Isolation and Identification of Highly Xylanase Producing Bacterium *Sphingobacterium* sp. SaH-05 from Soil

Younes Ghasemi, Hajar Sadeghi, Ahmad Gholami, Milad Mohkam, Mohammad Kargar

Abstract— A newly xylanolytic *Sphingobacterium* strain, designated as SaH-05, was screened from farmland environment of Fars province, Iran. Optimization of cultural conditions for production of xylanase was conducted by keeping all of parameters constant except the one which was studied. One hundred and fifty bacterial strains were isolated from different samples collected and screened for production of xylanase enzyme. One isolate designated as SaH-05 was identified as *Sphingobacterium* sp. based on morphological and biochemical characteristics along with 16s rRNA partial sequence analysis. This strain showed highest xylanolytic activity in media containing xylan as sole carbon source. The production of xylanase by this organism was optimized using different substrate concentrations, pH, temperature, incubation period and nitrogen sources. The maximum enzyme production (40 U/ml) by *Sphingobacterium* sp. SaH-05 was observed in wheat bran 0.9%, ammonium sulfate 0.01%, pH 8, 37 °C and after 24 h of incubation. Therefore, this indigenous isolate could be considered as promising xylanase producer with low requirement of carbon and nitrogen substrate, suitable for food industrial applications.

Index Terms— *Xylanase*, *Screening*, *Sphingobacterium*, Xylan, 16srRNA, Identification, xylanolytic

1 Introduction

XYLANS, the second-most plentiful polysaccharide in environment, are 1,4-linked β-D xylose heteropolysaccharides, which are the chief constituent of hemicelluloses in plant cell walls [1, 2]. Xylanase (EC 3.2.1.8), the enzyme responsible for degradation of xylans, have enormous commercial and industrial potential [2]. Xylanases in associated with the action of various hydrolytic enzymes involve in biodegradation of xylans [2]. According to their amino acid sequence of catalytic domain (http://pfam.sanger.ac.uk/), xylanases are principally categorized into two glycoside hydrolase (GH) families [3].

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The discovery of xylanase by Viikarri et al. (1986), led to increasing demand for their uses in industries [2]. Since then there has been much focused toward searching more novel microbial isolates, the xylanases from which can be used in the pulp and paper industries [2]. Moreover, xylanases are also used extensively as food additives to poultry [1], in wheat flour for improving dough suitable for making bread and characteristics for better quality of baked products [4], for the extraction of coffee, starch, and plant oils [5], and in combination with cellulase and pectinase for clarification of fruit juices [6]. Furthermore, their hydrolysis products can be transformed into xylitol (a non-cariogenic sweetener), or can be applied in the food industry as fat substitutes or thickeners [7]. In addition, xylo-oligosaccharides, chiefly xylobiose, demonstrate prebiotic properties that have a promoting influence on the growth of the gut bacterium *Bifidobacterium* [8].

There have been such reports on microbial production of xylanase from various fungi and bacterial species [2]. However, there have been a few studies on xylanase production by *Sphingobacterium* spp [3]. A soil isolate identified as *Sphingobacterium* sp. SaH-05 produced interestingly large amounts of xylanase enzyme. In this study we aimed to really appreciate the screening and isolation of highly xylanase-producing bacteria from soil in different area of farmlands in Fars province, Iran. The effect of various parameters on xylanase production was also studied.

2 Material and methods

2.1 Isolation and screening xylanolytic bacteria

Different samples of agriculture wastes in farmland area in Fars province, Iran, were collected and isolated by an enrichment culture method in order to detection of xylanase producing bacteria. The soil sample (1 g) was put into 100 ml of XC broth in a 500 ml Erlenmeyer and incubated on a rotary shaker at 150 rpm at 37 °C for 24 hours. The XC-broth medium containing 0.04% K_2HPO_4, 0.01% $MgSO_4$, 0.002% KCl_2, 0.0002% $FeSO_4$, 0.005% $(NH_4)_2SO_4$ and 0.03% xylan beech wood (w/v) (PH7). The subculturing was repeated three times with one milliliter of the previous culture and transferred into fresh XC broth. The enriched cultures were diluted (0.1 ml) and plated onto enrichment XC medium agar plates to isolate pure colonies. Then, the grown colonies on XC-agar plate were flooded with 0.1% (w/v) Congo red solution and kept for 30 min, and then washed by 0.1M NaCl solution. The colonies surrounded by clearance zone were opted as xylanase-producer strains and were purified on XC-agar plate for further studies [9].

2.2 Xylanase production

The selected isolates were incubated in XC broth medium for 18 h up to 24 h at 37 °C at 150 rpm. Then each strain with 0.1 OD was transferred to 250ml Erlenmeyer flasks containing 30ml liquid medium and incubated at 37^{0}C for 4 days on rotary shaker (150 rpm). A 2 ml sample of culture media was removed every 24h and centrifuged at 10000 g for 10 min at 4 °C then, the supernatant were collected and used for extracellular enzyme assay.

For obtaining the intercellular fraction of the xylanase, the cell pellet was washed three times with sodium phosphate buffer (pH7). After centrifugation at 10000 g for 10 min, the pellet was resuspended in the same buffer and sonicated using an ultrasonic cell disrupter in order to disperse bacterial aggregates. Then, the cell debris was removed by centrifugation at 10000×g for 10 min. The cell–free supernatant was used for intercellular enzyme assay.

2.3 Xylanase assay

Xylanase activity was determined base on the method of Miller with a little modification by measuring the amount of reducing sugars released from birch-wood xylan [10]. Briefly, 500 µl enzyme solution was added to 500 µl of phosphate buffer containing xylan at pH 7. After incubation at 37 °C for 30 min, the equal volume of 3, 5-dinitrosalicylic acid (DNS) reagent was added. The resultant mixture was then boiled for 5 min and cooled down to room temperature and its absorbance was measured at 550 nm. One unit of xylanase activity was defined as the amount of enzyme yielding 1 µmol of xylan within 1 min under the assay condition [9].

2.4 Identification of xylanase producer bacteria

The morphological, physiological and biochemical traits of xylanolytic bacteria were done according to Bergey's Manual of Systematic Bacteriology [11]. The identification of selected bacterium at the gene level, was conducted using the partial 16s rRNA gene of the selected strain was amplified by PCR. Isolation of genomic DNA and PCR amplification were adopted from Ghasemi *et al* [12]. The nucleotide sequence 16s rRNA of the tested isolate was compiled and compared with sequences in NCBI using a BLAST program. Finally, the nucleotide sequences of 16s rRNA genes were deposited to GenBank under the accession numbers JQ666256.1.

2.5 Optimization of chitinase production

In present study, the effects of cultural conditions and media composition on xylanase production by *Sphingobacterium* sp. SaH-05 were performed by using one-factor-at-a-time method, in which holding all the factors steady except the one which was studied. The investigated factors include several nitrogen and carbon source with their various concentrations (ammonium sulfate, ammonium nitrate, potassium nitrate, sodium nitrate yeast extract , tryptone and peptone), time period of incubation (24h-96h), initial pH and various fermentation temperatures (25, 37, 45^{0}C). For each of factor, three sets of experiment were conducted and the mean of the values was reported.

3 Results and discussion

3.1 Isolation and identification

A total of 150 samples were collected from various farmland area in Fars province, Shiraz, Iran, of these, 70 isolates were capable of using birch-wood xylan as a sole carbon and energy source. Thirty isolates which produced large and clear zones in shorter time (less than 48 h) were opted and transferred into liquid medium. The xylanase activity was measured by DNS method. Among the potent xylanase producers, strain SaH-05 showed the highest xylanolytic activity (35.21 U/ml) at 24 h after incubation. Therefore, strain SaH-05 was opted for future study and identification through 16s rRNA sequence analysis and phenotypic characterization. The taxonomic analysis of isolated SaH-05 was identified to the genus *Sphingobacterium* on the basis of Bergey's Manual of Systematic Bacteriology. The phenotypic tests showed this bacterium is gram negative, rod shaped, mobile, catalase and oxidase positive (Table 1). Further 16s rRNA sequence analysis also confirmed that this bacterium is belong to a member of *Sphingobacterium* sp with more than 98% nucleotide sequence homology.

3.2 Effect of incubation time on xylanase production

Production of xylanase by *Sphingobacterium* sp. SaH-05 at 37°C was followed by estimating the xylanase activities at

Table 1: Biochemical properties of *Sphingobacterium* sp. saH -05 strain

Biochemical properties	Result
Catalase	+
Oxidase	+
Motility	+
Urease	-
Voges–Proskauer	+

Hydrolysis of Starch and Tween 80	+
Utilization of	
Raffinose	-
Sucrose	+
D-Fructose	-
D-Glucose	+
Utilization of citrate	+
Nitrate reduction	-
Growth in NaCl	
0-4 %	+
5-7 %	-

Results represent the average ±SD of three replicates.

various time intervals (Fig. 1). The xylanase production rose rapidly from 8 to 24 h with a maximum xylanase yield of 40 U/ml and remained constant until 72 h, then declined sharply at 96 h of incubation. In addition, the isolate SaH-05 showed the highest xylanase activity (35.21 U/ml) within 24 h of cultivation, which was noticeably superior than *Bacillus pumilus* (7.67 U/ml after 24) *Aspergillus niger* (30 U/kg after 72 h), *Trichoderma* sp (60 U/kg after 72 h) and *Streptomyces* 1b 24D (8.32 U/ml after 4 days) [4, 13-15]. Moreover, the short growth time (24 h) of *Sphingobacterium* sp. SaH-05 was in a similar way to *Bacillus subtilis* cho40, but considerably lower than most of the reported microorganisms including fungi and bacteria [16]. The rapid xylanase production for the newly isolated bacterium is appropriate property in industrial processes and portrays a benefit of this on the other microorganisms already proposed for such purpose including food industries.

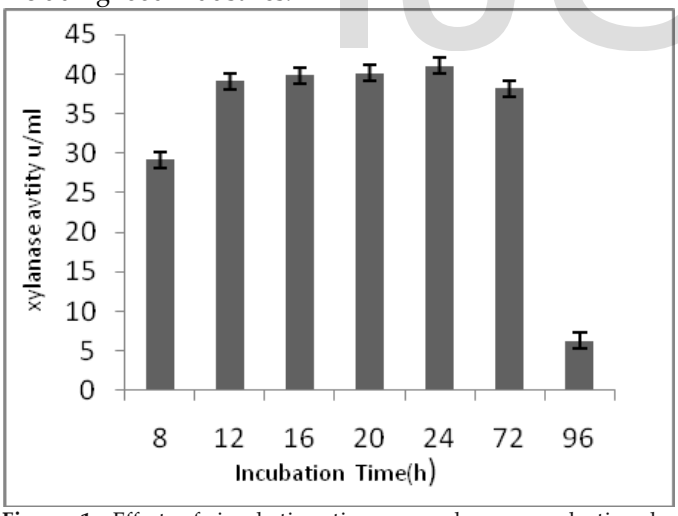

Figure 1: Effect of incubation time on xylanase production by *Sphingobacterium* sp. saH -05. Results represent the average ±SD of three replicates.

3.3 Effect of pH and temperature on xylanase production

The effect of pH and temperature on xylanolytic enzyme production by *Sphingobacterium* sp. SaH-05 was investigated by growing culture at different temperature (25, 37, 45 °C) and initial pH between 5 and 11. A high level of xylanase activity was obtained in the culture medium with pH 8 (Fig. 2) and optimum temperature at 37 °C (Fig. 3). The obtained results are in inconsistence with several researchers who reported neutral or slightly acidic conditions are favorable for xylanase production [6, 17, 18]. However, there were no reports on initial alkaline condition for optimal xylanase production. The optimal temperature for xylanase production of *Sphingobacterium* sp. SaH-05 was similar to *Sphingobacterium* sp. TN19, but also several reports on various optimal temperatures for xylanase production were reported [3].

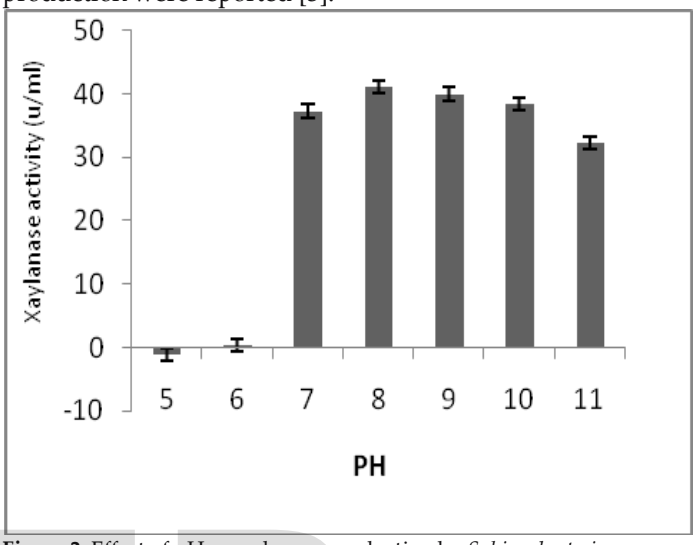

Figure 2: Effect of pH on xylanase production by *Sphingobacterium* sp. saH -05. Results represent the average ±SD of three replicates.

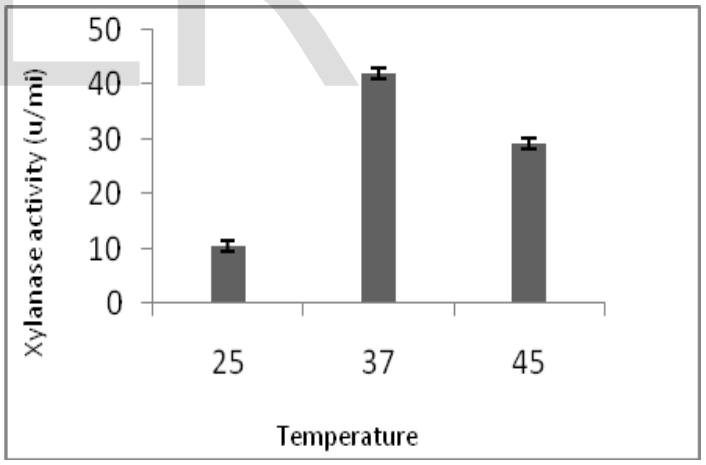

Figure 3: Effect of temperature on xylanase production by *Sphingobacterium* sp. saH -05. Results represent the average ±SD of three replicates.

3.4 Effect of nitrogen sources on xylanase production

The influence of additional nitrogen sources on xylanase production was studied by supplementing different inorganic (0.01%, w/v) and organic nitrogen sources (0.1%, w/v). Among the various nitrogen sources in the basal medium, ammonium sulfate was the most effective additives resulting in the increase of the enzyme production (Fig. 4) and then tryptone and ammonium nitrate came after as an organic and inorganic nitrogen source, albeit with a significant difference to ammonium

sulfate. Therefore, ammonium sulfate was selected d as optimal nitrogen sources for xylanase production by *Sphingobacterium* sp. SaH-05. Based on our knowledge this is the first report indicating that the ammonium sulfate lonely had significantly effect on xylanase production rather than such organic nitrogen sources. In contrast, Akhavan Sepahy et al and Sa-Pereira et al reported that ammonium sulfate has a strong repression effect on xylanase biosynthesis on *Bacillus* spp [19, 20]. This phenomenon maybe due to the reducing level of protease activity was directly in charge of the maintenance of xylanase activity [15].

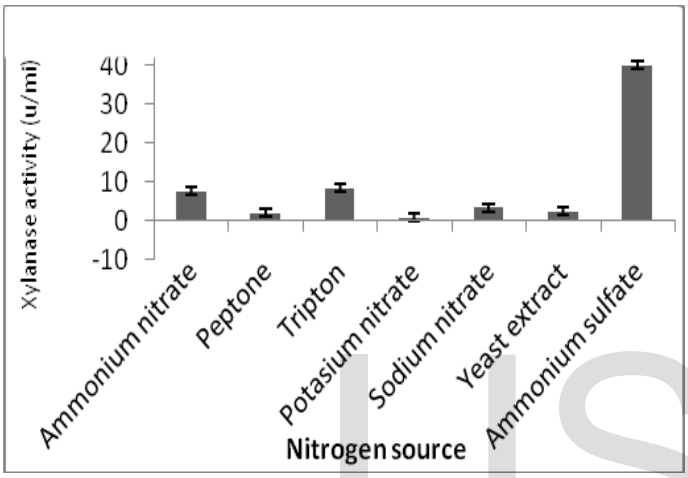

Figure 4: Effect of various nitrogen sources on xylanase production by *Sphingobacterium* sp. saH -05. Results represent the average ±SD of three replicates

3.5 Effect of xylan concentration on xylanase production

The production of xylanase by *Sphingobacterium* sp. SaH-05 was investigated in different concentrations of xylan (0.3 to 1%). The results revealed that culture medium supplemented with 0.9% xylan, produce maximum enzyme activity and then remained constant although with a little reduction in its activity (Table 2). These outcomes were in inconsistence with the finding of Akhavan Sepahi *et al.*, who reported that the production of the xylanase from *Bacillus mojavensis* was at highest rate with 2% xylan concentration [19]. In another study, Ellouse *et al.* reported that the maximum of xylanase prodsuction by *Sclerotinia sclerotiorum* was in 3% of xylan [17]. The Metabolizable saccharides such as xylose, glucose, sucrose, galactose and mannose did not have significantly repression on xylanase production (data not shown). It seems that wheat bran systems are resistant to catabolic repression even at high concentrations of such metabolizable saccharides [21]. Therefore, this bacterium could be considered as a suitable industrial choice for commercial production of xylanase in an inexpensive media due to its low requirement for carbon and inorganic nitrogen source.

Table 2: Effect of different carbon sources on xylanase production in *Sphingobacterium* sp. saH-05

Xylan Concentration (%)	Xylanase activity (U/ml)	Final pH
0.3	23±0.5	6.0±0.02
0.5	27±0.8	6.1±0.03
0.7	34±0.4	5.8±0.03
0.9	40±0.6	5.7±0.01
1	37±0.5	6.0±0.03

Results represent the average ±SD of three replicates.

4 Conclusions

Our results could be a step towards the identification of newly isolate capable to produce high amount of xylanase from such cheap carbon and nitrogen sources. The maximum xylanase was obtained at wheat bran (0.9%) and ammonium sulfate (0.01%) as an inorganic nitrogen source without the need of yeast extract and peptone. These feature reflect the potential use of *Sphingobacterium* sp. SaH-05 in a range of industries where seeking for low cost substrates. To our best knowledge, this is the first report of xylanolytic bacterium able to produce high level of xylanase along with unique properties such as non catabolite repression for xylanase production in addition the need for inorganic nitrogen source rather than organic one. In future by purification and study of biochemical characterization of SaH-05 isolate could determine further its potential for food industrial application.

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