JOURNAL OF PURE & APPLIED MICROBIOLOGY, Nov. 2014. Vol. **8**(Spl. Edn. 2), p. 491-498

Bacterial Production of Xylanase using *Nichotina tabaccum* Leaf Dust as Substrate

Komal P. Acharya* and Prateek Shilpkar

Department of Microbiology, M.D. Gramseva Mahavidyalaya, Gujarat Vidyapeeth, Sadara, India.

(Received: 06 August 2014; accepted: 29 October 2014)

The xylanase producing bacteria were isolated from soil. Among which three isolates KA01, KA02 and KA03 were used for further studies. From optimization study it was observed that, xylanase production is maximum at pH 5 and 30°C when 2% (v/v) inoculum of bacterial isolate KA01 was inoculated for 36 hours at 4% concentration of *Nichotina tabaccum* leaf dust provided with potassium nitrate (1% w/v) as nitrogen source and xylose as carbon source in nutrient medium under submerged condition at 150 rpm. Isolate KA 02 gives maximum xylanase production at optimized pH of 7, fermentation temperature 25°C, substrate concentration 5%, inoculum size 5%, incubation time 36 hrs, N- source urea and peptone (0.75%) and xylose as sugar source. Bacterial isolate KA03 reproduced maximum xylanase at pH 7.5, incubation temperature 50°C, concentration of substrate 3%, inoculum size 2%, incubation time 48hrs, N- source potassium nitrate and fructose as sugar. Inoculum age of 12 hrs was found optimum for all the three isolates. Further the xylanase production was found highest (1.77 U/mL) under KA 01 fermented under its optimized conditions compared to other two isolates in their optimized conditions.

Key words: Fermentative production, optimization, enzyme production, plant leaf dust**.**

Xylan is a heteropolysaccharide lignocellulosic polymer with a homopolymeric backbone chain of β-1,4 linked D-xylo-pyranose units. This backbone is commonly substituted with L-arabinofuranose, glucuronic acids or 4-O-methyl-D-glucuronic acid[1]. Xylan is mainly found in the secondary cell wall of plants and form an interface between lignin and other polysaccharides. Xylan is the most important for fiber cohesion and plant cell wall integrity.

* To whom all correspondence should be addressed.
TeleFax:+91-79-23274274;
E-mail: komal.ach13@gmail.com

Complete degradation of xylane requires the combined action of a complex enzymatic system known as xylanases. Xylanases are produced by a number of microorganisms including bacteria, yeast and filamentous fungi [2]. Xylanases are being applied in many industrial process like for bioconversion of lignocelluloses to sugar and alcohol [3]; pretreatment of lignocellulosic biomass; paper and pulp bleaching; textile and food processing industries etc [4]. Currently fruit juices are also being clarified using xylanases.

Since xylan is found abundantly in plant parts so it can be obtained by microbial fermentation process of these plants. Present study involves use of tobacco plant leaf as substrate in place of pure xylan and xylanase was produced by their bacterial fermentation. Bacterial species were

isolated locally from soil and screened for maximum xylanase production. Xylanase production was also optimized for various fermentation parameters.

MATERIALS AND METHODS

Chemicals

All chemicals used during the experimental work were of analytical grade. Brichwood xylan was purchased from SRL, India. *Nichotina tabaccum* leaf dust was used as substrate, was obtained from Bhadran, Kheda district, Gujarat.

Isolation, screening and identification of xylanolytic bacteria

Bacteria having xylanolytic acticity were isolated from soil samples collected from composting and dumping sites of Gujarat Vidyapith Center, Sadra, Gandhinagar, Gujarat by serial dilution method using nutrient agar medium contaning 1% brichwood xylan (Peptone 10g/L, Meat extract 3g/L, NaCl 5g/L, Brichwood xylan 10g/L, pH 7). The isolated colonies were further purified and then used for screening. For screening above N-agar medium was used and potential bacterial degraders were screened out on the basis of diameter of distinct clear zones around their colonies. Three potent bacterial species were selected and identified by cultural, morphological and biochemical characteristics. They were named KA 01, KA 02 and KA 03.

Optimization of fermentation conditions for xylanase production

Selection of fermentation medium

All the three bacterial isolates were inoculated separately in broth medium of Mandel and Sternberg medium(MS) (g/L, Urea- 0.3, ammonium sulphate-1.4, KH_2PO_4-2, $CaCl_2$-0.3, $MgSO_4$-0.3, protease pesptone-1, $FeSO_4.7H_2O$-0.005, $MnSO_4.7H_2O$-0.0016, $ZnSO_4.7H_2O$-0.0014, $CoCl_2$-0.002, tween 80-0.1%(v/v), pH-7), Mendel and Weber medium (MW) (g/L, urea-0.3, , ammonium sulphate-1.4, KH_2PO_4-2, $CaCl_2$-0.3, $MgSO_4$-0.3, protease pesptone-1, $FeSO_4.7H_2O$-0.005, $MnSO_4.7H_2O$-0.0016, $ZnSO_4.7H_2O$-0.0014, $CoCl_2$-0.002,pH-7) , salt medium (g/L, $MgSO_4$-0.01, $(NH_4)_2SO_4$-0.1, KH_2PO_4-0.2, K_2HPO_4- 0.7, sodium citrate-0.05, glucose-0.1, pH-7), Berg's medium(g/L, $NaNO_3$-2, $MgSO_4$-0.5, KH_2PO_4-0.05, $FeSO_4$-0.01, $CaCl_2$-0.02, $MnSO_4$-0.02, pH-7), fermentation medium (g/L, L-glutamic acid-0.3, NH_4NO_3-1.4, Tween 80-0.2%, KH_2PO_4-2, $CaCl_2$-0.3, $MgSO_4$-0.3, protease peptone- 0.75, $FeSO_4.7H_2O$-5, $MnSO_4$-1.6, $ZnSO_4.7H_2O$-1.4, $CoCl_2$-2, pH-7) and brichwood xylan containing Agar medium NaCl-5, meat extract-3, brichwood xylan-10, pH-7) and incubated at 37°C for 24hrs. After incubation time all the medium were centrifuged and the supernatant were analysed for xylanase activity following DNSA method.

Optimization of substrate concentration

Brichwood xylan in above optimized fermentation medium was substituted by *Nichotina tabaccum* leaf dust at different concentrations of 1, 2, 3, 4 and 5% (w/v). Bacterial isolates were inoculated in these medium and incubated for above mentioned conditions. After incubation xylanase activity was measured by DNSA method.

Effect of agitation

The effect of static and agitating conditions on fermentative xylanase production from tobacco leaf dust was checked. Two sets of three conical flasks having 100 mL fermentation medium containing 1% tobacco leaf dust were prepared and inoculated with above isolated three bacterial cultures. One set of three flasks was incubated in orbital shaker at 150 rpm and another set was kept in static condition at 37°C for 24 hr. After incubation, the supernatant of all the flasks was analysed for their xylanase activity by DNSA method.

Optimization of pH

To optimize the pH for highest xylanase production, optimized medium containing different pH viz. 5, 5.5, 6, 6.5, 7 , 7.5 and 8 were prepared and inoculated with respective three cultures. After incubation at 37°C for 24 hr, xylanase activity was checked by DNSA method.

Optimization of temperature

To find out optimum temperature, fermentation medium inoculated with bacterial cultures and incubated at 20, 25, 30, 35, 40, 45 and 50°C for 24 hr. and xylanase activity was measured thereafter.

Optimization of incubation time

Isolated bacterial cultures were incubated in their optimized medium at above optimized conditions for 12h, 24h, 36h, 48h, to 60h followed by measurement of xylanase activity.

Optimization of inoculum size

Bacterial cultures were added in their respective optimized medium @ 1%, 2%, 3%, 4% and 5% (v/v) and incubated for optimized time followed by measurement of xylanase.

Optimization of inoculum age

To determine the age of inoculum that can give optimum xylanase production, inoculum were prepared from12h, 24h, 36h, 48h and 60h old bacterial cultures and incubated for 24hr. After incubation, xylanase activity was checked for all the sets by DNSA method.

Optimization of nitrogen source

To know the optimized nitrogen source in medium for highest xylanase production, different nitrogen sources like ammonium sulfate, potassium nitrate, urea, peptone (0.5, 0.75, 1.0, 1.25 and 1.5%, w/v) and ammonium nitrate were used. The other components of medium were remained constant and inoculated with cultures. Xylanase production in all media was checked after incubation using DNSA method.

Effect of sugar

Different sugars were used as carbon source in fermentation medium to find out the best carbon source for maximum xylanase production. Cultures were inoculated in medium having sugars like xylose, sucrose, fructose and glucose. After incubation, xylanase activity was checked by DNSA method.

Enzyme production and estimation

After optimization, enzyme production by all three bacterial isolates was done under submerged condition as per optimized conditions. After incubation, fermentation media were centrifuged at 10,000 rpm for 15 minutes at 4^0C. Then the supernatant was used as enzyme source to perform xylanase assay by DNSA method. Xylanase activity was measured by using 1% (w/v) brichwood xylan or *Nichotina tabaccum* leaf dust as substrate and the release of reducing sugar xylose in 60 minutes at 50^0C was measured using DNSA (3,5- Dinitrosalicilic acid) method [5].Reaction mixture was containing 1 mL of 0.1M citrate buffer; pH 8, 0.5 mL enzyme and 1 mL substrate. O.D. of the reactive mixture was measured at 540 nm using spectrophotometer. One unit of xylanase activity (U/mL) is defined as the amount of enzyme liberating 1 µmole of xylose per minute.

RESULTS AND DISCUSSION

Isolation and screening of xylanase producing bacteria

Different soil samples were diluted serially and inoculated in xylan containing N-agar medium plates followed by incubation at 37°C for 24hrs. Isolated colonies from all the plates were purified and screened on above medium plates based on diameter of solubilization zone. Three isolates showing measurable solubilization zone were screened and named KA01, KA02 and KA03 (Table 1).

Table 1. Diameters of solubilization zone of bacterial isolates

S. No.	Bacterial isolate	Diameter of soliubilization zone (mm)
1	KA01	35
2	KA02	12.5
3	KA03	26

Identification of potent xylanolytic bacteria

On the basis of morphological, cultural and biochemical characteristics, the bacterial cultures were identified (Table 2 to 5). KA01 and KA02 were identified as *Bacillus sp*. and KA03 was identified as *Pseudomonas sp*.

Table 2. Microscopic observation

Isolates	KA01	KA02	KA03
Size	Big	Big	Small
Shape	Bacilli	Bacilli	Short rod
Arrangement	Single, chain	Single single	Cluster,
Gram reaction	+ve	+ve	-ve

Optimization of fermentation condition

Selection of fermentation medium

The optimum fermentation medium for potent xylanolytic bacteria was selected by observing their xylanase activity in different fermentation medium. Among which, bacterial isolates gave highest xylanase activity in nutrient broth containing 1% brichwood xylan (Fig 1). These results show that, among all other medium,

Table 3. Cultural characteristtics of KA01, KA02 and KA03

Isolates	KA01	KA02	KA03
Size	Intermediate	Large	Intermediate
Shape	Round	Round	Round
Margin	Entire	Entire	Irregular
Texture	Smooth	Smooth	Smooth
Elevation	Flat	Flat	Flat
Opacity	Translucent	Translucent	Translucent
Pigment	Nil	Nil	Nil
Cosistency	Moist	Dry	Dry

Table 4. Results of biochemical tests KA01, KA02 and KA03

Biochemical test	Reagent	KA01	KA02	KA03
MR test	Methyl red	-ve	-ve	+ve
VP test	5% α-napthol, 40% KOH	-ve	-ve	-ve
Simmon citrate	Bromothymol blue	+ve	+ve	+ve
Triple suger Iron	-	+ve	+ve	+ve
Urea hydrolysis	-	-ve	-ve	-ve
Gelatin hydrolysis	-	+ve	+ve	-ve
Starch hydrolysis	Lugol's iodine	+ve	+ve	+ve
Catalase	$3\%H_2O_2$	-ve	+ve	+ve
Oxydase	-	+ve	+ve	+ve
Mobility test	-	+ve	+ve	+ve
2% peptone	Lead acetate paper	-ve	-ve	-ve

Table 5. Results of biochemical sugar test for culture KA01, KA02 and KA03

Biochemical test	KA01	KA02	KA03
Fructose	+ve	+ve	+ve
Sucrose	+ve	+ve	+ve
Glucose	+ve	-ve	+ve
Xylose	-ve	-ve	-ve
Rhmanose	-ve	-ve	-ve
Raffinose	+ve	-ve	-ve
Galactose	-ve	-ve	-ve
Manitol	+ve	+ve	+ve
Arabinose	-ve	-ve	-ve
Trihalose	+ve	+ve	+ve
Ribose	-ve	-ve	-ve
Mannose	+ve	+ve	+ve
lactose	+ve	-ve	-ve

KA03 does not show xylanase activity in Mandel and Sternberg medium; KA02 is suppressed in Mandel and Weber medium, Salt medium and Fermentation medium and; KA01 is suppressed in Mandel and Weber's medium and Fermentation medium.

Optimization of substrate concentration

To obtain highest xylanase production, different concentration of *Nichotina tabaccum* leaf dust as a substrate was used. It was observed that, enzyme activity was increased up to 0.266 U/mL at 4%, 0.410 U/mL at 5% and 0.0801 U/mL at 3% for K01, KA02 and KA03 respectively (Fig 2) and

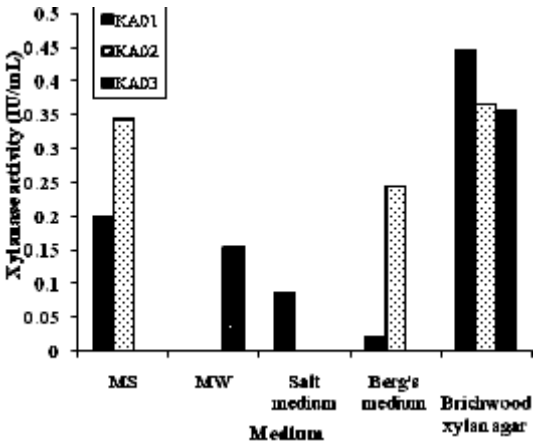

Fig. 1. Effect of medium on enzyme activity

thereafter enzyme activity decreased with increasing substrate concentration. It might be due to inhibition of enzyme or cell growth by some component of substrate. Similar results of decreased enzyme production were reported previously for *Bacillus coagulans* using wheat straw as substrate [6].

Effect of agitation

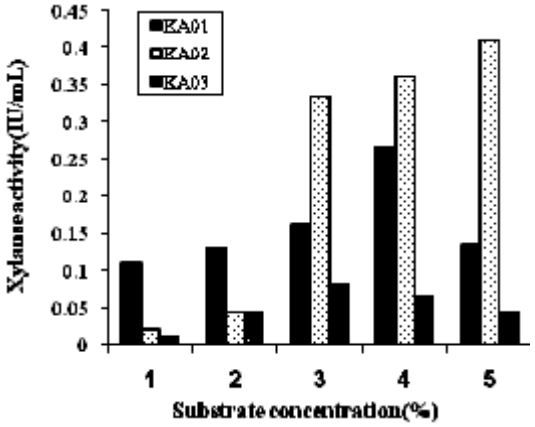

Fig. 2. Effect of substrate concentration on enzyme activity

Bacterial isolates KA01, KA02 and KA03 gave higher enzyme activity i.e. 0.222, 0.177 and 0.194 U/mL respectively under agitating condition than static condition (Fig 3). This is might be due to supply of enough oxygen and uniform mixing of nutrients during agitation fermentation conditions and these purposes are not fulfilled in static condition. Apart from this, clump formation was also observed in fermentation medium in static conditionalso reported that The agitation induces the xylanase production from *Bacillus subtillis* and *Bacillus subtillis* SV65S respectively[7,8].

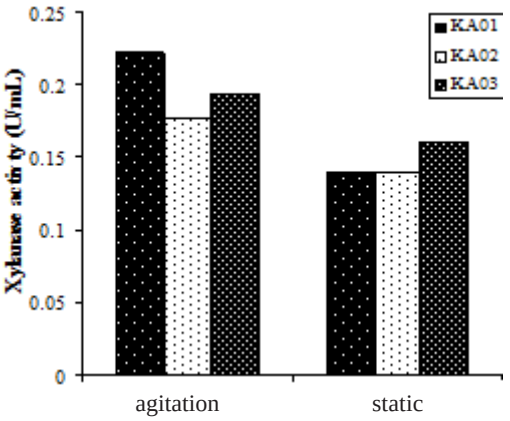

Fig. 3. Effect of agitation on enzyme activity

Optimization of pH

The medium's pH is one of the most regulatory parameters during the fermentation. In this study, maximum xylanase production was observed with pH 5 for KA01, pH 7 for KA02 and 7.5 for KA03 and showed maximum activity of 0.288, 0.177 and 0.488 U/mL respectively (Fig 4). This is because each organism holds different pH range for its optimum growth and activity. Substantial xylanase production is observed between 5 to 8 for *Bacillus pumillus* SV8 [9], at pH 9.0 for *Chromobacter* TPSV101 [10] and at pH7 for *Bacillus subtilis* [7]

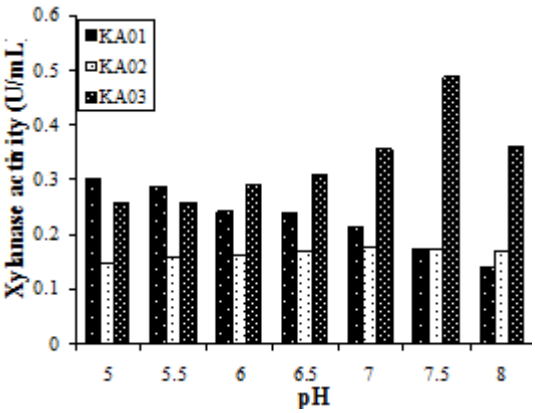

Fig. 4. Effect of pH on enzyme activity

Optimization of temperature

The highest xylanase activity was found at 25^{0}C for KA01 and KA02 while for KA03, optimum temperature was 50^{0}C (Fig 5). Any change either increase or decrease in temperature resulted in the gradual decrease in enzyme activity. The higher temperature above optimum may alter cell membrane composition and cause cell death. Maximum xylanase production by *Aspergillus niger* and *Trichoderma viridae* at 30^{0}C [11] and by *Pseudomonas thermophila* J18 [12] and for *Sporotricum thermophila* [13] at 50^{0}C was reported earlier.

Optimization of incubation time

Xylanase production by KA01, KA02 and KA03 was monitored up to 60h of incubation. Samples were withdrawn at regular interval of 12h for estimation of xylanase activity. KA01 and KA02 gave the highest activity at 36h i.e. 0.155U/mL and 0.133 U/mL respectively. While for KA03, maximum activity of 0.143 U/mL was found at 48h (Fig 6). The incubation time shows the duration needed

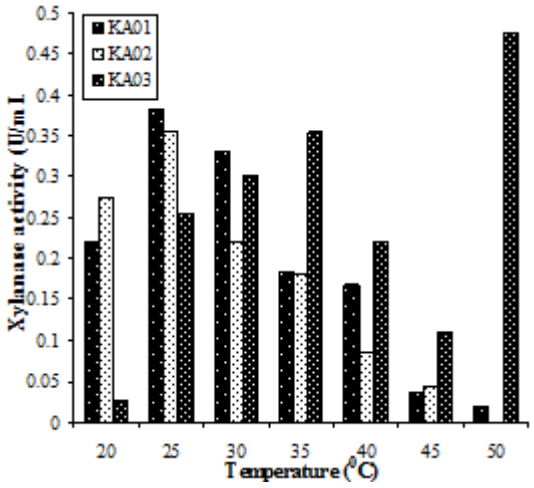

Fig. 5. Effect of temperature on enzyme activity

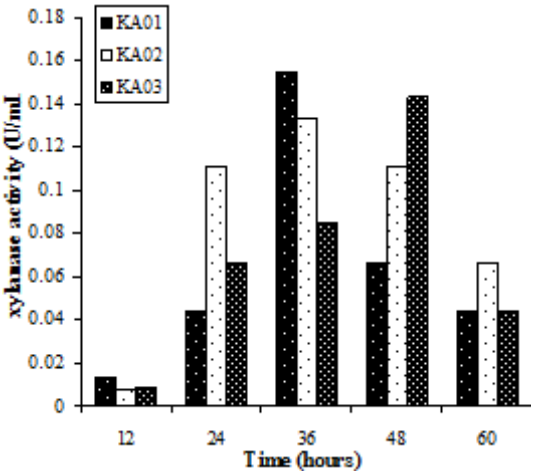

Fig. 6. Effect of time on enzyme activity

for maximum activity of microorganisms and it varies with species to species. Decrease enzymatic activity at time higher than optimum may be due to depletion of nutrients from fermentation medium. An identical incubation time of 36h was also reported for *Bacillus subtillis* for maximum xylanase activity [7] whereas for *Bacillus megaterium* it was 96 h [14].

Optimization of inoculum size

Xylanase production was highest i.e. 0.544 and 0.690 U/mL when 2% (v/v) inoculums of 24 h old cultures of KA01 and KA03 were added to the fermentation medium having 1% tobacco leaf dust respectively and it decreased with increase in inoculums size beyond 2% (Fig 7). For KA02, 5% inoculums size gave the highest enzyme activity of 0.377 U/mL (Fig 7). It is reported that maximum xylanase activity is obtained at 2% inoculums size for *Bacillus subtilis* [7] while 5% inoculum size was found to be optimum for *Bacillus subtillis* [15].

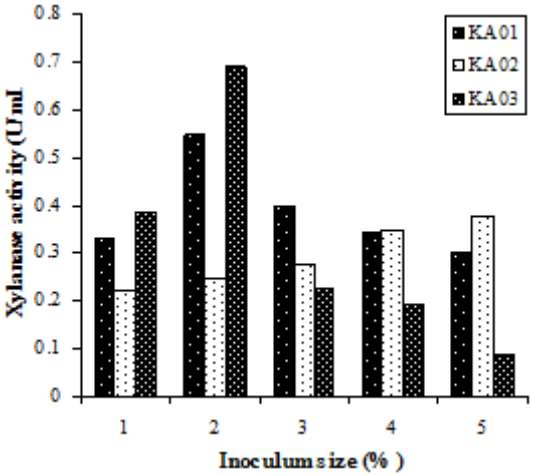

Fig. 7. Effect of inoculum size on enzyme activity

Optimization of inoculum age

The effect of inoculums age was studied by inoculating 50 mL of the production medium with 1% inoculums of 12 to 48 h old culture of KA01, KA02 and KA03. Highest enzyme activity was observed with 12 h old inoculums and it declined then after. The enzyme activity with 12h old inoculums of KA01, KA02 and KA03 were 0.755, 0.841 and 0.743 U/mL respectively (Fig 8). This is might be because of the cells may undergo stationary phase followed by decline phase. Maximum xylanase production from 18h old inoculum of *Bacillus pumillus* [8] and *Bacillus subtilis* [7] were obtained previously.

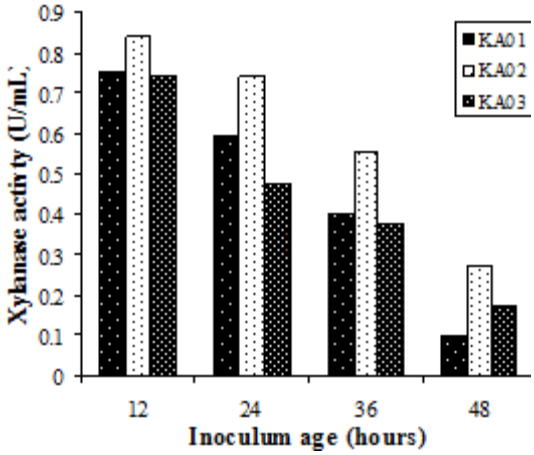

Fig. 8. Effect of inoculum age on enzyme activity

Optimization of nitrogen source

The production of primary metabolites by organisms is highly influenced by their growth which depends on the nutrients provided. Maximum xylanase activity for KA01 and KA03 was observed when 1% (w/v) potassium nitrate was provided. In case of KA02, maximum activity of 0.222 U/mL was found when urea (1% w/v) and peptone (0.75 w/v) were provided (Fig 9). Positive influence of inorganic source of nitrogen on xylanase production was also reported for *Bacillus pumilus* SV 85S by [8] whereas ammonium sulfate and sodium nitrate were found to be suitable nitrogen source for *Escherichia coli* and *Straptomyces cyaneus* SN32 respectively [16]

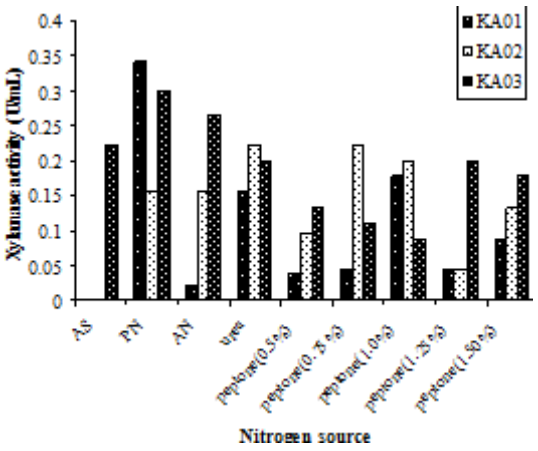

Fig. 9. Effect of nitrogen source on enzyme activity

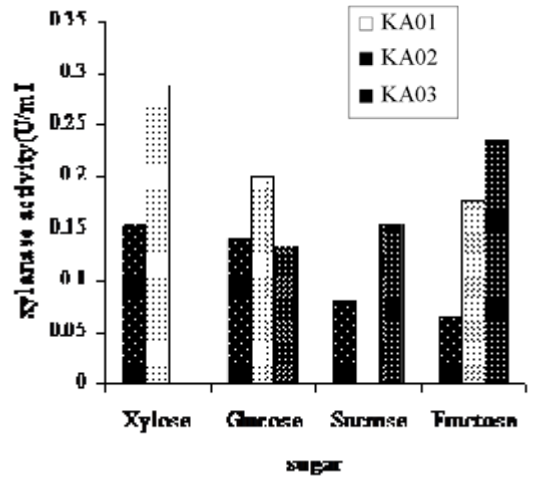

Fig. 10. Effect of sugar on enzyme activity

Effect of sugar on xylanase activity

Bacterial cultures KA01, KA02 and KA03 were grown in presence of xylose, glucose, sucrose and fructose. Among all, xylose acted as inducer for KA01 and KA02 and fructose for KA 03 (Fig 10). Xylose as the best sugar source is reported for *Bacillus sp.* [17], *Melanocarpus albomyces* [18] and *Pleuritus eryngii* [19] for xylanase production.

Production and estimation of xylanase

All the cultural conditions for enzyme production were optimized and the 12h old cultures of KA01, KA02 and KA03 were inoculated in fermentation medium using *Nichotina tabaccum* leaf dust as substrate as per optimized condition. Among all these three isolates, KA01 produced the maximum amount of xylanase (1.77 U/mL) after 36h incubation (Table 6).

Table 6. Xylanase activity of three bacterial isolates in optimizatied conditions

S. No.	Bacterial isolate	Xylanase activity (U/mL)
1	KA01	1.77
2	KA02	0.98
3	KA03	1.65

CONCLUSION

From the present study it can be concluded that, the isolate *Bacillus sp.* can produce xylanase by using *Nichotina tabaccum* leaf dust as substrate. The study indicates that, optimization of fermentation conditions can enhance the xylanase production.

ACKNOWLEDGMENTS

Authors are grateful to the Head, Department of Microbioogy, Sadara and Gujarat Vidyapith, Ahmedabad for providing necessary analytical facility to conduct this research.

REFERENCES

1. Silva, L., Carmona, E. Production and characterization of cellulose free xylanase from *Trichoderma inhamatum*. *Appl. Biochem. Biotechnol.*, 2008; **150**:117-125.
2. Gupta, V., Gaur, R., Gautam, N., Kumar, P., Yadav, I. and Darmwal, N. Optimization of

xylanase production from *Fusarium solani* F7. *Ame. J. Food Technol.*, 2009; **4**(1):20-29.

3. Viikari, L., Kantelinen, J., Sundquist, and Linko, M. Xylanase in bleaching from an idea to industry. *FEMS Microbiol. Rev.*, 1994; **13**:335-350.
4. Sa-pereira, P., Mesquita, A., Duarate, J., Barros, M. and Costa-Ferreira, M. Rapid production of thermostable cellulose free xylanase by a strain of *Bacillus subtilis* and it's properties. *Enzyme Microb. Technol.* , 2002; **30**:924-933.
5. Miller, G. L. Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Anal.Chem.*, 1959; **31**: 426-428.
6. Chaudhary, K., Kapoor, M. and Kuhad, R. Production of xylanase by *Streptomyces sp.* M83 using cost-effective substrates and it's application in improving digestibility of monogastric animal feed. *Indian. J. Microbiol.*, 2006; **46**:109-119.
7. Sanghi, A., Garg, N., Sharma, J., Kuhar, K., Kuhad, R. and Gupta, V. Enhanced production of cellulose free xylanase by alkalophilic *Bacillus subtilis* ASH and it's application in biobleaching of kraft pulp. *Bioresources* 2009; **4**(3):1109-1129.
8. Nagar, S., Gupta, V., Kumar, D., Kumar, L. and Kuhud, R. Production and optimization of cellulose free, alkali stable xylanase by *Bacillus pumillus* SV85S in submerged fermentation. *J. Microbiol. Biotechnol*., 2010; **37**:71-83.
9. Gupta, U. and Kar, R. Xylanase production by a thermotolerant *Bacillus* species under solid state and submerged fermentation. *Braz. Arch. Biol. Technol.*, 2010; **52** (6):1363-1371.
10. Prakash, S., Veeranagouda, Y. L. and Sreeramulu, K. Xylanase production using inexpensive agriculture wastes and it's partial characterization from halophilic *Chromohalobacter sp.* TPSV 101. *World J. Microbiol. Biotechnol.*, 2009; **24**:633-640.
11. Ikram-ul-Haq, Javed, M. and Khan, T. An innovative approach for hyper production of cellulolytic and hemicellulolytic enzymes by consortium of *Aspergillus niger* MSK 7 and *Trichoderma viridae* MSK-10. *Afr. J. Biotechnol.*, 2006; **5**(8):609-614.
12. Purkarthofer, H., Sinner, M. and Steiner, W. Cellulase free xylanase from *Thermomyces lanuginosus*, optimization of production in submerged and solid state culture. *Enzyme Microb. Technol.* , 1993; **15**:677-682.
13. Topaskas, E., Katapodis, P., Kekos, D., Macris, B. and Christakopoulous, P. Production and partial characterization of xylanase by *Sporotricum thermophile* under solid state fermentation. *World J. Microbiol. Biotechnol.*, 2003; **19**: 195-198.
14. Sindhu, R. Fungal xylanase production under solid state and submerged fermentation conditions. *Afr. J. Microbiol.*, 2008; **2**:82-86.
15. Sharma, A., Pujari, R. and Patel, P. Charactrization of thermoalkalophilic xylanase isolated from *Enterobacter* isolates. *Ind. J. Biotechnol.*, 2008; **8**:110-114.
16. Ninawe, S. and Kuhad, R. Use of xylan rich cost effective agro residues in the production of xylanase by *Streptomyces cyaneus* SN 32. *J. Appli. Microbiol.*, 2009; **99**:1141-1148.
17. Lopez, C., Blanco, A. and Pastor, F. I. J. Xylanase production by a new alkali-tolerant isolate of *Bacillus*. *Biotechnol. Lett.*, 1998; **20**: 243–246.
18. Sarawat, V. and Bisaria, V. S. Biosynthesis of xylanolytic and xylan-debranching enzymes in *Melanocarpus albomyces* 11S 68. *J. Fermentation and Bioeng.*, 1997; **83**:352-357.
19. Altaf, S. A., Umar, D. M. and Muhammad, M. S. Production of xylanase enzyme by *Pleurotus eryngii* and *Flamulina velutipes* grown on different carbon sources under submerged fermentation. *World Appl. Sci. J.*, 1957; **8**: 47-49.