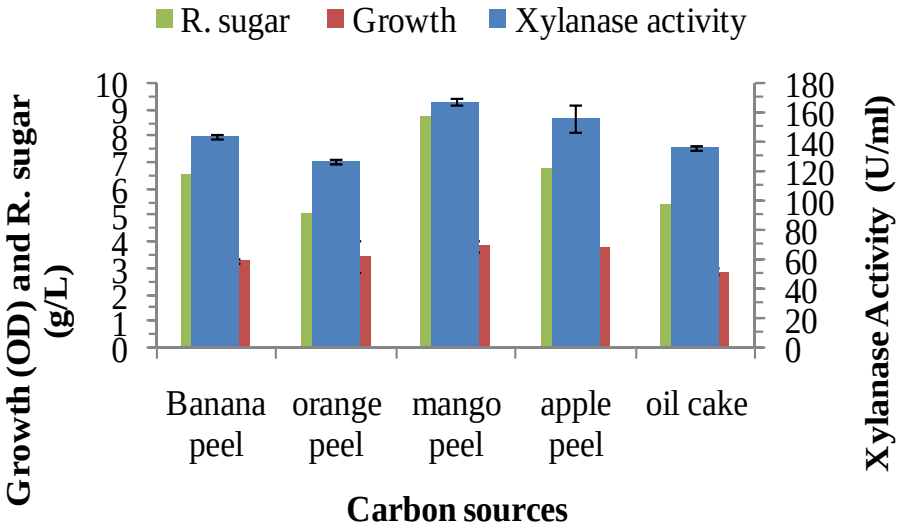

Figure 2. Effect of the carbon source (50 g/L initial concentration) on biomass concentration, xylanase activity and residual reducing sugar level at 24 h of fermentation (37°C, initial pH of 6.0).

with the increasing inoculum size. The highest xylanase activity was noted by using 10% inoculum (743 IU/ml), results are demonstrated in Figure 5. The effects of initial pH of fermentation on enzyme production and microbial growth are shown in Figure 6. The fermentations in Figure 6 were conducted for 48 h in a mineral medium that contained mango peel (10% initial concentration) yeast extract (5 g/L initial concentration) and peptone (2.5 g/L initial concentration). Clearly, the optimal initial pH for xylanase production and growth of *B. megaterium* was at pH 8.0 (Figure 6).

The effect of fermentation temperature on production of biomass and xylanase activity is shown in Figure 7. Clearly, 50°C was the best fermentation temperature for xylanase activity whereas maximum growth was observed at 45°C, further increase in temperature decreased the xylanase activity and growth which might be due to denaturation of enzyme at high temperature. Temperature is one of the important factors, which affects normal functioning of microorganism and enzyme production. The *B. megaterium* BRL-0101 is a commercially available substrates like oat spelt xylan (1887 IU/mL induced xylanase production; Figure 8). Xylan induced enzyme production at large extent, it is well known that xylan from various sources, are excellent inductors for xylanase production. Xylanase production was measured in the

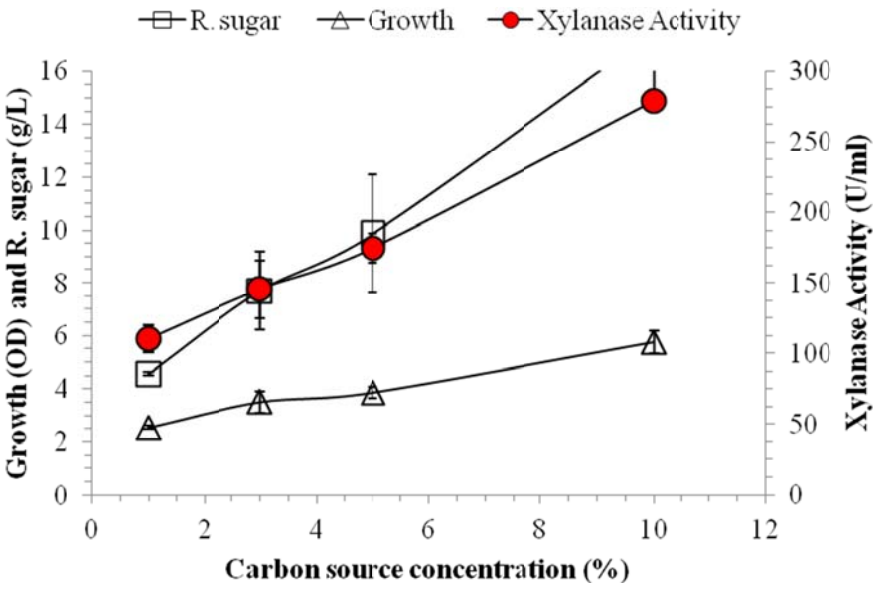

Figure 3. Effect of initial concentration of mango peel on final xylanase activity, biomass concentration and residual sugar concentration at 24 h of fermentation (37°C, initial pH of 6.0).

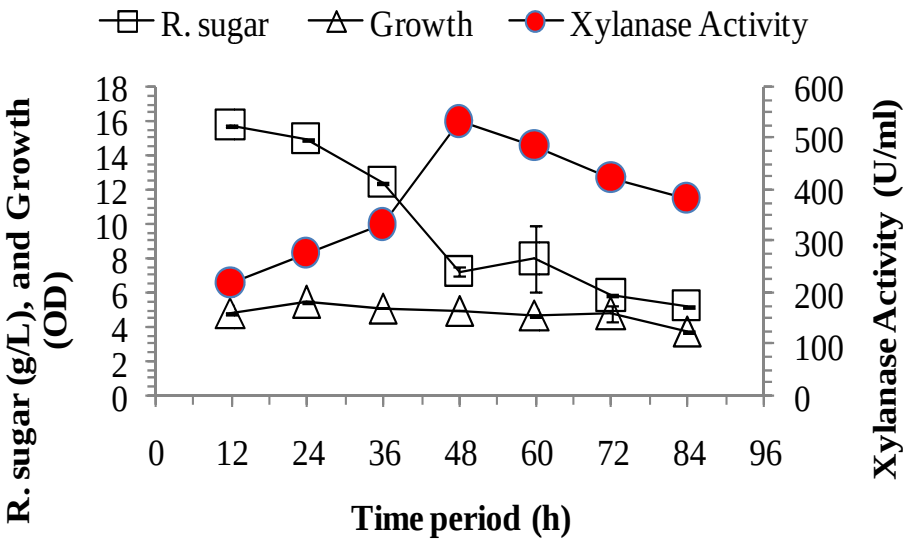

Figure 4. Batch profiles of biomass growth, xylanase activity and reducing sugar concentration during enzyme production (37°C, initial pH of 6.0).

presence of several organic and inorganic nitrogen sources separately and in combination using 10.0% (w/v) mango peel as a carbon source. Figure 9 showing the combination of peptone, yeast extract and sodium nitrate stimulated the highest xylanase production (2876.0 IU/mL) followed by a combination of peptone and yeast extract (2280.0 IU/mL). The combination of yeast extract with $NaNO_3$ and peptone with $NaNO_3$ produced 1955 and 1935 IU/ml, respectively.

DISCUSSION

Xylanases production demands are increasing due to wide applications in paper and pulp, bioethanol production and various pharmaceutical and chemical industries (Kuhad et al., 2010; Nagar et al., 2010; Rojas et al., 2011; Cavka et al., 2011; Lopez et al., 2011; Abdeshahian et al., 2010). This study could be economically feasible for industrial scale xylanases production by using inexpensive

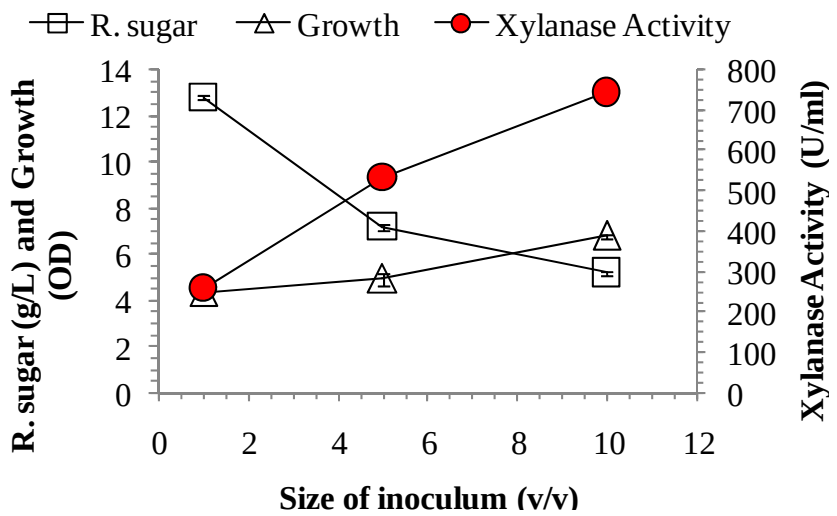

Figure 5. Effect of inoculum size on xylanase production, growth and residual reducing sugar level at 48 h of fermentation (37°C, initial pH of 6.0) in a mango peel (10% initial concentration) mineral medium.

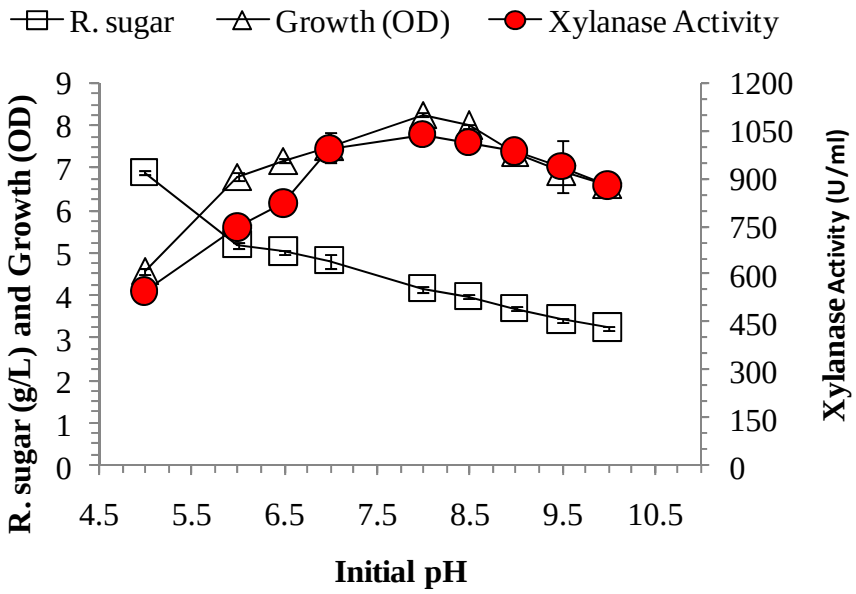

Figure 6. Effect of initial pH on xylanase production, biomass growth and residual sugar concentration at 48 h of fermentation (37°C) in a mineral medium containing mango peel and at initial concentration of 10%.

waste materials as carbon source. Agricultural wastes are abundant, cheap and inexhaustible substrates for value added products formation (Nagar et al., 2012). Carbon source is one of the essential constituents of the microbial growth and fermentation medium which significantly affects the overall cellular growth and metabolism. Mango peel, inexpensive agricultural residue; would affect the cost of the enzyme production directly. The use of pure sugars is uneconomical for xylanase production at large scale, while agricultural wastes are cost effective substrate for xylanase production (Ninawe and Kuhad, 2005). Xylanase activity increased with the passage of time to certain extent and prolonged incubation decreased xylanase titer. The reduction in the xylanase activity with the time of incubation might be due to reduction of nutrients, proteolysis and or change of pH

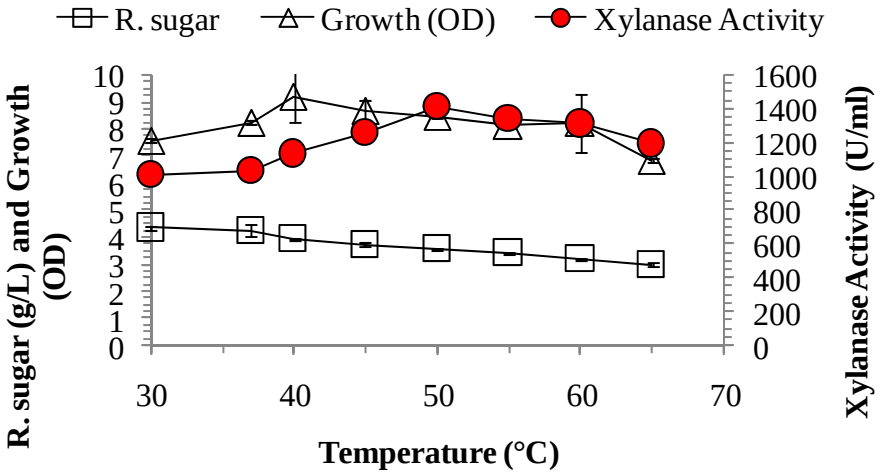

Figure 7. Effect of fermentation temperature on xylanase production, biomass concentration and residual sugar concentration at 48 h. The medium initially contained 10% mango peel. The initial pH was 8.0.

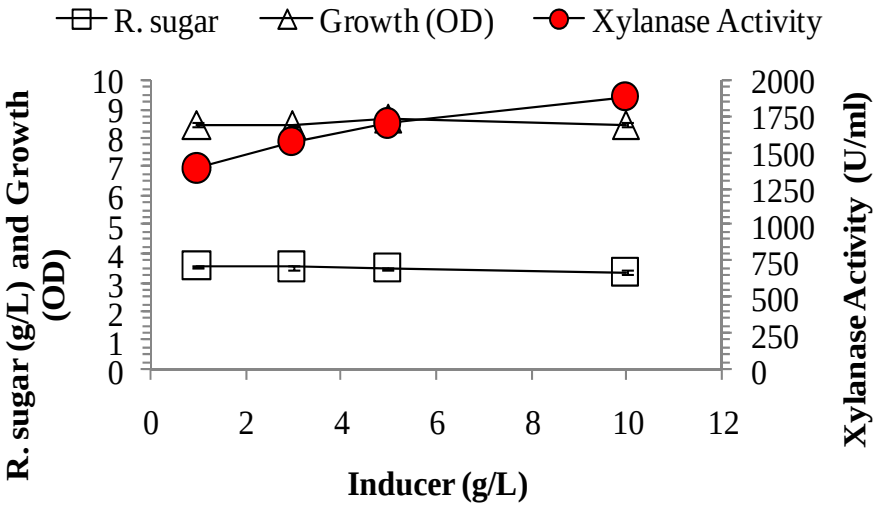

Figure 8. Effect of xylan as inducer on xylanase activity.

because of organic acids formation (Flores et al., 1997). *B. amyloliquefaciens* produced maximum xylanase activity in the culture broth after 48 h of incubation (Lincoln, 1960). *Streptomyces* Sp RCK-2010 secreted highest xylanase activity after 48 h by using wheat bran as carbon source (Kumar et al., 2012). *Bacillus* SSP-34 produced maximum xylanase activity (380 IU/mL) when grown for 96 h (Subramaniyan and Prema, 2000). In contrast to bacteria, fungi take more time such as *Trichoderma reesei* SAF3 produced maximum xylanase after 72 h of growth at 30°C under submerged conditions (Kar et al., 2006). Many researchers have reported hyper xylanases production from 1.0-5.0% (v/v) inoculum (Nagar et al., 2010; Battan et al., 2007; Kar et al., 2006). Low inoculum size is preferred for the synthesis of microbial products at commercial scale due to economical concerns (Lincoln, 1960). Higher inoculum size may increase moisture content and lead to decrease in growth and enzyme production, in the case of reduced inoculum size, desired production will take longer time (Baysal et al., 2003; Kashyap et al., 2002; Farga et al., 2009) an appropriate inoculum and nutrients could produce maximum product. In addition to this, the use of 10 % inoculum size for maximum xylanase production by *Bacillus* sp NCIM 59 has been reported (Kulkarni and Rao, 1996).

The genes involved in the production of certain enzymes in at least some microorganisms are known to

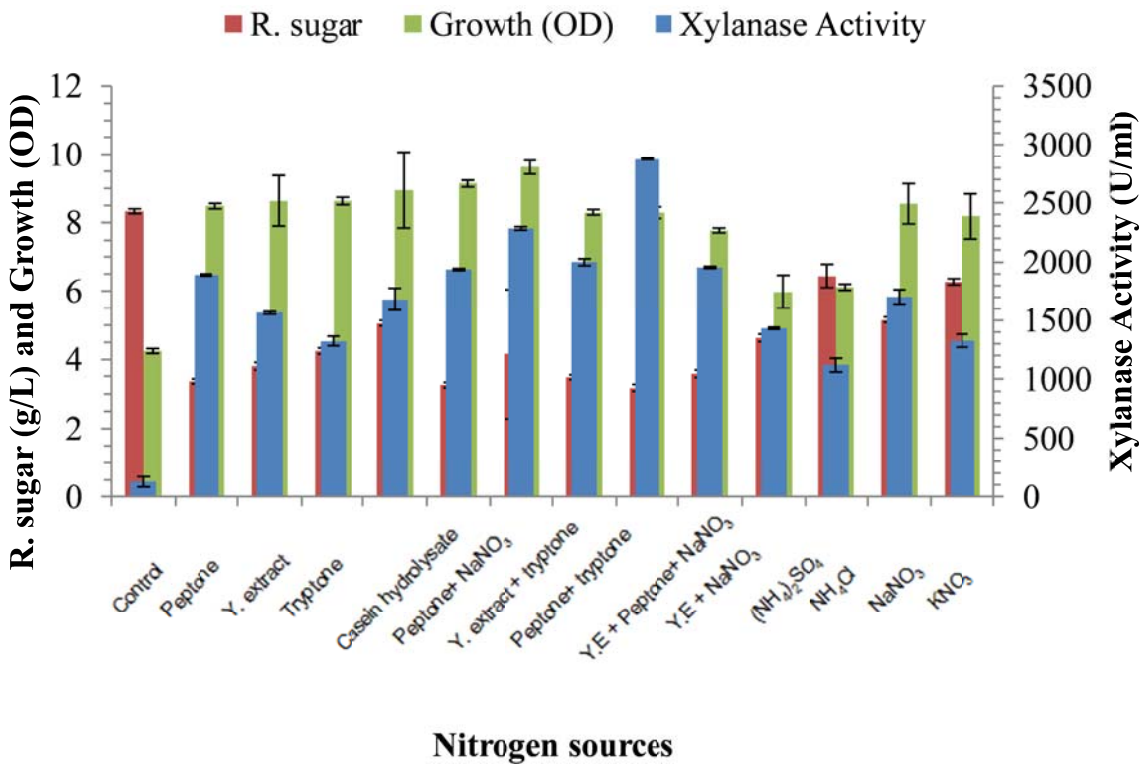

Figure 9. Effect of nitrogen source (10 g/L initial concentration) on xylanase production, biomass concentration and residual reducing sugar level at 48 h of fermentation (50°C, initial pH of 8.0) in a mango peel (10% initial concentration) mineral medium.

be pH regulated (Young et al., 1996). The optimal initial pH value for producing xylanase of course depends on the microorganism. Generally, fungi produce xylanase at acidic pH while bacteria produce xylanase at higher alkaline pH (Dhiman et al., 2009). However, some fungi such as *Aspergillus fischeri* Fxn 1 and *Aspergillus nidulans* KK-99 produced alkaline xylanases (Taneja et al., 2002). Kumar et al. (2012) reported xylanase production from *Streptomyces* sp. RCK-2010 at optimum pH 8.0. Xylanase production is inducible with the nature of substrate used (Nwodo-Chinedu et al., 2008). Highest xylanase production (251 IU/mL) by *Bacillus* SSP-34 was achieved when yeast extract and peptone each at 0.25% was supplemented in the medium as nitrogen source (Subramaniyan and Prema, 2000). However, tryptone was the best nitrogen source for xylanase production by *Bacillus circulans* AB16 (Dhilon and Khana, 2000) and *Geobacillus thermoleovorans* (Sharma et al., 2007). Yeast extract in combination with peptone significantly enhanced xylanase production in *Bacillus* sp. (Ruckmani, 2001). *Streptomyces* sp RCK-2010 produced highest xylanase activity from combination of peptone and beef extract (Kumar et al., 2012). The enhanced xylanase production in the presence of beef extract as well as peptone may be attributed to organic nitrogen source mediated regulation of microbial growth and metabolism (Gupta et al., 2000).

Conclusion

The low-cost process for xylanase production was developed in the present study, agro-industrial residues are interesting for this purpose due to their high availability associated with their low cost. However, these materials usually need treatments to become more susceptible to microbial action, fruit waste were treated with dilute acid to fermentable sugars. The results obtained herein proved mango peel as suitable substrate for xylanase production by *B. megaterium.* Under optimal conditions, xylanase levels as high as 2876 IU/mL was obtained. This study proved practical approach for xylanase production from fruit waste.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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