Epiphytic bet-mannanase producing bacterial strains

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Abstract. Dry remains of the herbal species of the plantain (*Plantago major*), the wormwood (*Artemisia vulgaris*) and the reed grass (*Calamagrostis acutiflora*) were used as a natural source for isolation of β-mannanase producing strains. They were isolated by using the carob gum as a single source of carbon and energy. Each chosen plant species was found to be colonized with a single dominant epiphytic group of microorganism, although the plants had been collected in the same location. *Bacillus circulans* was only found in *P. major*, *Bacillus subtilis* on *A. vulgaris*, whereas Pantoea sp. was found in *C. acutiflora.* Identification of the taxonomy affiliation of the isolated β-mannanase producers allowed using the formerly proposed primers for PCR cloning of β-mannanase genes previously occurred in the respective bacterial species. This approach let us cloning 330 bp fragment of β-mannanase genes from *B. circulans* and *B. subtilis* and 1000 bp fragment of β-mannanase gene from Pantoea sp. Testing the enzymatic activity of the isolated strains by staining the carob gum hydrolysis zones on the plates with Congo Red was carried out. As a result, the maximum activity was found in Pantoea sp.

1 Introduction

β-Mannanases are currently considered as the most promising type of the feed enzymes. However, they are not still readily available as commercial products and the physiological value of the reported prototypes has not been sufficiently characterized [1]. The substrate of β-mannanases is 1,4-β-mannan, which is found in grains in proportion ranging from 0.5 to 2% of the total dry weight [2]. Average share of β-mannan in the wheat, rye and barley grain is ~0.2% of the dry weight; the corn, triticale and bran contain ~0.6%; soybean meal and oilcake of different cultures contain 1.6-2.5% β-mannan.

A relevant approach to using β-mannanases in the feeding is establishing feed additives with immunomodulatory properties for piglets and other farm animals [3, 4]. Analyzing the reasons for the well documented high efficiency of β-mannanases in premixes leads to the conclusion that it is related with modulation of the water-retention ability is 1,4-β-D-mannan. The water-retention ability of free 1,4-β-D-mannan 10-folds or more exceeded one in the denatured starch and other well characterized polysaccharides [5]. First, partial enzymatic hydrolysis sharply increases water retention ability of β-mannan in the seed shells of the grain (it attains a value of 1:40 - 1:110), and then rapidly drops up to a negligible value. The swelling of β-mannan in the intestine hinders the motility of the chime and absorption of nutrients, which substantially decreases the daily weight gain. That is why β-mannan plays a significant role in the apparent feed efficiency of the grain not proportional to its relatively low content in the diet. Obviously, the mechanism of action of the β-mannanases on the feed components (first of all, the grain), requires an in-depth studies that can lead to a revolutionary improvement in the feed efficiency of the existing raw materials for animal husbandry.

β-Mannanases are commercially available for manufacturing premixes mostly as complex feed preparations containing enzyme blends. Examples are Sunzyme (Wuhan Sunhy Biology Co., Ltd, China) – fungal preparation containing nutrients ~200 U/g. CTC ZYME (CTC Bio, Southern Korea) is a recombinant enzyme from *B. lentus* with activity 800 U/g. AveMix XG 10 and AveMix ®02 CS (AVEVE Biochem NV, Belgium/China) are enzyme blends derived from Trichoderma spp. and Aspergillus spp. with a specific activity 80 U/g and 120 U/g respectively.

There are many recent reports about purification and characterization of novel β-mannanases from microbial isolates [6], optimization of their pH- and thermal resistance [7] and engineering recombinant producers of these enzymes [8]. Taken together, this survey suggests conclusively that biodiversity of the non-studied β-mannanases remains broad and there is no commonly acknowledged biotechnological decision made relatively to requirements to β-mannanase. Therefore, our work

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pursued isolation of β-mannanases from epiphytic microbes associated with cereals and other plant species.

2 Materials and methods

2.1 Growth media and growth conditions

Liquid selective medium for isolation of β-mannanase product contained 1.5 g/L of carob gum (LLC Uspekh, Russia), 1 g/L of yeast extract, 1 g/L of bactopeptone, 1 g/L NH_4Cl, 1.4 g/L KH_2PO_4, 0.2 g/L $MgCl_2$, 1% v/v of pipeline water as a source of microelements. The solid selective medium contained 9 g/L M9 of minimal salts (Difco, USA), 1.0 g/L of carob gum and 15 g/L of bacto agar (Difco, USA).

The isolated β-mannanase producing strains were supported on a solid medium. The cultivation was carried out for 24-48 hrs. at 30°C.

2.2 Biological material for isolating β-mannanase-producing microorganisms

Plant material was collected in December 2020 in Shumyatino village (Kaluga region, Russia, GPS co-ordinates 54.993598, 36.336314). Fragments of dry stems with fruit remains were collected from plantain (*Plantago major*), wormwood (*Artemisia vulgaris*) and reed grass (*Calamagrostis acutiflora*). The plant material was kept in sterile plastic bags with zip-locks until delivered to the laboratory.

Specimens of the plant material (~2 g each) were cut by sterile scissors and placed on 750 ml Erlenmeyer flask containing 50 ml of the selective medium with carob gum as a single source of carbon and energy. The flasks were then incubated in a thermostat without shaking for 24 hrs. at 30°C.

5 ml aliquots of the cumulative cultures were spread by Drigalsky spatula at 90 mm on the Petri dishes with the solid selective medium and cultured for 72 hrs. at 30°C.

The appeared colonies were smeared by microbiological loop at 90 mm Petri dishes with the solid selective medium of the above-mentioned composition and cultivated for 48 hrs. at 30°C for producing separate colonies. Three subsequent passages were carried by the same way out for obtaining pure cultures.

2.3 Genomic DNA purification

Pure cultures were inoculated to 3 ml of the liquid full medium (5 g/L yeast extract, 10 g/L bactopeptone, 10 g/L NaCl) and incubated for 18 hrs. at 30°C with agitation at intensity 180 rpm.

The bacteria were precipitated by centrifuge for 1 min at 13,000 g and thoroughly re-suspended in 100 µl 50 mM EDTA, pH 8.0. 300 µl lysis buffer (CTAB 1%, β-mercaptoethanol 1%, NaCl 4%, Tris-HCl 50 mM, EDTA 50 mM, pH 8.0) was added and mixed with the microbial suspension. 300 mg glass beads with diameter 0.5-1.0 mm were added and the tubes were subjected to an intensive vortexation for 2 min. The tubes were heated at 60°C for 30 min. Equal volume of the chloroform was added, the tubes were subjected to an intensive vortexation and the phases were separated by centrifugation for 5 min at 13,000 g. The upper (water) phase was collected and placed to an empty 1.5 ml Eppendorf tube. The volume was determined and 3-fold excess of 96% ethanol was added. The tubes were kept at -20°C for 10 min and centrifuged for 5 min at 13,000 g. The pellet was dried and dissolved in 200 µl deionized water. 200 µl chloroform was added, the tubes was then subjected to vortexation and centrifuged for 5 min at 13,000 g.

The upper (water) phase was collected and placed to an empty 1.5 ml Eppendorf tube. The volume was determined and 2-fold excess of 96% ethanol and 1/10 v of saturated ammonium acetate were added. The tubes were centrifuged for 5 min at 13,000 g and the pellet was dried and solved in 50 µl deionized water.

2.4 16S-rDNA amplification and sequencing

Formerly described primers 8F AGAGTTTGATCCTGGCTCAG and 926R CCGYCAATTYMTTTRAGTTT for PCR amplification of 16S-rDNA gene fragment were used (Srivastava et al., 2008). Dream Taq DNA polymerase (Thermofisher Scientific, USA) was used in accordance with the manufacturer's instructions. 1 µl DNA was used as a template per 30 µl of the reaction mixture. PCR program was run at Thercyc MC-16 thermocycler (DNA-Technology, Russia) as following: preliminary denaturation 94°C – 2 min; 30 cycles (94°C – 30 sec, 45°C – 45sec, 72°C – 30 sec); completing the synthesis at 72°C for 2 min.

The amplified 960 bp long 16S-rDNA gene fragments were purified with a Silica Bead DNA Gel Extraction Kit (Thermofisher Scientific, USA) in accordance with the manufacturer's instructions. DNA sequencing was carried out by Eurogen Company (Russia) as a customer service. Both 8F and 926R primers were used for sequencing. The DNA sequences obtained by oncoming sequencing where merged and manually checked.

2.5 Taxonomic assignment of the microbial isolates

16S-rDNA sequences where uploaded to N-Blast service available on line (9). The most similar sequences were selected and used for reconstruction of a dichotomy tree.

2.6 Cloning β-mannanase gene from Bacillus circulans and Bacillus subtilis

Previously described primers S1 AAGTHCATGAYGCYACRGG- and S2 CCWGCATAYTCRTACATATGG (H means A, C, or T; Y - C or T; R - A or G; W - A or T) corresponding to conserved regions of β-mannanases from bacteria belonging to genus Bacillus were used for PCR cloning

of β-mannanases fragments from *B. subtilis* and *B. circulans* [10].

Previously described primers pEG-F CGCGGATCCATGAGTACTTTTACTGTAGTACC CGC and pEG-R CCGCTCGAGTTAACTCAGAACGCTGCC [11] specific to mannanase genes from Pantoea were used for cloning the fragments of the gene of interest from Pantoea ssp. siolate.

The PCR was carried out at Thercyc MC-16 thermocycler (DNA-Technology, Russia) under the following conditions: 30 cycles at 94°C in 40 sec, 50°C in 30 sec, 72°C – 40 C.

2.7 Enzymatic assay of β-mannanase activity

The enzymatic activity of β-mannanase was assessed qualitatively on the Petri dishes with the selective medium of the abovementioned composition with the carob gum as described previously [12]. Briefly, the inoculated Petri dishes were cultured for 48 hrs., soaked in 1% (w/v) Congo Red solution (LenReactiv, Russia) for 15 min and discolored with 1 M NaCl for 2 hrs.

3 Results and discussions

Searching for microbial isolates harboring β-mannanase genes was carried out during winter season when plant vegetation was impossible. The herbal species conserving fruit remains where chosen. Between these, the plantain (*P. major*), the wormwood (*A. vulgaris*) and the reed grass (*C. acutiflora*) were the most abundant in the chosen geographical location. For this reason, the dry stems with the fruit remains of these species putatively containing β-mannans were collected and used for isolation of the plant-associated microorganisms.

Using a massive (50 ml) cumulative culture with subsequent plating of the germinated microorganisms onto selective solid medium, containing the carob gum (almost pure β-1-4-mannan) as the single source of the carbon and energy allowed to isolate the bacterial species exhibiting the properties listed in Table 1. The taxonomical classification of the appeared isolates was carried out on the basis of their 16S-rDNA sequencing.

The data in Table 1 supplemented with observation of the cultural properties of the isolates, allowing the following conclusions:

1. Each chosen plants species was colonized mostly with a single dominant epiphytic group of microorganisms exhibiting a β-mannanase activity although the plants grew in the same location.
2. Bacilli (*B. subtilis* and *B. circulans*) has been reported as sources of β-mannanase genes many times, whereas genus Pantoea (Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacterales; Erwiniaceae; Pantoea) was appeared among potential producers of these enzymes only in a few works [13].

Table 1. Properties of bacterial species isolated from dry steams and fruit remains of herbal species using carob gum as a single source of the carbon and energy

Source plant species	Morphology of microbial colonies	The closest bacterial isolate annotated in NCBI GenBank		Taxonomical assignment on the basis of 16S-rDNA sequencing
		Accession number and description	% of 16S-rDNA identity	
P. major	Small white colonies with small prominences	*Bacillus circulans* strain FDAARGOS_783, **CP053989**	100	*B. circulans*
A. vulgaris	Opaque white colonies with long coiling prominences	*B. subtilis* from soil specimen picked up from a hot water spring, **LC065158**	98,5	*B. subtilis*
C. acutiflora	Mucous yellow/orange colonies	*Pantoea sp.*, **MK602498**	98,1	Pantoea sp.

The identification of taxonomic position of the isolated β-mannanase producers allowed using the formerly proposed primers for PCR cloning of the respective genes. This approach allowed obtaining 330 bp fragment of β-mannanase genes from *B. circulans* and *B. subtilis* and 1000 bp fragment from Pantoea sp.

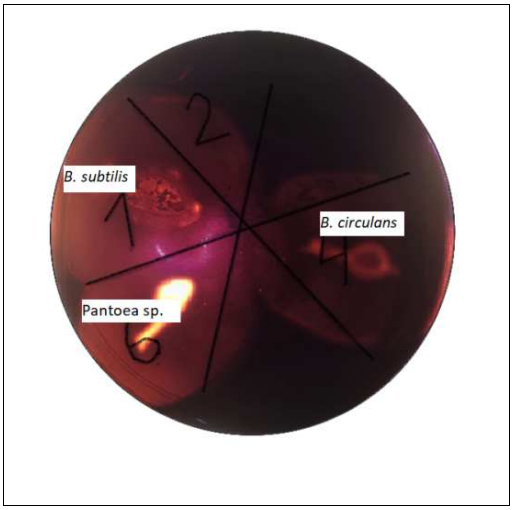

Fig. 1. Petri dish with the solid selective medium containing 1 g/L carob gum as a single source of the carbon and energy with smeared culture of *B. circulans*, *B. subtilis* and Pantoea sp. after cultivation for 48 h, staining with 1% Congo Red and distaining with 1 M NaCl. The appeared halo demonstrates presence of the specific β-mannanase activity.

Testing β-mannanase activity in the obtained bacterial isolates (Fig. 1) led to the conclusion that the

highest activity was found in Pantoea sp. found at *C. acutiflora* since this bacterial isolate grew well on a medium containing the carob gum as a single source of energy and exhibited the greatest halo in plate test for the mannanase activity with Congo Red.

B. circulans exhibited just a slow growth at the selective medium was, therefore just a low β-mannanase activity was suggested in it. *B. subtilis* grew well, however it exhibited a relatively poor substrate hydrolysis in the plate enzymatic test with Congo Red.

Polysaccharides containing linkages of the β-mannose are broadly found in the plant cell walls within the seeds (including the grain and the beans). These are mostly derivatives of β-1,4-mannan including pure β-mannan, glucomannan, galactomannan, and galactoglucomannan. These polymers are composed of a backbone constituted by chains of β-1,4-linked mannose residues and randomly decorated with α-glucose and β-mannose side chains linked via α-1,6-galactose residues [14]. Introducing β-mannan-degrading enzymes to the animal and poultry diet is beneficial for improving accessibility of its organic and mineral components. Mannooligosaccharides appearing as a result of β-mannan hydrolysis are beneficial as prebiotics and stimulators of the immunity. The exhaustive hydrolysis of the grain and bean β-mannan into monomers requires a combinations of several enzymes *e.g.* β-mannanase (EC 3.2.1.78), β-mannosidase (EC 3.2.1.25), β-glucosidase (EC 3.2.1.21), and α-galactosidase (EC 3.2.1.22). β-mannanase can randomly hydrolyze the linkages β-D-1,4-mannopyranoside and has the highest significance for the plant β-mannan and overall diet assimilation [15].

β-Mannanases are broadly distributed in all kingdoms of the living organisms *i.e.* Eubacteria and Archae, filamentous and yeast-like fungi, plants. Structural properties of the amino acid sequences particularly catalytic center allow attribution of each known the β-mannanases to one of three GH-families glycoside hydrolase: 5, 26, or 113 [16].

Bacterial β-mannanases usually have a moderate thermal stability (max. 50-60°C) and pH optimum about 6.0 or higher. The most popular in practice β-mannanases from *B. circulans*, *B. subtilis* and other Bacilli belong to GH5 family. They exhibit the thermal stability up to 60°C and pH optimum in the range of 6.0-10.0. These enzymes are usually highly stable towards the thermal and chemical denaturation due to a presence of disulfide bonds.

β-Mannanases from *Pantoe agglomerans* and other gram-negative bacteria belong to GH26 family. They have thermal stability up to 50°C and pH optimum in range 6.0-6.5 [10]. Practically used recombinant β-mannanases often contain artificially introduced mutations beneath the active center e.g. Gly267Ser and His134Lys in the Man26P from *P. agglomerans* [10]. This allows improving the maximum catalysis rate up to 2.5-3.5 times versus the parental wild type enzyme.

The fungal β-mannanases may be members of both GH5 and GH26 families. They usually exhibit a higher catalytic rate but narrower substrate specificity versus their bacterial homologues. The fungal β-mannanases belonging to GH5 usually attack the native high molecular mass polysaccharide substrate and exhibit a low molar activity whereas members of GH26 usually responsible for the final degrading of the dextrin and oligosaccharides and work with a high rate.

Members of GH113 family are relatively rare found in the natural microbial isolates. Most of them were found in extremophile bacteria. Typically, the enzymes from this family are characterized with the higher thermal stability and broader substrate specificity than in other GH families but with a relatively low molar activity. They demonstrate a downward trend of activity toward galactomannan (guar and locust bean gum) with a high share of galactosyl. In contrast, their activity towards glucomannan from the konjac flour is high [17].

Most fungal β-mannanases from GH5 and GH26 families exhibit a higher activity on locust bean gum than on the konjac flour. The bacterial β-mannanases from both families have relatively looser structure of the active centers that facilitates recognition of different substrates.

4 Conclusions

The chosen methodology allows a rapid and labor-efficient screening for β-mannanase producing strains and cloning of β-mannanase genes from them. Remains of the herbal stems and fruits were found to be an optimal source of the strains producing these type of the enzymes. The cloned genes are good for engineering recombinant producers of β-mannanase and direct testing the pure enzymes in the animal feeding experiments.

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