Lin et al. (2017) Can J Biotech 1(3): 73-79
https://doi.org/10.24870/cjb.2017-000112

Canadian Journal of Biotechnology

ISSN 2560-8304

ORIGINAL RESEARCH PAPER **OPEN ACCESS**

Bacterial Xylanase in *Pseudomonas boreopolis* LUQ1 is Highly Induced by Xylose

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Received: Sep 10, 2017; Revised: Oct 25, 2017; Accepted: Oct 26, 2017

Abstract

A xylanase producing bacterium was isolated from paper mill sludge in Thunder Bay, Canada. The newly isolated bacterium was identified as *Pseudomonas boreopolis* according to its 16S rRNA gene sequence and designated as LUQ1. The zymogram analysis indicated that there was one band of protein with xylanase activity, and the molecular weight of the enzyme was about 20 kDa. This xylanase works best at pH 6.0 and 65°C. The xylanase can be induced to express by xylose. The expression was enhanced on increasing the concentration of xylose, which reached its highest activity at 12 mM of xylose in fermentation. Wheat bran was the best carbon source in submerged fermentation. The highest xylanase activity of 25.61 U/ml was obtained at 96 h using wheat bran feedstock. When barley straw and wheat bran were used as feedstocks, the addition of 10 mM of xylose increased xylanase activity by ~50% and ~15%, respectively. The results showed that the strain LUQ1 has a great potential to produce xylanase for industrial applications.

Keywords: Xylanase, Xylose, *Pseudomonas boreopolis*, Induction

Running Title: Xylanase in *P. boreopolis* LUQ1 is inducible

Introduction

Xylanases (EC 3.2.1.8) are glycosidases that catalyze hydrolysis of xylan into short xylooligosaccharides [1]. These enzymes are useful tools in several industries; for example, they are used as animal feed additives and in the manufacturing of food, drinks, and bread [2]. In biomass conversion, xylanases can help to improve the efficiency of bioconversion. A recent report revealed that a novel bacterium possessing xylanase and other hydrolyzing enzyme can convert hemicellulosic xylan into butanol in a consolidated process without the addition of saccharolytic enzymes [3]. In pulp and fiber processing industries, xylanases have been widely used to remove residual lignin from pulp and bleach paper pulp [4]. Using xylanases in pulp bleach helps to increase pulp fibrillation, reduce beating times in the original pulp, and increase the freeness in recycled fibers. Thus, using enzymes for pulp biobleaching provides an alternative to chemical bleaching methods. The pulping processes usually work at high temperatures and pH. Therefore, the xylanase used for pulp bleaching applications should be thermostable and active at a neutral or alkaline pH. To minimize cellulose damage, the xylanases used in this application should be cellulase-free [5]. Many xylanases can partially meet the requirements. Hence, screening such xylanases is still a worthy area of research.

Xylanases are generally produced by fungi and bacteria. Fungal xylanases are often found to be contaminated with cellulases [6]. Many bacteria are reported to produce cellulase-free thermostable xylanase such as *Bacillus pumilus* SV-205 [7], *Paenibacillus sp.* ASCD2 [8], *Bacillus pumilus* ASH [4], *Bacillus subtilis* [9], *Geobacillus thermoleovorans* [10], and *Bacillus pumilus* SV-85S [11]. In this paper, we have described the isolation and identification of a potential xylanase producer: *Pseudomonas boreopolis* LUQ1. It was found that xylanase expressed by *P. boreopolis* LUQ1 was

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inducible, and xylose can significantly improve the expression of xylanase. Xylanase produced by this organism is cellulase-free and potentially applied in the selective hydrolysis of the hemicellulose component in the pulp and paper industry. Furthermore, the characterization of this enzyme is also presented.

Materials and Methods

Isolation and identification of *P. boreopolis* LUQ1

Pseudomonas boreopolis LUQ1was isolated from paper mill sludge collected in Thunder Bay, Ontario, Canada. Ten grams of samples were suspended in sterile distilled water. After serial dilutions, suspensions were spread onto a medium containing (g/l): beechwood xylan powder 5.0 g, $MgSO_4.7H_2O$ 0.2 g, KH_2PO_4 1.0 g, $K_2HPO_4.2H_2O$ 1.3 g, NH_4NO_3 1.0 g, $FeSO_4.7H_2O$ 0.05 g, $CaCl_2$ 0.02 g, and agar 15.0 g. The colonies selected were streaked on separate plates until purity. After purification, isolates were transplanted to beechwood agar plates, incubated for 48 h at 30℃ and flooded with Congo red solution followed by washing with 1 M NaCl. The bacterial xylanase-producing ability was confirmed by the size of a transparent halo on beechwood agar plates. The transparent halo on beechwood agar plates were measured and compared. The LUQ1 strain was selected for further study because it had the largest halo zone. To identify LUQ1, its genomic DNA was extracted using a genomic extraction kit (Bio Basic, Canada). Partial 16S rRNA gene was amplified by a polymerase chain reaction (PCR) using HAD-1 (5´-GACTCCTACGGGAGGCAGCAGT) and E1115R (5´-AGGGTTGCGCTCGTTGCGGG) primers. The PCR products were purified and sequenced. BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was performed on the sequences to determine the identity of the bacterial isolate. The partial 16S rRNA gene sequence determined in the present study has been deposited in the GenBank database under accession NO. KU973537.

SDS-PAGE and zymogram analysis

SDS-PAGE was performed according to the BioRad instruction manual for the Mini-Protean II system using 10% (w/v) gels containing 0.1% (w/v) beechwood xylan. Following centrifugation at 10,000 g for 10 min, culture supernatants were used as crude enzyme samples for SDS-PAGE and zymogram analysis. The crude enzyme samples were mixed with loading buffer and heated in a boiling water bath for 1 min. 15 µL crude enzyme samples were subjected to SDS-PAGE analysis, and 5µL samples were electrophoresed on SDS-PAGE gel for zymogram analysis. After electrophoresis, the gel was divided into two parts. One part, which contained the sample and the molecular markers (Fermentas, SM1811, Canada), was stained with Coomassie Brilliant Blue R-250. The other part of the gel was soaked for 30 min in 1% (v/v) Triton X-100 to remove the SDS and to renature the proteins in the gel. It was further incubated in the citrate buffer (pH 6.0) for 5 min at 65°C. The gel was soaked in a 0.1% (w/v) Congo red solution for 15 min and washed with 1 M NaCl until the excess dye was rinsed away from the active bands. Dipping the gel in a 4% (v/v) acetic acid solution stopped the reaction. The background turned dark, and clear bands in areas exposed to xylanase activity were observed.

Enzyme assay and characterization of crude enzyme

The xylanase activity was assayed according to the method of Bailey [12], and the amount of reducing sugars released from the beechwood xylan (1%, w/v) was measured using the 3, 5-dinitrosalicylic acid (DNS) method [13]. The 200 µl reaction mixture containing 10 µl of diluted crude enzyme and 190 µl of a 1% (w/v) suspension of beechwood xylan in a citrate buffer (pH 6.0) was incubated at 65°C for 5 min. Next, 200 µl of DNS reagent was mixed with the enzyme reaction mixture and boiled for 5 min. A control was run simultaneously that contained all the reagents, but the reaction was terminated prior to the addition of crude enzyme. The Carboxymethyl-cellulase (CMCase) activity was determined according to Ghose's method [14]. One unit of activity was defined as the amount of enzyme required to release 1µmol of reducing sugar per minute under these conditions.

The pH optima of enzymes were determined by measuring their activities at various pH values using different buffers, such as citric acid-Na_2HPO_4 (pH 3.0-7.0), sodium phosphate (pH 8.0), and glycine-NaOH (pH 9.0-11). Xylanase and CMCase activities were measured at different temperatures (45, 50, 55, 60, 65, 70, 75 and 80°C) under standard assay conditions using beechwood xylan and carboxymethyl cellulose as substrates to determine the optimum temperature of the reaction.

Production of xylanase induced by monosaccharides

The bacteria grown in Luria Bertani (LB) liquid media (10.0 g/l peptone, 5.0 g/l yeast extract, 5.0 g/l NaCl), up to the late exponential phase of growth were supplied with arabitol, sorbitol, galactose, xylose, mannose, glucose, mannitol, arabinose, and xylitol separately to reach a final concentration of 50 mM. The mixtures were incubated in a rotary shaker at 30°C and 200 rpm. After 24, 48, 72, 96 and 120 h, the culture was harvested by centrifugation at 10,000 g for 10 min, and the supernatant was used as a crude enzyme to determine the xylanase activity.

Optimal concentration of xylose for inducing xylanase expression

To determine the effects of xylose induction concentrations on the bacteria, different concentrations (0 mM, 2 mM, 4 mM, 6 mM, 10 mM, 12 mM, 14 mM) of xylose were added to the LB medium when the bacteria had reached the late

exponential phase. The samples were collected after 24, 48, and 72 h. The supernatants were used to determine the xylanase activity under standard assay conditions.

Production of xylanase using different feedstocks (agricultural residues)

The effects of various carbon sources on xylanase production were assessed by culturing bacteria in a mineral salt medium (3.0 g of KH_2PO_4, 3.0 g of $NaNO_3$, 0.5 g of $CaCl_2$, 0.5 g of $MgSO_4.7\ H_2O$, 7.5 mg of $FeSO_4.7H_2O$, 2.5 mg of $MnSO_4.H_2O$, 2.0 mg of $ZnSO_4$, and 3.0 mg of $CoCl_2$ in 1000 ml of water) at 30°C. Wood dust, xylan, agave, wheat bran, or barley straw was used as the sole carbon source (1%, w/v) individually in a liquid medium. Samples were collected at 24 h intervals during the cultivation period. The samples were centrifuged at 10,000 g for 1 min and the supernatants were analyzed for the xylanase activity as described.

Effect of xylose on xylanase production

To determine the induction effect of xylose on xylanase production, the bacterium was cultivated in 250 mL Erlenmeyer flasks containing 50 mL of the mineral salt medium with 1% (w/v) wheat bran or barley straw and maintained at 30°C under agitation at 200 rpm. After 24 h of culture growth, xylose was added to the fermentation medium at a final concentration of 10 mM. The xylanase activity was estimated after another 24 h. A control was run in parallel under the same conditions but without adding xylose to the culture medium.

Results and Discussion

Isolation and identification of *P. boreopolis* LUQ1

For this study, the isolate LUQ1 showed maximum xylanase activity on a xylan agar plate. The strain was identified as *Pseudomonas boreopolis* based on its 16S rRNA gene sequence. The 16S rRNA sequences of the strain showed 99% similarity with *Pseudomonas boreopolis* SR1 (Accession no. KM103098.1), *Pseudomonas boreopolis* S2-s-PMWA-6 (Accession no. AJ864722.1) and *Pseudomonas boreopolis* strain SR3 (Accession no. KM103100.1). The partial sequence of the bacterial 16S rRNA gene of *P. boreopolis* LUQ1 has been submitted to GenBank at NCBI, and its accession number is KU973537. This strain was used for further research.

Characterization of the crude enzyme

The highest xylanase activity of *P. boreopolis* LUQ1 was observed at pH 6.0, and it demonstrated 60.6% and 56.2% of maximum activity at pH 5.0 and 9.0, respectively (Fig. 1A). The optimum temperature was 65°C. The crude xylanase from *P. boreopolis* LUQ1 showed a 64.2% residual activity even at 75°C (Fig. 1B). The crude enzyme produced by *P. boreopolis* LUQ1 is completely cellulase-free as no CMCase activity could be detected at various pH levels and temperatures (Fig. 1A, B). Thermal stability studies showed that the crude enzyme from *P. boreopolis* LUQ1 was stable at 50°C. The residual activity of the crude enzyme was less than 20% after 30 min of heating at 70°C (Fig. 1C). The xylanase produced by *P. boreopolis* LUQ1 is thermostable, cellulase-free and active at a neutral pH. The xylanase can selectively hydrolyze the hemicellulose component and minimize the damage of cellulose which meets the need of paper pulp biobleaching.

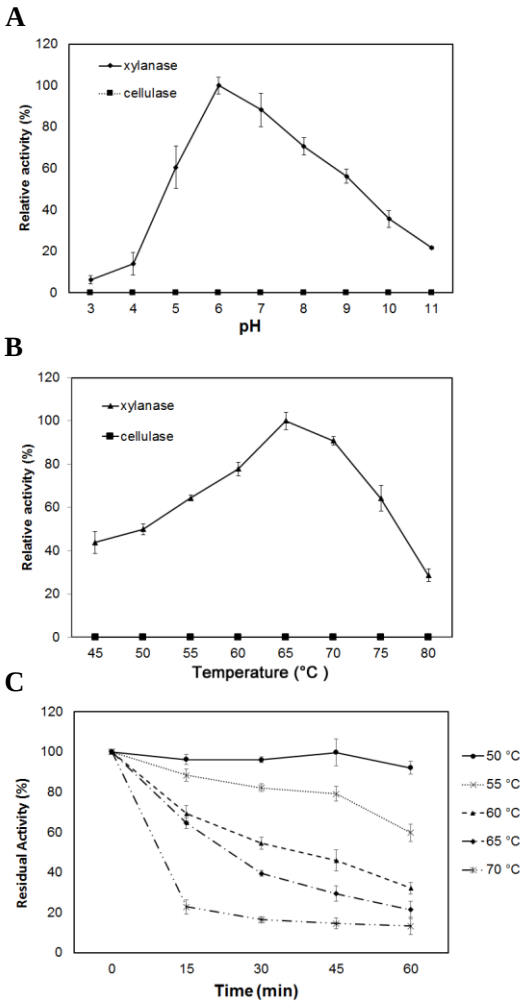

Fig. 1: Influence of temperature and pH on enzyme activity. **(A)** The optimal pH for enzyme activity in the crude enzyme was measured at different pH levels from 3.0 to 11.0. Data are the mean of three replicates and bars indicate their standard deviation. **(B)** The optimal temperature for enzyme activity was measured at different temperatures from 45°C to 80°C. Data are the mean of three replicates and bars indicate their standard deviation. **(C)** The enzyme was diluted in a citric acid-Na_2HPO_4 buffer (pH 6.0, 0.05 M) and incubated at different temperatures. Residual activity was measured using standard assay after time intervals of 15, 30, 45 and 60 min.

SDS-PAGE and zymogram analysis

The concentration of soluble protein in culture supernatant was low. Although the loading samples in SDS-PAGE gel were three times more than in zymogram analysis gel, the protein bands in SDS-PAGE were weak (Fig. 2). A single band was detected on the activity staining gel, and the molecular mass of xylanase was estimated to be approximately 20 kDa.

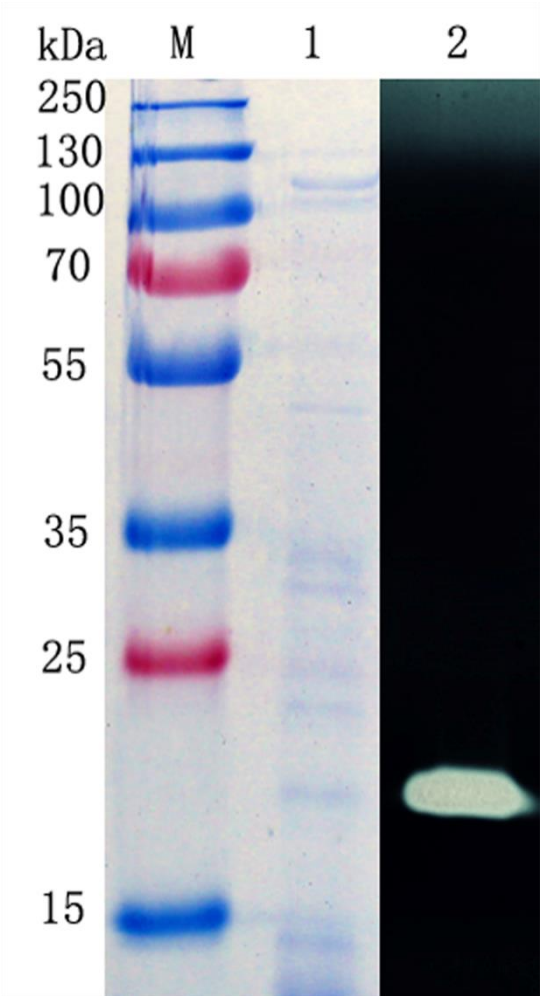

Fig. 2: SDS-PAGE and zymogram of the supernatant expression of *P. boreopolis* LUQ1. Lane 1: Coomassie blue staining of the SDS-PAGE gel; Lane 2: Congo red staining of the SDS-PAGE gel containing 0.1% beechwood xylan; M: standard protein molecular weight markers.

Production of xylanase induced by monosaccharides

It was reported that xylanase produced by various bacteria and fungi is inducible. Xylan has been proved to be an excellent carbon source since it can induce the microorganism to produce more xylanase [6]. But since xylan is a large polymer which cannot penetrate the cells, it must be assumed that xylanase is induced by low molecular mass saccharides digested from xylan. Small molecular weight carbohydrates such as xylose, arabitol, sorbitol, galactose, mannose, glucose, mannitol, arabinose, and xylitol were used to screen the potential inducer for xylanase expression of *P. boreopolis* LUQ1. Monosaccharide was added in the culture medium independently as an inducer after the bacteria had reached the late exponential phase of growth in the LB medium. The xylanase production improved dramatically when xylose was used as an induction chemical, but other monosaccharides did not have significant induction effects (Fig. 3A). Xylose usually has a dual role as a repressor and as an inducer that depends on its concentrations [15]. In some microbes, such as in *Aspergillus nidulans*, xylose acts as an inducer at low concentrations and works as an inhibitor at higher concentrations [16].

To determine the optimum concentration of xylose required for maximal xylanase induction, xylose was added to the media in varying concentrations after the exponential growth phase of bacteria in LB medium. It was observed that xylanase activity increased alongside the xylose concentrations and attained a steady level when xylose concentrations reached around 12 mM (Fig. 3B).

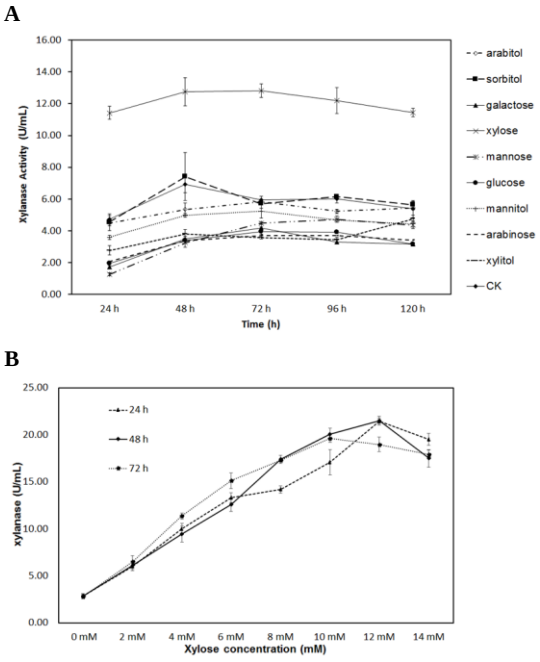

Fig. 3: (A) Time course of xylanase production by *P. boreopolis* LUQ1 in LB medium supplied with different monosaccharides. Monosaccharides are used as inducers with the final concentration of 50 mM in the LB medium. The mixtures were incubated in a rotary shaker at 30°C and 200 rpm. The xylanase activity was measured using standard assay after time intervals of 24, 48, 72, 96 and 120 h. **(B)** Effect of concentration of xylose on xylanase production. *P. boreopolis* LUQ1 was precultured for 24 h in an LB medium and induced by xylose with different concentrations. Xylanase activity was measured after 24 h induction.

Effect of different carbon sources on the production of xylanolytic enzymes

The results of a time course experiment for the production of xylanase with different carbon sources in the flasks are presented in Fig. 4. Wheat bran was found to be the most effective for xylanase production in the submerged fermentation. Similar results were reported in *Bacillus pumilus* [7]. There are at least two main reasons for this. One is that wheat bran has lower lignin content and 18% protein, thus serving both as a carbon and a nitrogen source [17]. The other reason is that wheat bran is easily degraded and releases low mass saccharides to induce the microorganism to produce more xylanase. The xylanase activities increased gradually after three days of culture. Maximum activity (25.61 U/ml) was reached on day four, and remained at a steady level when wheat bran was used as a carbon source. This activity is comparable to the previously reported microbial strains for natural xylanase production (Table 1). With xylan medium, the activities of the xylanases increased by day three and reached a maximum on day six. Most xylanase activity did not diminish greatly after it reached the highest level. Negligible xylanase activities were detected in wood dust, starch, agave, and barley straw (Fig. 4). The highest xylanase activity was detected in the medium using wheat bran as the sole carbon source which can be attributed to its degradable xylan, starch, and protein components. *P. boreopolis* LUQ1 in a wheat bran medium can grow better than in any other medium.

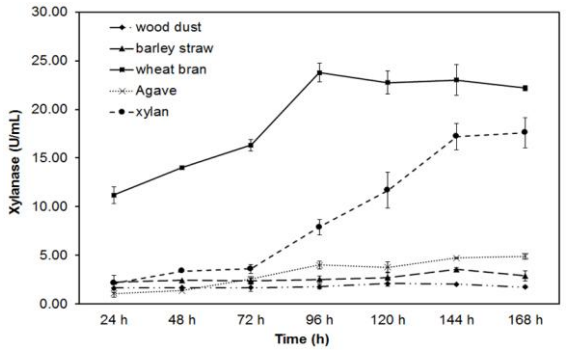

Fig. 4: Effect of various carbon sources on xylanase production in submerged fermentation by *P. boreopolis* LUQ1. Different agricultural residues individually were used as the substrate in submerged fermentation shaking for 7 days at 30°C.

When xylose was used as an inducer in the fermentation, the induction effects differed according to the feedstock, but it was not as significant as in the LB medium which did not contain xylan. Xylanase activity increased about 50% in the barley straw media with 10 mM xylose. When wheat bran was used as a feedstock, the xylanase activity increased by ~15% after induction (Fig. 5). The xylanase production by *P. boreopolis* LUQ1 did not show high activity in fermentation condition, since the optimal temperature of xylanase is around 65°C and much higher than fermentation temperature. In wheat bran medium, the xylan in wheat bran is easier to be digested and release more xylose to enhance xylanase expression by *P. boreopolis* LUQ1. So, *P. boreopolis* LUQ1 produced more xylanase in wheat bran media than it did in the barley straw.

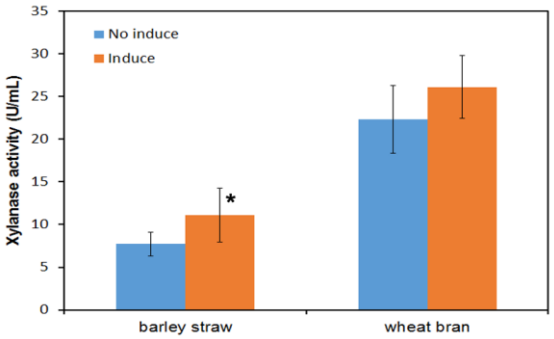

Fig. 5: Effect of induction on xylanase production under submerged fermentation. *P. boreopolis* LUQ1 was induced by 10 mM xylose at the late exponential phase in the medium using barley straw or wheat bran as the sole carbon source. Xylanase activity was measured after 24 h induction. Results are presented as mean ± SD and differences were analyzed with Students t-test, *: indicated significant difference ($p < 0.05$).

Conclusion

The results of this study indicate that *P. boreopolis* LUQ1 can produce cellulase-free and thermostable xylanase. Wheat bran was the best carbon source to produce xylanase by submerged fermentation, and the extracellular secretion of xylanase can be significantly improved when xylose is used as an inducer. Future research to optimize the fermentation condition and induction parameters would be helpful for the industrial applications of the strain LUQ1.

Acknowledgements

This work was supported by "the Fundamental Research Funds for the Central Universities" and Canada NSERC Grant to WQ.

Conflict of Interest

No conflict of interest declared.

Table 1: Comparison of xylanase from *P. boreopolis* LUQ1 with other known bacteria.

Stain name	Mol. wt	pH_{opt}	$Temp_{opt}$ (℃)	activity (U/mL^{-1})	Reference
Cellvibrio mixtus strain J3-8	38	6	50	10.1	[20]
Bacillus halodurans S7	43	9	75	5.1	[19]
Bacillus. coagulans	-	7	55	18	[21]
Bacillus subtilis	-	6	60	12	[9]
Paenibacillus campinasensis BL11	41	9	55	26.8–29.39	[18]
Geobacillus thermoleovorans	-	8.5	80	5–8	[10]
P. boreopolis LUQ1	20	6	65	25.61	This study

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