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XYLANASE PRODUCTION BY A NEW *Bacillus pumilus* SY30A UNDER SOLID STATE FERMENTATION AND ITS APPLICATION IN OIL PALM BIOMASS PULP BLEACHING

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Abstract: In the current study, the production of extracellular xylanase by *Bacillus pumilus* SY30A was investigated in a solid state fermentation. The effects of temperature and pH on xylanase production were tested. The best physical parameters observed for the enzyme production by *B. pumilus* SY30A were 37 °C and pH 9 respectively. Results showed that wheat bran was found to support the highest yield of xylanase among various carbon sources tested. The maximum enzyme production was obtained 1438 IU/g after 72 h of fermentation. The crude xylanase was then used to pretreat the unbleached oil palm soda-anthraquinone pulp. Results showed that with only 20 U of *B. pumilus* SY30A crude extract xylanase of 55 °C for 1 hour, decreased the kappa number of 43% while increased the pulp viscosity of 10%. This preliminary study on paper pulp bleaching disclosed the potential use of *B. pumilus* SY30A xylanase as biobleaching agent, which is also a more environmental sustainable method in comparison to those conventional chemical bleaching method.

Keywords: *Bacillus sp*, biobleaching, solid culture, xylanase.

Introduction

As hydrolytic enzymes, xylanases catalyze the endohydrolysis of 1,4-beta-D-xylosidic linkages in xylan, the major hemicellulose in hardwoods and non-wood fibers. Started from the middle of 1990s, the beneficial effect of xylanases in various industries was substantially reported (Javier Pastor *et al*., 2007). For instances, the addition of xylanases in the blended animal fat diet improved the body weight gains and feed absorption of the livestock due to better digestibility. On the other hand, the application of xylanases in baking assists the flour hemicellulose degradation and resulted in improving bread volume, crumb structure, and bread antistaling effect. Furthermore, xylanase was also being used for bio-conversion of lignocelluloses, especially xylan in wastes from agriculture or food industries into xylose and biofuel. The increasing environmental concern associates with the conventional chlorine-based pulp bleaching plans have driven the application of more eco-friendly bleaching agents in pulp and paper industry. The employment of xylanases for pulp bleaching could reduce or minimize the use of chlorine and other chlorine-containing chemicals and thus, improving the environmental sustainability of the pulp and paper industry.

Many bacteria and fungi have been studied for xylanase production (Heck *et al*., 2002; Kinegam *et al*., 2007; Bakri *et al*., 2010). *Bacillus* species play an important role in applied microbiology as industrial microorganisms for a very long time. For example, *Bacillus subtilis* has used by Japanese for the solid-state fermentation of soybeans in the production of natto for even more than a thousand years ago. *Bacillus* species are favourable for industrial purposes due to several reasons, which including they require short fermentation cycle times owing to their high growth rates and their capacity to secrete extracellular proteins into the medium. Schallmey *et al*. 2004 reported that *Bacillus*

spp. enzymes contribute about 50% of the total enzyme market.

For the production of any industrial enzyme, an inexpensive substrate and an efficient fermentation process are essential for commercial viability. It is well accepted that solid-state fermentation has several advantages over submerged fermentation. Generally, due to smaller volume of solvent required by the former for product recovery, it results in higher productivity per unit volume, lower contamination and foaming problems, and better exploitation of various agro-residues as substrates in comparison to the latter (Gupta & Kar, 2009).

In our laboratory, a novel strain of *Bacillus pumilus* SY30A was isolated to produce a high level of cellulase-free xylanase under solid-state fermentation (SSF). Taking into account the importance of xylanase in industry, the present study focused upon the improvement of xylanase production by the strain and its application for paper pulp bleaching.

Material and Methods

Microorganism

The organism used was *B. pumilus* SY30A, an alkaliphile isolated in our laboratory from Syrian soil (Ammoneh *et al*., 2014). The isolate was routinely cultured on nutrient agar plates (NA). The plates were incubated at 30 ºC until bacterial colonies developed, and then they are kept at 4 ºC and subcultured every fifteenth day.

Inoculums' Preparation

5 ml of medium containing nutrient broth was transferred to a 50 ml tube and sterilized in an autoclave at 121°C for 20 min. After cooling, a loopful of bacterial culture was aseptically transferred and rotated at 200 rpm (30 °C) in a shaking incubator overnight. 1% of this culture was used to inoculate 20 ml of the same medium in 100 ml flask and incubated in orbital shaker at 30 °C until the optical density at 600 nm (OD_{600}) reached 0.15 (cell density about $2x10^8$ colony-forming unit (CFU)/ml).

Solid State Fermentation

Enzyme production was carried out in 100 ml Erlenmeyer flasks containing 5 g of wheat bran and nutrients. Distilled water was then added to adjust the moisture to 60%. The fermentation medium consisted 1 g/L of K_2HPO_4; 3 g/L of NaCl; 0.3 g/L of $MgSO_4.7H2O$; and 3 g/L of yeast extract and 5 g/L of peptone, as nitrogen source. 1 ml of the prepared inoculum from *B. pumilus* SY30A strain was transferred into the solid medium and placed in the incubator. Flasks were removed from the incubator after 3 days of cultivation. Then, 25 mL of distilled water containing 0.1% Triton X-100 was added to the fermented material and the suspension in flasks were stirred for 90 min by a magnetic stirrer.

The enzyme was extracted as the clear supernatant after centrifugation at 9800 x g for 15 min.The pH of the basal media for the strain cultivation was varied from 3.0 to 11.0 to examine the effect of initial medium pH on the xylanase production. The influence of incubation temperature was also investigated by carrying out the fermentation at different temperature viz. 30, 37, 40, 45 and 50 °C.

Enzymatic Assays

Xylanase activity was determined by using 1% birchwood xylan as the substrate as described by Bailey *et al*. (1992). The solution of xylan and the enzyme at an appropriate dilution were incubated at 55 ºC for 5 minutes. The reducing sugars produced were determined according to the dinitrosalicylic acid (DNS) procedure by using xylose as the standard (Miller 1959).

The released xylose was measured by spectrophotometer with the wavelength of 540 nm. One unit (U) of enzyme activity is defined as the amount of enzyme capable of releasing 1 µmol xylose per ml per minute under the described assay conditions. On the other hand, carboxymethylcellulase activity was assayed similarly as xylanase activity, wherein 1% of carboxymethylcellulose solution (sodium salt, ultra-low viscosity) was used. DNS method

was used to assay the reducing sugars released as well while filter paper activity (FPA) was assayed according to the method recommended by Ghose (1987). One international unit of FPA is the amount of enzyme which forms 1µmol glucose (reducing sugar as glucose) per min during the hydrolysis reaction. Results given are the mean of triplicate experiments.

Biobleaching

Preparation of pulp

The oil palm empty fruit bunch (EFB) fibre was obtained from EcoFibre Bhd., Johore, Malaysia. The EFB was soaked in water for one day and then washed to remove contaminants such as sand, dust and oil. The EFB was air-dried before pulping. The EFB pulp was produced by using a 4 L stationary stainless steel digester (with neither external circulation mixing nor internal agitation), manufactured by NAC Autoclave Co. Ltd., Japan, fitted with a microcomputer-controlled thermocouple at the Department of Bioresource, Paper and Coating Technology, School of Industrial Technology, USM.

Biobleaching of pulp with crude extract enzyme

The unbleached EFB pulp at 5% consistency (W/W) was treated with *B. pumilus* SY30A crude extract enzyme obtained under optimized conditions for xylanase production. The experiments were carried out in flasks at pH 5 and incubated at 55 ºC. After the completion of the treatment, the pulp was filtered and rinsed with distilled water. The resultant pulp was determined for Kappa number and viscosity.

Pulp analysis

The effect of lignin removal from pulp was analyzed by determining the kappa number according to TAPPI UM-246. The viscosity of pulp was examined based on JPRI Standard 3015 (a modified method of TAPPI Standard T230 su-66) to indicate the average degree of polymerization of cellulose, which gives a relative indication of the cellulose degradation.

Results and Discussion

Effect of Different agro-residues on Xylanase Production

The production cost of xylanase is one of the major obstructions in wide spreading its application. Hence, having low cost production systems for this enzyme is a necessity (Bae *et al*. 2008). To achieve the aim, the utilization of low cost agro-residual waste as a substrate was targeted and the developments of more efficient bioprocess strategies were intended to obtain high enzyme titre (Biswas *et al*. 2010). The effect of various agro-industrial substrates, including soy cake, wheat bran, wheat straw, cotton seed cake, corn cobs and beet pulp, on xylanase production were shown in (Figure 1).

Nevertheless, it was obvious to see that not all the agro-residues were suitable to be utilized as substrates for the xylanase production

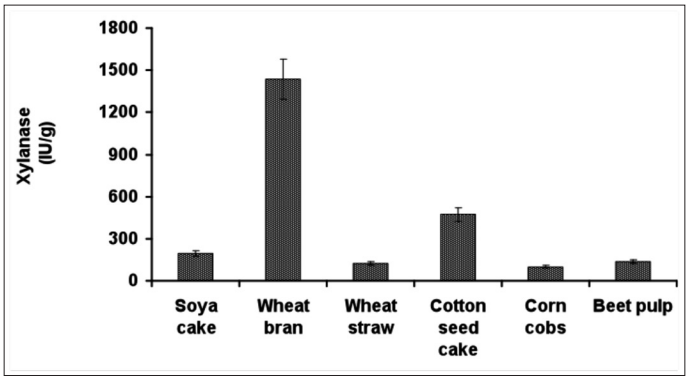

Figure 1: Xylanase production by *Bacillus pumilus* SY30A using agro-industrial substrates under solid state

by the *B. pumilus* SY30A. Figure 1 clearly demonstrated that wheat bran was found to be the best substrate for endoxylanase production (1438 IU/g) in the present study. Moreover, its xylanase production was even about three times higher than that of cotton seed cake (482 IU/g), which was the second highest xylanase production substrate. The suitability of wheat bran for xylanase production by solid-state was reported by several workers (Gessesse & Mamo, 1998; Malarvizhi *et al*., 2003; Ninawe and Kuhad, 2005). For instance, Gessesse and Mamo (1998) had found that a high level of xylanase was produced by *Bacillus* sp. AR-009 when wheat bran was used as a solid support. On the other hand, Ninawe and Kuhad (2005) claimed that wheat bran was an enhancer for xylanase production by *Streptomyces cyancus* SN32.

Xylanase Production by Bacillus pumilus *SY30A on Wheat Bran*

Effect of medium pH

Result in Figure 2 shows the effect of initial medium pH on xylanase production from *Bacillus pumilus* SY30A by solid-state fermentation (SSF) at 37 ºC for 72 hours. It was obvious to see that the production of xylanase was increased by the increase of pH from 3.0 to 9.0 and the production slightly declined when pH was further increased to 10.0 and 11.0. This indicated that the production of xylanase by *B. pumilus* SY30A pH was very sensitive to pH changes. The result showed that mild alkaline of pH 9.0 was the optimum for *B. pumilus* SY30A to have the highest xylanase production (1438 IU/g). The result was in agreement to those xylanase productions by *Bacillus* sp. (Anuradha *et al*., 2007) and an alkalophilic *Bacillus* strain MK001 (Kapoor *et al*., 2008), wherein the maximum yield of xylanase was also reported at the initial medium pH of 9.0.

As reported by many researchers, the optimum pH of the medium is varied among different microorganisms as they require a range of pH for growth and activity. Verification of the optimum pH for a certain microorganism is necessary because many enzymatic systems and the transport of various species of enzymes across the cell membrane are strongly influenced by the pH (Kapoor *et al*., 2008). The use of unfavourable pH during the cultivation of the organisms might limit the growth; furthermore, the xylanase production was also retarded due to the substrate inaccessibility. Basically, xylanase production by bacteria is preferable at mild alkaline medium, whereas acidic medium of pH 4-6 favours fungal xylanases production (Poorna & Prema, 2006).

Effect of Incubation Temperature

On the other hand, Figure 3 shows that the fermentation incubation temperature had a substantial effect on xylanase production

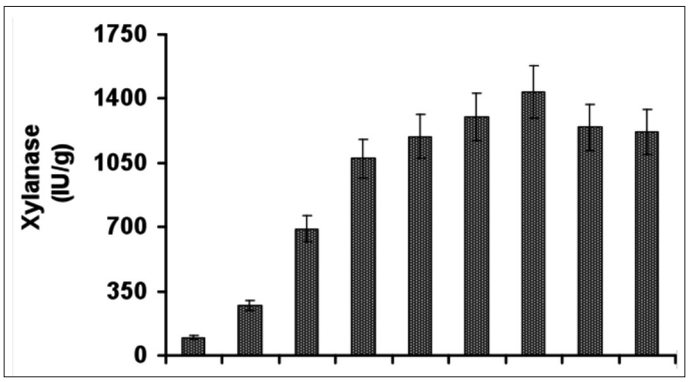

Figure 2: Effect of initial pH of the medium on xylanase production from *Bacillus pumilus* SY30A under solid state fermentation after 72h of incubation at 37C

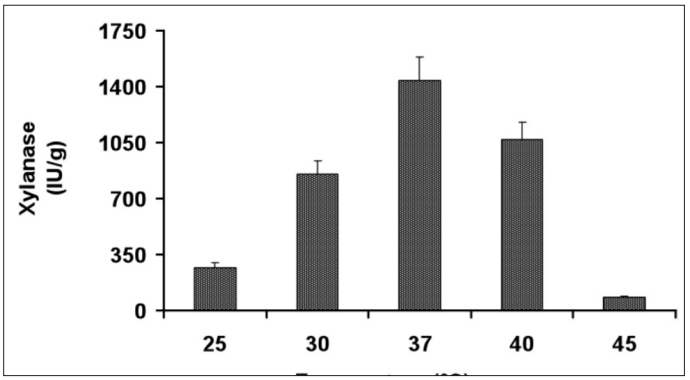

Figure 3: Effect of incubation temperature on xylanase production from *Bacillus pumilus* SY30A under solid state fermentation after 72h of incubation

by *Bacillus pumilus* SY30A under solid-state fermentation. A markedly increase of xylanase yield from 267 IU/g at 25 ºC to 833 IU/g at 30 ºC. A further increase of temperature to 37 ºC even increased the yield up to 1438 IU/g. Nevertheless, beyond 37 ºC, although with merely 3 ºC increment of temperature, the xylanase yield showed a dramatic decrease to 1050 IU/g and finally dropped to 81 IU/g at 45 ºC. Corresponded to our result, the incubation temperatures lower than 37 ºC or over 40 ºC were not favourable for enzyme production. The result was in agreement with Sanghi *et al*. (2009) and Hidayah *et al*. (2008) findings. The former verified that the temperature for optimum xylanase production from *B. subtilis* ASH was at 37 ºC, and the latter found that endoglucanase from *B. pumilus* EB3 was maximally secreted at 37 ºC.

Other enzymes activities

Other enzymes viz. carboxymethyl-cellulase (CMCase) and filter-paperase (Fpase) productions by *B. pumilus* SY30A were also examined in this study. *B. pumilus* SY30A strain demonstrated very low of cellulase activities. In which, the activities of CMCase and Fpase were only 0.05 IU/g and 0.016 IU/g, respectively. This indicating that enzymes released from *B. pumilus* SY30A is cellulase-free nature and it is suitable to be used in bleaching of paper pulp because cellulose hydrolysis can be minimized.

Biobleaching of Oil Palm EFB Pulp

Effect of incubation time

The effect of different incubation times for enzymatic pretreatment of pulp with crude xylanase from *B. pumilus* SY30A was studied. The unbleached EFP pulp with 5% consistency was pretreated with enzyme dose of 30 U/g of moisture free pulp for different time intervals (1-5 h). A control sample of pulp (not treated with xylanase) was also processed under identical conditions. Figure 4 shows the maximum reduction in kappa number (45%) was achieved with 2 hours incubation time. However, it is obvious that the reduction of kappa number could reach about 43% over the maximum reduction of kappa number with only 1 hour incubation. On the other hand, a prolonged incubation with more than 2 hours did not give any beneficial effect on enzymatic delignification of pulp.

Apart from that, it was interesting to see that the viscosity of pulp increased after the first hour of incubation and then decreased to a constant viscosity with further extend of incubation times (Figure 4). The increase of pulp viscosity was mainly due to the hydrolysis of hemicellulose, majorly xylan, with lower degree of polymerization from the pulp and hence indirectly increased the average degree of polymerization of cellulose (Suurnäkki *et al*., 1997). Nevertheless, the decrease of the pulp viscosity with the incubation times of two

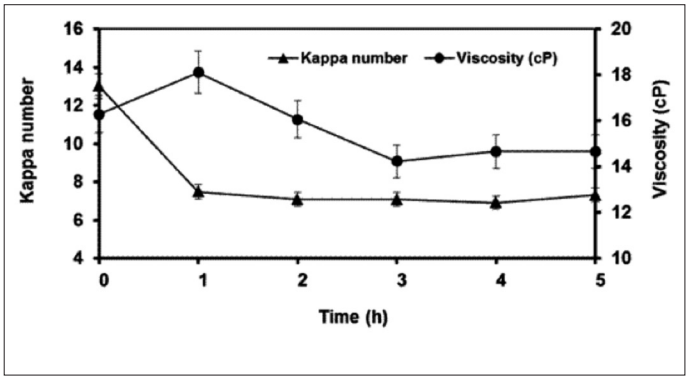

Figure 4: Effect of incubation time on biobleaching of EFB pulp using crude xylanase (30 IU/g of dry pulp) from *Bacillus pumilus* SY30A

and three hours indicated that the degradation of cellulose chain was possibly occurred. Since the prolonged incubation with three hours up to five hours yielded pulp with a constant viscosity, it might imply that there was a minimum amount of cellulolytic enzymes present in the extract of *B. pumilus* SY30A xylanase.

In short, the results designated that xylanase pre-treatment with *B. pumilus* SY30A xylanase for 1 hour was sufficient and optimum to attain a pulp with substantially low kappa number and retained high pulp viscosity.

Effect of enzyme dose on bleaching of EFB pulp

The bleaching efficiency using different xylanase doses (10-60 U/g) of crude xylanase from *B. pumilus* SY30A was also carried out with 1 hour incubation time. (Figure 5) clearly shows that the increase of the xylanase dose up to 20 U per g of dry pulp decreased the kappa number significantly. However, xylanase doses beyond 20 U/g did not exhibit any beneficial effect on kappa number reduction. The result was in agreement with findings reported by Suurnäkki *et al*. (1997) and Terrasan *et al*. (2013), that above a certain enzyme concentration, even though the degree of hydrolysis increased, only small additional benefits were observed. The effect of xylanase treatment on the removal of lignin of oil palm EFB pulp was very promising because with only 20 U of *B. pumilus* SY30A crude extract enzyme per gram of dry pulp at 55 °C for 1 hour, the reduction of kappa number reached 43.8%. The result was more effective than treatment with *Arthrobacter* sp. MTCC 5214

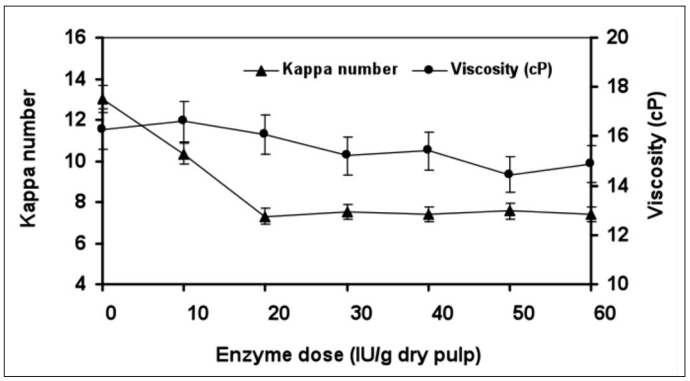

Figure 5: Effect of xylanase dose (IU/g dry pulp) from *Bacillus pumilus* SY30A for biobleaching of EFB pulp

(Khandeparker & Bhosle, 2007) and *Bacillus* sp. XTR-10 (Saleem *et al*., 2009), where the former merely achieved 20% reduction in kappa number of unbleached pulp with 20 U/g, whether the latter attained 60% reduction in kappa number with 40 U/g.

On the other hand, it was very interesting to see that the increase of enzyme dosage to 60 U/g only caused a slightly decrease of the pulp viscosity. The result ascertained that the presence of cellulolytic enzyme in the *B. pumilus* SY30A was very little. As a result, the selectivity between the delignification (due to the hydrolysis of xylan) and cellulose degradation by this enzyme treatment was rather high.

Conclusion

The present study reveals that *Bacillus pumilus* SY30A strain is a highly potential and promising microorganism as it produced a high level of xylanase under SSF improved conditions. Besides, the crud xylanase from this strain was found beneficial in prebleaching of EFB pulp. The prebleaching with xylanase was very promising towards oil palm EFB pulp by giving rather high kappa number reduction and retaining high pulp viscosity. The present work has verified the potential of xylanase enzyme in oil palm EFB pulp prebleaching process at laboratory scale. However, scale up of this environmental sustainable technology is required to ensure the process is applicable in pulp and paper industry.

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