



## Isolation of Endopyhtic and Xylanolytic *Bacillus pumilus* Strains from *Zea mays*\*

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**Abstract:** In this study, two endophytic xylanolytic bacteria, named M1 and M2, were isolated from surface sterilized *Zea mays* stem and leaf, respectively. Isolates were identified as *Bacillus pumilus*. Microorganisms showed different morphology on agar plates. Xylanase production level and profile varied between isolates, as well. Maximum xylanase production level of  $188.0 \pm 20.0$  and  $5.6 \pm 1.1$  U/ml were achieved by *Bacillus pumilus* M1 and *Bacillus pumilus* M2 in a liquid medium containing 3% corn cobs as a sole carbon source and inducer, respectively. Isolates produced very low level of cellulase in crude enzyme extract. *B. pumilus* M1 xylanase was partially characterized. Enzyme had a maximum activity at pH 8.0 and 65°C, seemed proper for pulp and paper industry, and required further characterization. In conclusion, this study indicated that inside part of *Zea mays* is a novel and good source for isolating xylanase producers. After detailed characterization, such enzymes could be used in various applications in paper and pulp, textile, food and feed industries.

**Key Words:** *Bacillus pumilus*, endopyhte, plant, xylan, xylanase producers.

### *Zea mays*'dan Endofitik ve Ksilanolitik *Bacillus pumilus* Suşlarının İzolasyonu

**Öz:** Bu çalışmada, yüzey sterilizasyonu yapılmış *Zea mays* gövde ve yapraklarından, sırası ile M1 ve M2 olarak isimlendirilen iki tane endofitik ksilanolitik bakteri izole edilmiştir. İzolatlar *Bacillus pumilus* olarak tanımlanmıştır. Mikroorganizmalar agar üzerinde farklı morfoloji sergilemiştir. İzolatlar arasında, ksilanaz üretim seviyesi ve profilinde de farklılıklar bulunmaktadır. İçinde %3 mısır koçanını tek karbon kaynağı olarak bulunduran sıvı besi yerinde, *Bacillus pumilus* M1 ve *Bacillus pumilus* M2 sırası ile maksimum ksilanaz aktivitesi olan  $188.0 \pm 20.0$  ve  $5.6 \pm 1.1$  U/ml değerlerine ulaşabilmiştir. İzolatlar, kaba fermentasyon özüünde çok düşük miktarda selüloz üretmiştir. *B. pumilus* M1 ksilanazı kısmi karakterize edilmiştir. Enzim pH 8.0 ve 65°C'de maksimum aktivite göstermiş, kağıt beyazlatma endüstrisine uygun görülmüş ve ileri derecede karakterizasyona ihtiyaç duymuştur. Sonuç olarak, bu çalışma *Zea mays*'ın iç kısmının, ksilanaz üreticilerinin izolasyonu için yeni ve iyi bir kaynak olduğunu göstermiştir. Detaylı tanımlama ardından bu enzimler, kağıt, tekstil, gıda ve yem endüstrisinde çeşitli uygulamalarda değerlendirilebilmesi mümkündür.

**Anahtar Kelimeler:** *Bacillus pumilus*, endofit, bitki, ksilan, ksilanaz üreticileri.

#### Introduction

$\beta$ -1,4-Xylan, mainly found in the secondary walls of plants, represents one-third of the renewable biomass on the earth (Bakir 2005). Xylan polymer largely consists of a  $\beta$ -D-1,4- linked D-xylose backbone substituted with acetyl, arabinosyl, and glucuronosyl side chains. Frequency and composition of the branches are dependent on the source of xylan. The complexity of the xylan molecules requires the action of the different hydrolytic enzymes. The most effective one is endo- $\beta$ -1,4-xylanase that cleaves the  $\beta$  -1,4 bonds of xylan backbone and produces xylo-oligosaccharides.

In recent years, interest in xylan degrading enzymes have been increased due to their applications in various agro-industrial processes, such as hemicellulosic biomass conversion to fuels and chemicals, paper pulp delignification, animal feedstock digestibility enhancement, xylo-oligosaccharide production, juices clarification, beer consistency improvement, fruit juices and wines aroma enhancement and pentose containing disaccharides synthesis (Rahman et al. 2001, Makkonen et al. 2005, Wong et al. 1988, Gunata et al. 1990, Spagna et al. 1998, Rémond et al. 2004). Thus, extensive

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biochemical analysis have been conducted on xylan degrading enzymes from *Rhizopus* spp. (Bakir et al. 2001), *Aspergillus* spp. (Kadowaki et al. 1996), *Scytalidium* spp. (Düsterhöft et al. 1997), *Thermomonaspora* spp. (Tuncer 2000) and *Bacillus* spp. (Degrassie et al. 2003, Avcioglu et al. 2005, Ersayin et al. 2005, Ersayin 2006).

Xylanase producers were mostly isolated from soil. Soil based isolation needs screening of many soil samples and microorganisms (Panbangred et al. 1983, Avcioglu et al. 2005, Rawashdeh et al. 2005, Kinagam et al. 2007), culture enrichment steps (Sanchez et al. 2005, Xu et al. 2005) and serial dilutions (Rawashdeh et al. 2005). Endopyhtes are defined as organisms inhabit inside of leaves, braches and roots without harming the plant (Hawksworth et al. 1995, Azevedo et al. 1999). Due to hemicellulosic (xylan) component of plant cell wall, existence of endophytic xylanolytic microorganisms is expected. Few endophytic xylanase producers have been reported up to date (Burke et al. 1997, Suto et al. 2002, Ersayin et al. 2007).

In this study, we screened stem and leaf parts of the *Zea mays* and isolated two xylanolytic *Bacillus pumilus* isolates. In addition, we determined the xylanase production level of isolates using agricultural byproduct, corn cobs and characterized enzyme partially.

## Material and Method

Leaf and stem samples of *Zea mays* were collected from field of Başkent University Institute of Transplantation and Gene Sciences. Healthy samples having no disease symptoms were harvested (August, 2007) and subjected to microorganism isolation studies.

**Isolation of xylanase producing bacteria and microbial maintenance** : Endopyhtic xylanase producer was isolated using the method of Suto et al. (2002) with some modifications. The leaf and stem samples were washed in running water for 10 min and cut into 0.5 cm pieces. Then, pieces were washed in sterile distilled water for extra 10 min and surface sterilized by soaking in 75% ethanol for 4 min to avoid surface originated microbial contamination. The sterilized samples, dried on sterile filter paper, were cut longitudinally into two pieces aseptically using sterile razor blade. Then, inside part of the stem and leaf samples were placed on a CRX agar (w/v: 0.5%; xylan, 0.5%; peptone, 0.5%; yeast extract, 0.1%;  $\text{KH}_2\text{PO}_4$ , 0.02%;  $\text{MgSO}_4$ , 0.015%; Congo red, 0.01%;  $\text{Na}_2\text{CO}_3$  and 2.0%; agar) and incubated at 35°C for 2 days. Due to enzymatic hydrolysis of xylan-Congo red complex in CRX agar, xylanase producing bacteria

formed a clear zones in a red background. Then, xylanase positive isolates were subjected to the purification and characterization steps.

To make sure that growth on plate indicated the microorganism originally located inside the plant, surface of the samples were also contacted to surface of CRX and screened for microbial growth.

In purification step, successive colonies were picked and serially (8-10 times) streaked on CRX plates and microscopically examined for purity. Pure isolate was maintained at 35°C on X agar plates (w/v: 0.5%; xylan, 0.5%; peptone, 0.5%; yeast extract, 0.1%;  $\text{KH}_2\text{PO}_4$ , 0.02%;  $\text{MgSO}_4$ , 0.015%;  $\text{Na}_2\text{CO}_3$  and 2.0%; agar). Xylanolytic nature of the isolates was further investigated by growing them in liquid media containing agricultural byproduct as a sole carbon source and inducer.

Isolates were stored at -20°C in (w/v): 1.0%; glucose, 0.5%; peptone, 0.5%; yeast extract, 0.1%;  $\text{KH}_2\text{PO}_4$ , 0.02%;  $\text{MgSO}_4$ , 0.01%;  $\text{Na}_2\text{CO}_3$  and 50% glycerol (v/w).

**Identification of bacteria:** The pure isolates were characterized by means of conventional procedures (Sneath 1986). The test characterizing the isolates included endospore staining, Gram staining, motility, oxygen requirement and catalase test. The tests were performed in triplicate. Gram staining result was confirmed using McConkey agar plates. Endospore production was confirmed through heat test at 80°C for 10 min (Slepecky and Hemphill 1992). Isolates were identified at genus level as a *Bacillus* according to Claus and Berkeley (1986) and Gupta et al. (1992), and then, they were further identified using the API 50 CH-API 50 CHB/E medium kit (BioMérieux).

**Xylanase production:** Xylanase production was performed in 250 ml shake-flask bioreactors containing 100 ml medium at 30°C, 175 rpm for 7 days. The medium contained 0.5% NaCl, 0.25% yeast extract, 0.1%  $\text{KH}_2\text{PO}_4$ , 0.02%  $\text{MgSO}_4$ , 0.1%  $\text{Na}_2\text{CO}_3$  and 3% steam hydrolyzed corn cobs powder, agricultural byproduct, as sole carbon source and inducer. Ground corn cobs were autoclaved for 30 min at 121°C and dried overnight at 100°C. The fermentation medium was centrifuged for 40 min at 11,000 x g and the supernatant was used as xylanase extract (crude enzyme extract).

**Enzyme assay:** Xylanase activity was determined according to Bailey et al. (1992) using 1% Birchwood xylan in 50mM phosphate buffer at pH 7.0 and 40°C. Cellulase (EC 3.2.1.4) activity was assayed in 50 mM phosphate buffer at pH 7.0 and 40 °C using 1 x 6 cm Whatman No.1 filter paper strips as substrate

(Miller et al. 1959). Dinitrosalicylic acid (DNSA) method was used to determine reducing sugar concentration in both assays by using either xylose or glucose as standard. Initial reaction rates were taken into account in activity measurements. One unit of xylanase (U) was defined as the quantity of enzyme necessary to produce 1  $\mu$ mole of xylose/glucose equivalents per min at 40°C under the given conditions. All the experiments were performed at least two times and the averages were given.

**Effect of pH and temperature on xylanase activity:** To determine the pH-dependance of the xylanase activity, the following buffers (50 mM) were used over the pH range 5.0-9.0 at 40 °C by standard activity assay; citric acid- $\text{Na}_2\text{HPO}_4$  (pH 5.0-6.0), sodium phosphate (pH 7.0-8.0), glycine-NaOH (pH 9.0) and carbonate-bicarbonate (pH 11.0). The effect of temperature on the xylanase activity was determined at 40-80 °C in 50 mM phosphate buffer at pH 7.0 using the standard xylanase assay.

## Results

**Xylanolytic microorganism isolation:** Surface sterilized, longitudinally cut stem and leaf samples from healthy *Zea mays* were placed on CRX agar and screened for successive xylanase positive colonies. To make sure that there was no external bacterial contamination, exterior part of the surface sterilized samples were also displayed for microbial growth. Since there was no surface originated microbial growth on CRX plates, xylanase positive colonies were considered to be from inside of the plant.

Therefore, two xylanolytic bacteria, M1 and M2, were isolated from inside of the stem and leaf part of *Zea mays*, respectively. They were identified at genus level. They were rod shaped, Gram positive, aerobic, motile, catalase positive and formed endospores (Table 1). Gram and endospore staining results were confirmed by growth test on McConkey agar and heat test (Slepecky and Hemphill 1992). On the basis of these properties, M1 and M2 were classified as strains of the *Bacillus* genus (Claus and Berkeley 1986). The identification was confirmed by utilization the method described by Gupta et al. (1992), as well. Then, isolates were identified further as a *Bacillus pumilus* strains according to API 50 CH-API 50 CHB/E medium kit (BioMérieux). Therefore, isolates will be referred as of this point as *B. pumilus* M1 and *B. pumilus* M2.

Table 2. Characteristics of M1 and M2 isolates on agar plate.

Isolate	Colony size (mm)	Colony shape	Colony edge	Colony appearance	Growth arrangement
M1	5-7	round-irregular	Lobate	shiny	swarm
M2	10-12	curled	Entire	opaque	spread

Isolates presented different morphology on agar plate as presented in Table 2. M1 colonies were 5-7 mm in size, irregular, lobate, swarm and shiny. However, colonies of *B. pumilus* M2 were rather bigger (10-12 mm) than *B. pumilus* M1, curled, unlobate, spread and opaque. Morphology comparison results pointed to possible strain difference between two isolates.

**Xylanase production:** In addition to qualitative determinations, xylanase production performance of isolates were quantitatively determined and comparatively evaluated. The production of xylanase was performed in shake flask fermentation media containing 3% of ground corn cobs as sole a carbon source and inducer.

Figures 1 and 2 present the xylanase production profile of organisms on 3% corn cobs. Both M1 and M2 were able to produce xylanase from low-cost byproduct (corn cobs), but xylanase production level and profile varied between isolates. When compared to *B. pumilus* M1, *B. pumilus* M2 produced very low level of xylanase and maximal xylanase activities of  $188.0 \pm 20.0$  U/ml and  $5.6 \pm 1.1$  U/ml were achieved by *B. pumilus* M1 and *B. pumilus* M2 strains after 3 and 4 days fermentation, respectively (Figures 1 and 2). Moreover, isolates had very low level of cellulase activity (0.002-0.003U/ml) in crude enzyme extract.

As shown in Figures 1 and 2, xylanase production profiles resembled the bacterial growth profile, and so we may conclude that there was a possible correlation between growth and the activity of the xylanase enzyme. A low level of xylanase activity was detected in the earlier stages of fermentation and the enzyme activity steadily reached its highest value. Then, probably because of the enzyme degradation by proteinase activity and xylanase inactivation, there was a decrease in the xylanase titers.

Table 1. Characterization of M1 and M2 isolates.

	M1	M2
Cell shape	Rod shaped	Rod shaped
Endospore	+	+
Gram	+	+
Growth on McConkey agar	-	-
Oxygen requirement	A <sup>+</sup>	FA <sup>+</sup>
Catalase	+	+
Motility	+	+

A: Aerobe, FA: Facultative aerobe.

Since *B. pumilus* M1 produced xylanase at reasonable rate without any optimization, its enzyme was partially characterized. The pH and temperature dependence of *B. pumilus* M1 xylanase was given in Figures 2 and 3, respectively. Enzyme had maximum activities at both pH 6.5 and 8.0, which may indicate the production of multiple, at least two, xylanases with acidic and basic pH optima, as stated from many microorganisms (Avcioglu et al. 2005). Enzyme had also maximum activity at 65°C. Most of the mesophilic xylanases are optimally active at temperature below 50°C and act in acidic and neutral pH ranges. Only few xylanases are presented to be active and stable at alkaline pH and high temperature (Collins et al. 2005).

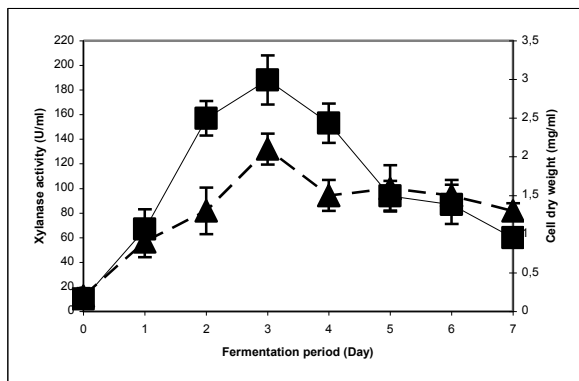


Figure1. Xylanase production profiles of *B. pumilus* M1 on 3% corn cobs. (The fermentation medium contained 0.5% NaCl, 0.25% yeast extract, 0.1% KH<sub>2</sub>PO<sub>4</sub>, 0.02% MgSO<sub>4</sub>, 0.1% Na<sub>2</sub>CO<sub>3</sub> and 3% corn cobs. The fermentations were carried out in an incubator shaker at 35 °C and 175 rpm.; ■: xylanase activity; ▲: cell dry weight).

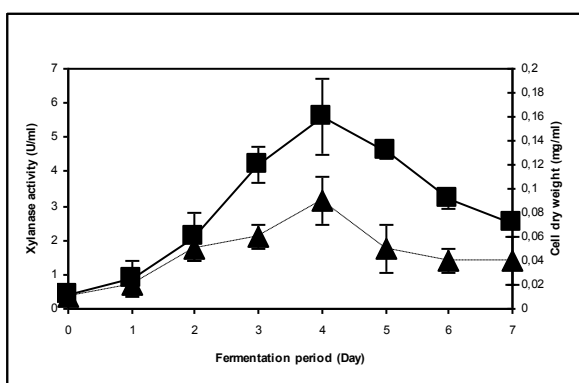


Figure 2. Xylanase production profiles of *B. pumilus* M2 on 3% corn cobs. (The fermentation medium contained 0.5% NaCl, 0.25% yeast extract, 0.1% KH<sub>2</sub>PO<sub>4</sub>, 0.02% MgSO<sub>4</sub>, 0.1% Na<sub>2</sub>CO<sub>3</sub> and 3% corn cobs. The fermentations were carried out in an incubator shaker at 35 °C and 175 rpm.; ■: xylanase activity; ▲: cell dry weight).

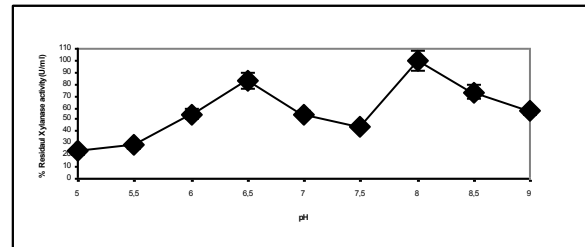


Figure 3. The pH dependency of *B. pumilus* M1 xylanase in crude extract. 100% residual xylanase activity was equivalent to 100U/ml.

It is also noteworthy to mention that existence of *B. pumilus* M1 and *B. pumilus* M2 within plant was found to be tissue selective which can be explained by xylan composition and structure variations through tissue to tissue within a plant (Coughlan and Hazlewood 1993) and nature of the microbial xylanolytic system such as substrate specificity and type of xylanolytic enzymes (Liab et al. 2000 and Ersayin 2006).

**Discussion:** Many bacterial and fungal endophytic microorganisms have been isolated from various types of vegetation such as cotton (McInroy et al. 1995), native tree species from national park in Thailand (Lumyong et al. 2002), banana (Cao et al. 2004) and rice (Naik et al. 2007). Roots and leaves of maize (*Zea mays* L.) were screened for endophytic actinomycetes (McInroy et al. 1995, De Aroujo et al. 1999).

Endophytes were mainly considered as producers of bioactive compounds (Strobel et al. 1996, and Strobel and Long 1998) and protective agent of plant against disease and insect (Bultman and Bell 2003, Cao et al. 2004). However, there are few studies reported on utilization of plants as sources of endophytic xylanase producer (Burke and Chairney 1997, Suto et al. 2002) and no work presented on xylanolytic endophyte isolation from maize. This is the first report presenting the screening of maize for xylanolytic bacteria isolation.

When compared to the data presented in literature, screening and isolation of xylanase producer from *Zea mays* (plant) was easier and quicker than that from soil (Panbangred et al. 1983, Avcioglu et al. 2005, Rawashadeh et al. 2005, Xu et al. 2005, Kinegam et al. 2007). In this study, we isolated two xylanase producers *B. pumilus* isolates. Together with morphological difference, due to variations in xylanase production profile and titer, *B. pumilus* M1 and *B. pumilus* M2 were considered to be two different isolates.

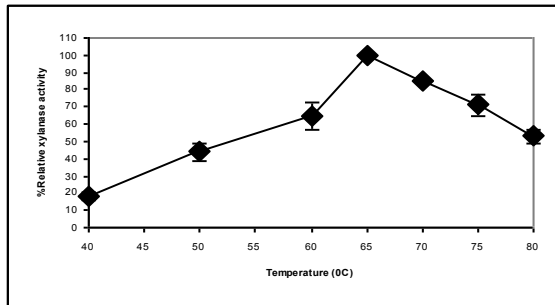


Figure 4. The temperature dependency of *B. pumilus* M1 xylanase in crude extract. 100% residual xylanase activity was equivalent to 100U/ml.

In the literature, different xylanase activities have been reported over a wide range, usually 2-1000 U/ml for *Bacillus* species (Bakır et al. 20005). Clearly the xylanase activity of our isolate M1,  $188.0 \pm 20.0$  U/ml, was not very high, but not low either. After optimization of enzyme production conditions, it is possible to increase xylanase production level. Significant xylanase production level increase (~50-80 folds) with enzyme production condition optimization was reported for *B. pumilus* (Poorna and Prema 2006), *Rhizopus oryzae* (Bakır et al. 2001) and *Aspergillus oryzae* (Szendefy et al. 2006). Accordingly, xylanase production by the our isolates may possibly be further increased by optimization of the enzyme production conditions such as nitrogen sources of medium, air flow, pH and stirring rate.

Xylanases from fungi, active in neutral or acidic pH, but bacterial xylanases generally have higher optimal pH and are stable at alkaline pHs which makes bacterial xylanases being more suitable for applications in the paper and pulp industry (Porna and Prema 2006). The most studied xylanase producers among bacterial sources are *Bacillus* species, due to their high yield and stability at alkaline pHs (Avcioglu et al. 2005, Duarte et al. 2000, Ersayın 2006). Having maximum activity at pH 8.0 and 65°C with no cellulase activity, *B. pumilus* M1 xylanase was thought to be a promising for paper and pulp industry. After detailed characterization, possible application area of *B. pumilus* M1 xylanase can be investigated.

In conclusion, we have shown that plants can be considered as a source of xylanolytic microorganism and indigenous leaf and stem part of the *Zea mays* could serve as a source for xylanolytic microorganism isolation. After detailed characterization, such enzymes could be used in various applications in paper and pulp, textile, food and feed industries. Due to wide range of application area, isolation of new xylanase

producers is a great value for both researchers and industry.

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