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Modern Trends in Agricultural Production and Environmental Protection

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UTILIZATION OF MISCANTHUS WASTE BIOMASS FOR XYLANASE PRODUCTION BY SOIL BACTERIUM *SINORHIZOBIUM MELILOTI*

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ABSTRACT

Miscanthus × giganteus, as a lignocellulosic material, consists of about 30% xylan and represents a good basis for research as a possible raw material in the production of xylanases. Among the soil bacteria, rhizobium is rarely investigated as an enzyme producer. For the first time, Sinorhizobium meliloti strain 207 was used to obtain xylanase during submerged and solid fermentation using miscanthus biomass as a substrate. During submerged fermentation (28°C), the maximum xylanase activity was achieved after 48 h with 10% inoculum and H_2SO_4 substrate modification. The maximum xylanase activity of 1.215 U/mL was obtained during solid-state fermentation (28°C) by using also H_2SO_4 modified miscanthus biomass which was moistened with distillate water. The crude enzyme, produced by strain 207, could be further used in eco-friendly processes of lignocellulose material bioconversion to useful products.

***Keywords**: agroindustrial waste, xylanase activity, solid-state fermentation, Sinorhizobium meliloti.*

INTRODUCTION

The xylanase enzymes (endo-1,4-b-xylanases) cleave the β-1,4-glycosidic linkage between the xylose residues in the backbone of xylans. Thus xylanases form a usable product such as xylose, xylobioza as xylo-oligosaccharides (Gomes et al., 2016). They are a widespread group of enzymes and are one of the most important ones, as their enzymatic activities are required for the depolymerization of the hemicellulosic constituent of the plant cell walls. The xylanase substrate, xylan, is the most abundant hemicellulose and represents approximately one third of the total renewable organic carbon on the earth (Chakdar et al., 2016). Xylan is found in large quantities in hard and soft wood, as well as in many years old plants. It is usually found in the secondary cell wall of plants (Collins et al., 2005). Due to its heterogeneity and complexity, complete hydrolysis of xylan requires a wide variety of enzymes of cooperative action. A complete xylanolytic enzymatic system is quite present among fungi, actinomycetes and bacteria, and some of the most important producers of xylanolytic enzyme include following genera: *Aspergillus, Trichoderma, Streptomyces, Phanerochaetes, Chytridiomycetes, Ruminococcus, Fibrobacteres, Clostridia* and *Bacillus* (Motta et al., 2013).

Xylanases have wide range of industrial and biotechnological applications. For instance, xylanases can be used in paper industry, in the improvement of the quality of animal feed, textile and food processing industries and also in the transformation of lignocellulosic materials in fermentable sugars for the production of second generation ethanol (Gomes et al., 2016; Chakdar et al., 2016).The production of xylanases must be improved by finding more potent fungal or bacterial strains or by inducing mutant strains to excrete greater amounts of the enzymes (Motta et al., 2013). Among bacteria, rhizobial species were rarely investigated as the producer of xylanase.

Agricultural–residues are usually used for enzymatic conversion and for the industrial production of xylanase enzymes, due to its complex structure. They also represent an example of cheap raw material for industrial production of enzymes (Nkohla et al., 2017). Fermentation is the primary technique for the production of various enzymes, and fungi and bacteria play a very important role in the fermentation process. There are two different fermentations, solid-state and submerged fermentation which were used for the production of certain enzymes. Submerged fermentation (SmF) is most often used in the production of bacterial enzymes due to the need for

higher water potential. Solid-state fermentation (SSF) is preferred when enzymes have to be extracted from fungi that require a lower aqueous potential. More than 75% of industrial enzymes are produced by SmF, one of the main reasons is that SmF supports the use of genetically modified organisms to a greater extent than SSF (Martins et al., 2011; Buntić et al., 2019). On the other hand, the main advantage of using SSF in the production of enzymes is to use waste lignocellulosic materials as a substrate and thus easily recycle.

Miscanthus × giganteus is a perennial rhizomous grass which is consists of cellulose (33.9%), xylan and araban (32.2%) and lignin (26.3%) (Lee and Kuan, 2015). It grows very rapidly, up to 4 m high, producing a high annual biomass yield of 20-26 t of dry matter/h. Miscanthus is a lignocellulosic plant that is being researched as a possible raw material for obtaining paper, energy, building materials, nursery and greenhouse substrates. Recently, miscanthus biomass attracted interest as a potential raw material for the production of ethanol because it is rich in carbohydrates and grows well even in poor soil (Huyen et al., 2010).

The aim of this study was to examine the hydrolytic potential of rhizobium *Sinorhizobium meliloti* strain 207 through the production of xylanase during SmF and SSF. A modification of the lignocellulosic substrate (Miscanthus × giganteus) was made and the influence of substrate modification on the production of xylanase during SmF and SSF was also tested.

Materials & Methods

Bacterial strain and inoculum preparation

Rhizobium working culture was prepared by using *Sinorhizobium meliloti* strain 207 from the Collection of the Institute of Soil Science (ISS WDCM375-Collection of Bacteria, Institute of Soil Science, Department of Microbiology). This strain was selected according to qualitative test using xylan agar plate (per liter: birch wood xylan 1 g, yeast extract 3 g, K_2HPO_4 3 g, KH_2PO_4 1 g, $MgSO_4$ 0.5 g and agar 6 g) (Buntić et al., 2019). A loopfull of purified culture of the strain 207 was transferred from yeast mannitol agar medium on selective agar medium and incubated (3-5 days, 28°C). After incubation, agar plate was flooded with Gram's iodine (2.0 g KI and 1.0 g I_2 in 300 mL distilled water) for 5 min. Clear zones around colonies indicated xylan hydrolysis (Mihajlovski et al., 2015). Microorganism working culture was grown in Erlenmeyer flasks containing yeast mannitol broth (YMB) in a rotary shaker (125 rpm, 48 h, 28°C) (Buntić et al., 2018).

Sample materials and their preparation

The miscanthus (*Miscanthus* × *giganteus*) solid waste material was collected from the local field experiment of Institute of Soil Science, dried and grounded to a particle size up to 3 mm (unmodified substrate). The modification of substrate was performed with 1% solution of following agents: H_2SO_4, NaOH or H_2O_2 in ratio 1:5 (w:v). After 2 h at room temperature (25°C), the solid phase was separated by a vacuum pump and washed with distilled water. The resulting modified substrate was dried for overnight in an oven at 105°C. Obtained miscanthus modified (M) substrate were: M-H_2SO_4, M-NaOH and M-H_2O_2.

Liquid fermentation

Batch experiments were carried out in 100 mL Erlenmeyer flasks which were placed in a rotary shaker at 28°C and under agitation of 125 rpm. Xylanase were produced by growing 1, 5 and 10% inoculum of the *S. meliloti* 207 in media containing waste lignocelluloses material – miscanthus (unmodified or modified). Experiment was conducted in triplicate under following conditions: 2% of predetermined substrate concentration during 24, 48 or 72 h of fermentation process. After incubation, the culture medium was subsequently centrifuged (6000 × g, 15 min) and the cell-free supernatant (enzyme sample) was tested for xylanase activity.

Solid-state fermentation

All experiments related to the production of the xylanase during the SSF were performed in 100 mL Erlenmeyer flasks with 2 g of different substrates (unmodified or modified miscanthus biomass). In order to moisten the substrate, before substrate sterilization at 121°C for 20 min, distillated water (solution 1) or mineral salts solution of K_2HPO_4 (3 g/L), KH_2PO_4 (1 g/L) and $MgSO_4$ 0.5 (g/L) (solution 2) was added in ratio 1:4 (w:v). Overnight bacterial culture in a concentration of 10%, grown in YMB, was inoculated into different sterile solid media and incubated in the thermostat at 28°C. After 2 days of incubation, 10 mL of 0.1 M acetate buffer (pH 4.8) was added for enzyme extraction. All the samples were filtrated and the liquid aliquot was centrifuged and analyzed as enzyme sample, as explained below.

Enzyme assay for xylanase

Xylanase activity was measured by the reduction of 3,5-dinitrosalicylic acid (DNS) in the presence of xylose released by enzymatic hydrolysis of xylan, according to the method of Miller (Miller, 1959). Crude enzyme extract (500 µL) was incubated with 500 µL of 1% solution of xylan (birch wood xylan for xylanase) in acetate buffer (0.1 M, pH 4.8) for 15 min at 50°C. After incubation, 1 mL of DNS reagent was added and the reaction mixture was boiled (90 °C) for 5 min, cooled down and diluted by adding 5 mL of distilled water. The absorbance was recorded on the UV/visible spectrophotometer (UV-160A, Shimadzu Corporation, Japan) at 540 nm against blank (non incubated enzyme). One unit of xylanase activity was defined as the amount of enzyme that released 1 µmol of xylose equivalents per minute. Units were calculated according to the following formulae (Irfan et al., 2016):

$$Xylanase\ activity\ (IU) = \frac{\text{Reductin sugars (mg/mL)} \times 1000}{\text{Incubation time (15 min)} \times 150}$$

Results and Disscusion

Xylanase qualitative test

Sinorhizobium meliloti strain 207 was grown on xylan agar plate for 4 days. The appearance of halo zones around bacterial colony on the plate indicated that strain 207 could hydrolyze xylan and use it as the sole carbon source (**Fig. 1**).

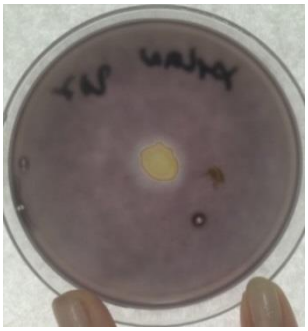

Fig.1. Hydrolysis of xylan agar by *Sinorhizobium meliloti* strain 207

Hydrolysis of xylan by *S. meliloti* has not been reported in the literature yet. Iannetta and authors (1997) reported xylanase activity of 0.41 and 0.76 U/mL in culture supernatant of *Rhizobium leguminosarum* bv. viciae.

This rhizobial species was grown in the presence of bean root and pectin as carbon source in liquid medium (Iannetta et al., (1997). Furthermore, Ivashina and Ksenzenko (2012) described *pssW* gene encoding for glycosyl hydrolase with endo-1,3-β-xylanase and endo-1,4-β-xylanase activities. As it has been determined that *pssW* gene has its counterparts in rhizobial strains such as *R. leguminosarum* and *R. etli*, it is possible that xylanase activity of *S. meliloti* could underline in the presence of similar gene (Ivashina and Ksenzenko, 2012).

Submersed fermentation using miscanthus waste biomass

Among all commercial xylanases which are can be from various origins, around 80–90% is produced in submerged culture. Xylanases are produced in response to xylans from various alterantive substrates: wheat bran, sugarcane bagasse, rice husks and wood pulp (Polizeli et al, 2005; Motta et al., 2013). On the other hand, miscanthus biomass was primarily used in the production of ethanol, but not in the production of enzymes. Considering that the production of enzymes during SmF or SSF occurs at a lower temperature than fermentation during ethanol production, the rest of biomass can still be used in the production of ethanol.

The optimal period for maximal xylanase production in the SmF process varies between different microorganisms. Different experiments were conducted to study this process parameter in enzyme production (Nagar et al., 2010; Anuradha et al. 2007; Breccia et al., 1998). The obtained results of this study indicate that fermentation period of 48 h was optimal for xylanase production by *S. meliloti* strain 207 (**Fig. 2**).

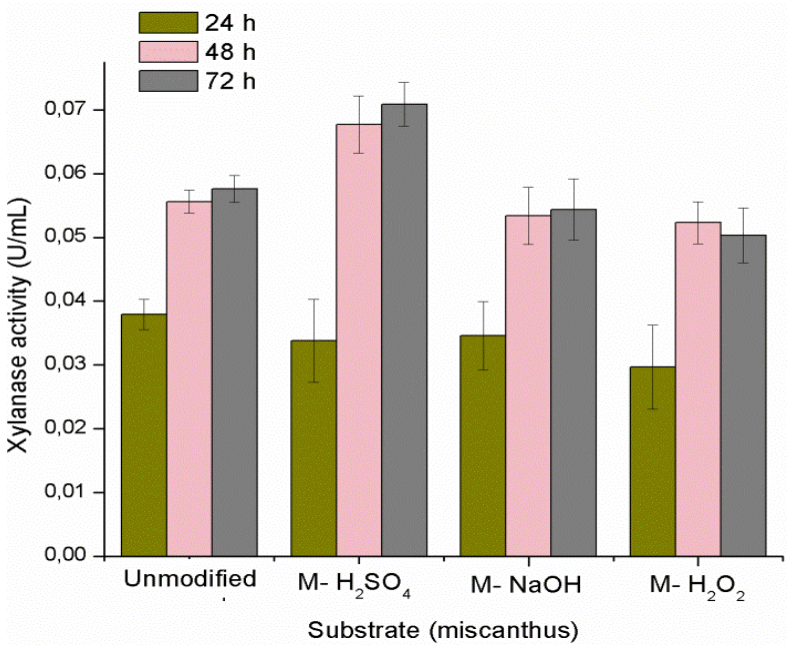

Fig.2. Xylanase production by *Sinorhizobium meliloti* strain 207 during different incubation period of submerged fermentation (5% inoculum, 28 °C, 125 rpm)

A slight increase in activity occurred after 72 h of using unmodified and modified biomass treated with H_2SO_4 and NaOH. That increase was not significant in comparison to the increase of enzyme activity between 24 and 48 h of the incubation time. It was 68%, 49.9%, 64.7% and 56.7% with using of unmodified, M-H_2SO_4, M-NaOH- and M-H_2O_2 modified waste biomass. In the literature, various microorganisms produced maximum xylanase activity after different incubation period. Thus, *Bacillus pumilus* SV-85S and *Streptomyces violaceoruber* produced maximum xylanase activity after 36 h, *Bacillus amyloliquefaciens* and *Bacillus circulans* D1 after 48 h and *Bacillus* SSP-34 showed maximum xylanase activity when grown for 96 h (Nagar et al., 2010; Khurana et al., 2007; Bocchini et al., 2002).

The effect of inoculum size on xylanase production by rhizobium *S. meliloti* 207 was also examined, and the results of obtained xylanase activities were presented in **Fig. 3**.

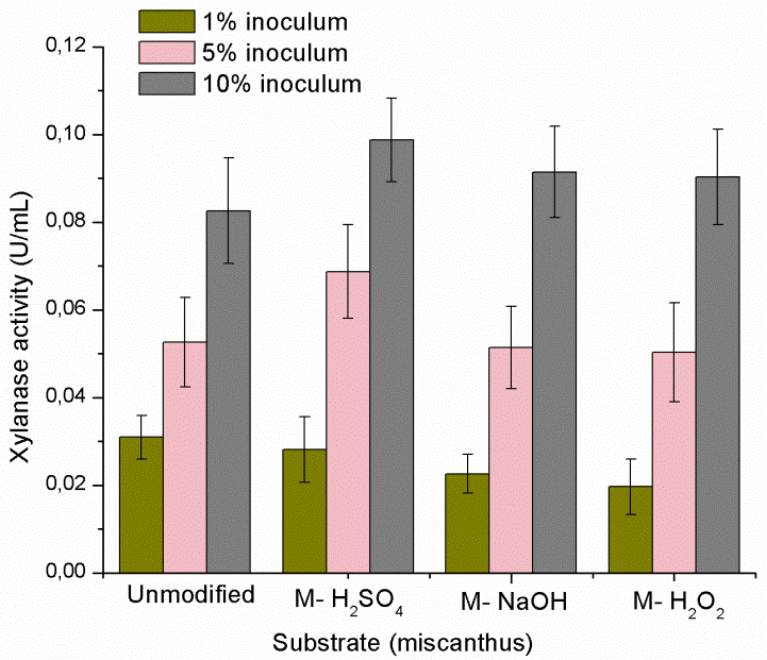

Fig.3. Xylanase production by Sinorhizobium meliloti strain 207 using different inoculum size during submerged fermentation (incubation period of 24 h, 28°C, 125 rpm)

With the increase of the inoculum size from 1 to 10%, xylanase activity was increasing as well. This increase appeared to be linear. Bearing in mind biomass modification, substrate modified with H_2SO_4 gave maximum xylanase activity of 0.0987 U/mL of crude enzyme extract. There were no differences between applying NaOH and H_2O_2 as modification agents on xylanase activity. The activities were similar with increasing of inoculum size. In addition, by comparing these two modified substrates with unmodified miscanthus biomass, there were no significant differences.

The inoculum size must be sufficiently large to colonize all the substrate particles (Shah and Madamwar, 2005). However, high concentration of inoculum is not preferred in industrial fermentation (Nagar et al., 2010). The size of the inoculums varying from 1 to 5% was insufficient to colonize all the substrate particles in the liquid medium. Therefore, they achieved lower xylanase activities.

Several researchers have reported the use of 1.0–5.0% (v/v) inoculum size for hyper production of xylanase (*Bacillus pumilus* ASH and *Trichoderma reesei* SAF3) (Nagar et al., 2010).

Solid-state fermentation using miscanthus waste biomass

Using miscanthus biomass (which on average consists of about 30% of xylan+araban) as a cost-effective substrate, rather than commercial pure xylan is a better economical strategy for the enzyme production (Lee and Kuan, 2015). In this way, problem of the waste biomass disposal could be solved. On the other hand, a huge amount of biomass is used to generate heat by combustion in large ovens. Instead of combustion, it can be used for the production of enzymes and further for the production of ethanol. This method of processing waste biomass can be considered as an acceptable way of protecting the environment.

The results of solid-state fermentation by using unmodified and modified miscanthus biomass for xylanase production are shown in **Fig. 4**.

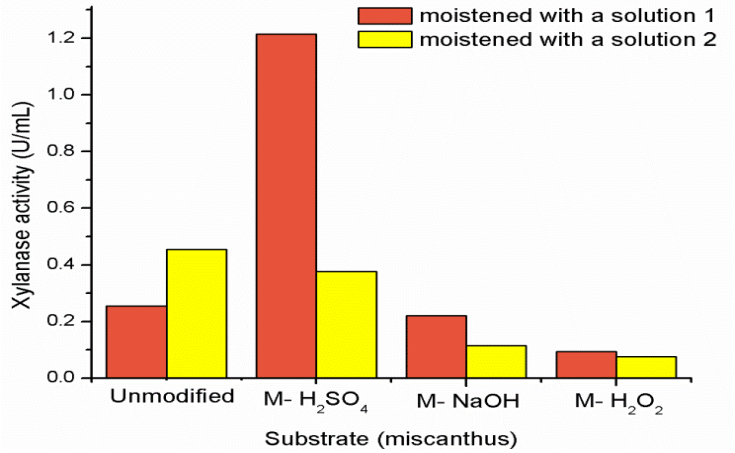

Fig.4. Xylanase production by *Sinorhizobium meliloti* strain 207 during solid-state fermentation (48 h, 28°C)

The maximum xylanase activity of 1.215 U/mL was obtained by using M-H_2SO_4 modified waste biomass by *S. meliloti* 207. Acid hydrolysis of miscanthus by using the dilute sulfuric acid (H_2SO_4) is the most

common option, among the chemical pretreatments (acids, alkalis, alcohols, organic acids, pH-controlled liquid hot water, or ionic liquids). During this pretreatment, carbohydrates (mainly hemicelluloses) are depolymerized in the liquid into oligosaccharides and monosaccharides (Lee and Kuan, 2015; Guo et al., 2012). The success of xylanase production was five times lower when the unmodified and M-NaOH modified biomass were used as an alternative enzyme substrate (0.254 and 0.221 U/mL, respectively). It is probably because dilute sulfuric acid more easily renders a yield of xylose than glucose from the biomass, while pretreatment in alkaline solutions like NaOH and ammonia causes solubilization and removal of hemicelluloses (Lee and Kuan, 2015). Furthermore, these values were obtained when the distillate water was used as substrate moistening agent. However, when solution 2 was used as a substrate moistening agent, the obtained xylanase activities were lower. This salt mixture only improved the production of enzyme during the use of an unmodified biomass of miscanthus.

Among xylanases-producing microorganisms, the lower activity was obtained by *Neocallimastix sp*. strain L2 (1.13 U/mL) using Avicel (PH 105) from Serva at 50°C in comparison with this study. However, higher xylanase activity was achieved by using wheat bran, bagasse hydrolysates and oat and urea by *Penicillium clerotiorum* (7.5 U/mL), *Bacillus circulan*s D1 (8.4 U/mL) and *Aspergillus niger* PPI (16 U/mL) respectively (Motta et al., 2013). Therefore, *Sinorhizobium melilot* strain 207 is not as potent in xylanases production, but is a rare case of rhizobial species with this ability.

Conclusion

The cost of producing xylanase has a significant impact on the economics of the waste biomass conversion process. On-site production of xylanase is a potential strategy which could be used in order to reduce costs. The use of the enzymes secreted from microorganisms grown on the same lignocellulosic material that will be further converted to ethanol can become an acceptable way of environmental protection. Therefore, utilization of miscanthus biomass for the production of xylanase was justified. Solid-state fermentation was a better choice for xylanase production by *Sinorhizobium*

meliloti strain 207. In addition, chemical pretreatment with H_2SO_4 improved xylanase activity in comparison to the unmodified substrate. The crude enzyme, produced by strain 207, could be further used in eco-friendly processes of lignocellulose material bioconversion to useful products, as well as in the manufacturing of animal feed, bread, food and drinks, textiles, cellulose pulp and paper industry.

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