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MICROBIOLOGY

Production of Endoglucanase by *Exiguobacterium mexicanum* OB24 Using Waste Melon Peels as Substrate

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Abstract: Millions of tons of agricultural waste are produced globally every year. A practical solution to this global problem is to convert this waste into value-added products. In this study, endoglucanase enzyme production was carried out by using waste melon peels as a carbon source. To use this important resource, its stubborn structure must be broken down. Rumen bacteria are regarded as unique for this job. Therefore, firstly endoglucanase producing rumen bacteria was isolated and the bacteria with the best activity (OB24) were identified by molecular methods (16S rRNA gene squencing). As a result of the sequence analysis, it was determined that isolate belonged to *Exiguobacterium mexicanum*. Then, by optimizing the culture conditions, the enzyme production potential was increased. The optimal conditions were determined as 50 g/L MPP, 2g/L yeast extract, 60 h incubation time, pH: 6.0, and 40°C temperature. Under optimized conditions the enzyme activity increased approximately 3.8-fold.

Key words: Endoglucanase production, waste melon peel, *Exiguobacterium mexicanum* OB24, agricultural wastes.

INTRODUCTION

The decrease in fossil fuel resources and the related environmental pollution are directing the world's energy policies towards the development of alternative energy resources (Liu 2020, Oni et al. 2020). In this regard, there is a global interest in developing industrial processes to produce biofuels from plant biomass such as natural oils, starch, sucrose and lignocelluloses (Hamann et al. 2015). Lignocellulosic materials, composed of lignin, pectin, hemicellulose, and cellulose(10-40% 5-10%, 20-40%, and 45-60%, respectively), are found as cell wall components in plants (Guzel & Akpinar 2019, Ravindran & Jaiswal 2016). Agricultural wastes are attractive sources for the production of new valuable materials from zero-value lignocellulose wastes (Hasanin & Hashem 2020). As the world population continues to increase, the production of fruits and vegetables around the world is also increasing (Bahadur et al. 2018, Liakou et al. 2018). Some types of fruit, such as melon, watermelon, pineapple, banana etc. are broadly acceptable for taste and nutritional value, but >40% of the total fruit mass, including seeds, peel, and pulp is not edible (Ben-Othman et al. 2020, Gomez-Garcia et al. 2021). Most of these waste materials are thrown into the environment. To counterbalance the waste issue, administer environmental sustainability and overcome the economic development model "take, make, and dispose," the concept of circular economy has been introduced by the application of sustainable and profitable technologies to

utilize byproducts (Villacis-Chiriboga et al. 2020). In modern food waste management, it is very important to evaluate by-products, obtain valuable products from them and/or develop new products with market value (Villacis-Chiriboga et al. 2021). Today, agricultural wastes provide low-cost cellulose for valuable products production to reduce cost and environmental problems (Hero et al. 2017, Hussain et al. 2019, Yang & Wyman 2008). Therefore, it is important to use these wastes and to produce valuable new products from them.

Melon (*Cucumis melo*), which grows in warm regions of the world, belongs to the *Cucurbitaceae* family and melon is one of the most consumed fruits in the world (Li et al. 2006). Although the pulp, seed and juice of the melon are used for human consumption, the peel, which constitutes 30% of the whole fruit, is disposed of as waste without any useful recycling (Broitman et al. 2018). It is estimated that the worldwide production of melon is about 40 million tons per year and 8 to 20 million tons of waste accumulates from its non-edible parts (peels and seeds) (Hasanin & Hashem 2020). Valorization of fruit by-products is considered as an important activity to minimize the harmful effect of food waste on the ecosystem, to recover value-added compounds and to create new income sources (Esparza et al. 2020, Sabater et al. 2020).

Despite the biologically recalcitrant nature of lignocellulosic biomass, it can be efficiently digested by rumen microorganisms in natural ecosystems (Vida & Tedesco 2017). The rumen environment has a diverse population of microbes that colonize and degrade lignocellulosic materials (Azizi et al. 2020, Krause et al. 2003). Digestion developed by rumen microbes has reached its greatest evolution in ruminants. Therefore, the rumen is one of the best environments for degradation of lignocellulosic material (Gharechahi et al. 2021, Xing et al. 2020). The selection of cheap substrate is considered as one of the most important criteria for reducing production costs in industrial fermentation process (Altun et al. 2020, de Castro & Sato 2015, Tuysuz et al. 2021). So, we hypothesized that lignocellulosic material-rich waste or by-products such as melon peels could be used as substrates for endoglucanase production.

In this study, for the first time, endoglucanase was produced from *Exiguobacterium mexicanum* by using waste melon peels powder.

MATERIALS AND METHODS

Substrate preparation

Melon peels were washed properly, followed by oven drying at 80°C till constant weight. The dried peels were ground into fine particles and termed as Melon Peel Powder (MPP) (Raji et al. 2017).

Isolation of Endoglucanase Producer Rumen Bacteria

The Rumen samples (0.5 g) were placed in falcon tubes containing 50 mL sterile 0.9% NaCl. A serial dilution (dilution series from 10^0 to 10^{-7}) was prepared and homogenized at 180 rpm for 1 hour at 35°C (Baltaci & Adiguzel 2016). Each of these dilutions spread on carboxymethyl cellulose (CMC) - Bushnell Haas Medium (BHM) agar plates (10 g/L CMC, 1 g/L K_2HPO_4, 1 g/L KH_2PO_4, 0.2 g/L $MgSO_4.7H_2O$, 1 g/L NH_4NO_3, 0.05 g/L $FeCl_3.6H_2O$, 0.02 g/L $CaCl_2$, and 20 g/L agar). The plates were incubated at 35°C for 48 h. At the end of the period, nine different colonies were selected and pure cultures were obtained. The colonies were stored in the Triptic Soy Broth (TSB) with 20% glycerol content at -85°C for further studies (Demir et al. 2018).

Screening of endoglucanase producing isolates

In this stage, nine bacteria were screened in terms of the ability to produce endoglucanase. The experiments were carried out in 250-mL flasks containing 100 mL of the screening medium composed of 10 g/L MPP and 1 g/L K_2HPO_4, 1 g/L KH_2PO_4, 0.2 g/L $MgSO_4.7H_2O$, 1 g/L NH_4NO_3, 0.05 g/L $FeCl_3.6H_2O$, 0.02 g/L $CaCl_2$ (pH 8.0). To prepare the seed culture, the test bacteria activated on Triptic Soy Agar (TSA) were transferred into 250-mL Erlenmeyer fasks containing 100 mL of TSB, and the flasks were incubated at 35°C for 24 h. One mL (OD_{600}nm =1.0) of the prepared seed culture was then employed for the inoculation of the screening medium. After the flasks were inoculated, they were left to the incubation at 35°C with agitation speed of 150 rpm. After 48 h, endoglucanase activities were analyzed and the most productive strain was selected for subsequent experiments.

Optimization of culture conditions for endoglucanase production by the best strain

The endoglucanase production was carried out in 250 mL fasks containing 100 mL of sterilized melon peel medium (screening medium) described above. During the experiments, different MPP concentrations (10–70 g/L), temperatures (25–50°C), initial pHs (4–10) and incubation times (with 12 h intervals up to 96 h) were tested to increase enzyme production.

The preliminary experiments were performed to determine the most suitable co-cultures and incubation time (12-96 h). Then, nitrogen sources (yeast extract, peptone, urea, and ammonium sulpfate) and concentration (0-4 g/L), temperature (25-50°C with 5°C intervals), and initial pH (4-10 with 1 interval) parameters were studied, respectively (Baltaci et al. 2020b). In the case of screening experiments, one milliliter of pre-culture was used for the inoculation of MPP-BHM medium (100 mL) in a 250 mL flask. Enzyme activities were detected by spectrophotometer OD540 nm according to the DNS method.

Molecular identifcation of the best enzyme producer isolate

For identification of the best isolate, genomic DNA isolation was performed according to the Promega WizardR Genomic DNA Purification Kit (A2360) protocol. 16S rRNA gene region, was amplified using 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') primers. 30 µL volume of PCR mixture containing, 18.1 µl ddH_2O, 3 µl 10X PCR buffer, 1.2 µl DMSO, 1.8 µl $MgCl_2$, 1µl (10 µM) forward primer (27F), 1µl (10µM) reverse primer (1492R), 0.6 µl dNTP, 0.3 µl Taq DNA polymerase and 3 µl template DNA (Adiguzel et al. 2020). PCR amplification was carried out according to the following protocol: initial denaturation for 5 min at 94°C followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 30 s, extension at 72°C for 30 s, and final extension at 72°C for 5 min. The PCR products were visualized on a 1% agarose gel (Baltaci et al. 2017). The amplified fragments were cloned into *Escherichia coli* JM101 strain with the pGEM-T Easy Cloning Vector (Promega, Southampton, UK) according to the instructions of the manufacturer. After the cloning, plasmid isolation was carried out and the 16S rRNA gene region was sequenced in the Macrogen Company (Netherlands). The 16S rRNA sequence was compared with the other bacterial series in GenBank and EzBioCloud (http://blast.ncbi.nlm.nih and https://www.ezbiocloud.net/), similarity rate between them was determined and GenBank accession numbers were received. A phylogenetic tree was constructed with Mega4 software based on the 16S rDNA sequences of the strains closer to isolates (Tuysuz et al. 2021).

Analysis of enzyme activity and cell growth

Endoglucanase activity of each culture was measured by DNS (3,5-dinitrosalicylic acid) method through determining the amount of reducing sugars liberated during hydrolysis (Miller 1959). CMC solution (1%) was prepared in 1 N citrate buffer (pH 7.0) which was considered as substrate. Then, 100 µl crude enzymes and 1 mL citrate buffer were added into the mixture of 1 mL CMC solution. The mixture was incubated at 35 °C for 30 min and DNS was added. Then the samples were boiled for 10 min, cooled in water for color stabilization and the optical density was measured at 540 nm (Rocky-Salimi & Hamidi-Esfahani 2010). One unit of endoglucanase activity was defined as the amount of enzyme that could hydrolyze CMC and release 1 µmol of glucose within 1 min of reaction. The amount of reducing sugar was determined using a glucose standard curve.The standart curve was given in Figure 2. Endoglucanase activity was calculated using the following formula:

$$Enzyme\,activity\left(\frac{U}{mL}\right)=\frac{m}{Vt}$$

m is the actual amount (µmol) of reducing sugar produced by enzymatic hydrolysis, V is the volume (mL) of the enzyme solution, and t is the hydrolysis time (min) of the enzyme.

Statistical analysis

Each experiment was repeated at least three times. The variance analysis was carried out according to the one-way ANOVA test using the Prism software 7.0 (GraphPad Software, San Diego, CA), and the averages were compared with the Duncan test at a confdence level of 0.05.

RESULTS AND DISCUSSION

Screening of endogluconase producer isolates

Preliminary experiments in this study focused on isolating a bacterium that produces maximal endogulcanase. For this, CMC was used as the sole carbon source in the isolation medium. Rumen samples collected from Erzurum slaughterhouses were used as the isolation source of endoglucanase-producing bacteria. The isolated strains were purified and bacteria thought to be different in colony structure and morphology were selected. In this way, a total of nine rumen bacteria were isolated from the samples taken. Then, they were screened for endoglucanase production abilities in sterile medium containing MPP and CMC-BHM agar plates. The data presented in Table I. show that OB24 strain has the highest OD_{600} value (1.29) and endoglucanase activity (12.6 U/mL) in culture among the nine isolates. Therefore, OB24 strain was selected for subsequent experiments. In addition, the hydrolysis zone image of OB24

Table I. Screening of Endoclucanase producer bacteria.

Isolates	Bacterial growth (OD_{600})	Endoglucanase enzyme activity (U/mL)
OB1	0.56	4.45
OB3	0.37	2.26
OB6	0.42	1.3
OB9	0.36	3.29
OB13	0.59	6.5
OB24	**1.29**	**12.6**
OB32	0.31	4.2
OB33	0.55	6.9
OB36	1.25	10.7

Screening conditions: Temperature 35 °C, initial pH 8.0, MPP concentration 10 g/L, and incubation time 48 h.
Bold values indicate the OB24 isolate to be used in the subsequent stages of the study.

was given in Figure 1. Next, strain OB24 was identified as *Exiguobacterium mexicanum* based on 16S rRNA sequencing analysis. This strain (GenBank Accession number: OK147925) had 1524 nucleotides (nt) and was 99% similar to *Exiguobacterium mexicanum*. The phylogenetic tree was constructed using the MEGA4 program (Figure 3). When the literature is examined, although there is only one study on cellulase production by *Exiguobacterium* sp. Alg-S5 (Mohapatra 2017), there is no study on cellulase production with *Exiguobacterium mexicanum*.

Optimization of culture conditions for endoglucanase production by *Exiguobacterium mexicanum* OB24

Many studies have shown that endoglucanase production is affected by a variety of physicochemical factors, including the type and composition of the substrate, the concentration and type of carbon and nitrogen sources, temperature, pH, incubation time, and agitation (de Almeida et al. 2014, Gastelum-Arellanez et al. 2014, Maharana & Ray 2015, Philip et al. 2020). Therefore, in this study MPP concentration, nitrogen source and concentrations, incubation time, temperature, and pH parameters were optimized by following one-variable-at-a-time approach. First, various concentrations of MPP (10-70 g/L) were tested, and the highest endoglucanase production was achieved with 50 g/L (21.9 U/mL). An increase in MPP concentration of more than 50 g/L led to a slight decrease in endoglucanase production (Figure 4). This might be because MPP contains some inhibitor compounds.

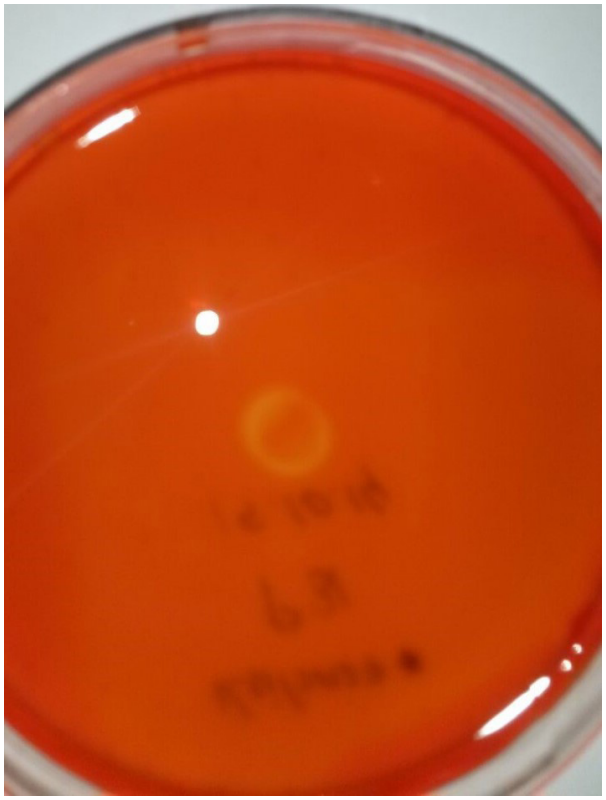

Figure 1. Orange zones of OB24 with cellulase activity on CMC-BHM medium.

It has been reported in the literature that concentrations of nitrogen sources significantly affect the amount of enzyme produced (Kachlishvili et al. 2006, Membrillo et al. 2008). Therefore, it is very important to determine nitrogen sources that give good results in enzyme production. As seen in Figure 5, the effect of four different nitrogen sources (amonnium sulphate, urea, yeast extract, and peptone) on endoglucanase activity at different concentrations (0-4 g/L) was investigated. Maximum enzyme production (27.6 U/mL) was achived supplemented with 2 g/L of yeast extract.

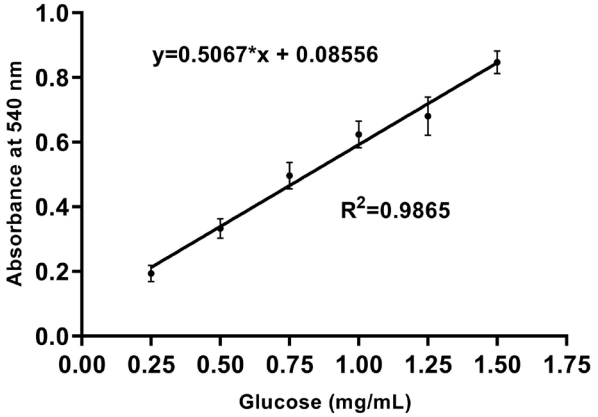

Figure 2. Glucose standard curve.

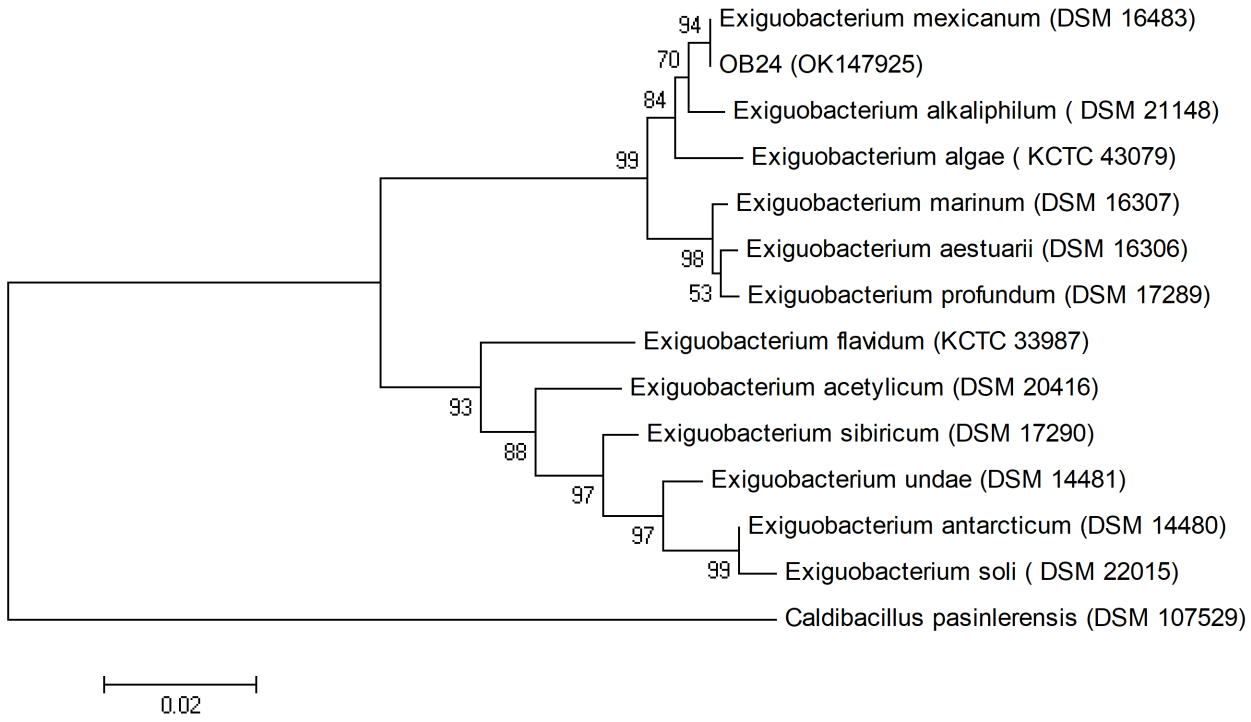

Figure 3. Neighbor joining phylogenetic tree on the basis of 16S rRNA gene sequence data of the *Exiguobacterium mexicanum* OB24 isolated from rumen samples. *Caldibacillus pasinlerensis* (Baltaci et al. 2020a) was used as out-group. Bootstrap values based on 1000 replications are listed as percentages at branching points. The accession numbers are given in parentheses. Only bootstrap values >50% are shown at nodes.

In previous studies, it was stated that yeast extract gave the best result in endoglucanase activity (Malik et al. 2021, Taherzadeh-Ghahfarokhi et al. 2021, Thakur et al. 2021). All concentrations of urea reduced endoglucanase enzyme activity. In previous studies, it has been reported that urea decreases the production of some industrial enzymes (Negi & Banerjee 2009, Yong et al. 2008). The possible explanation for this is that urea competes with endoglucanase peptide chains for hydrogen bonds, thereby disrupting the secondary structure of the endoglucanase protein and causing it to lose endoglucanase activity.

Temperature plays an important role in enzyme production by directly affecting the physiology and growth of microorganism. As shown in Figure 6, although strain OB24 produce enzyme in a wide temperature range from 25 to 50 °C, the maximal endoglucanase activity was determined at 40 °C (34.3 U/mL) . Since the rumen environment is 39 °C (Kamra 2005), it is not surprising that OB24 (isolated from rumen) has the best activity at 40 °C.

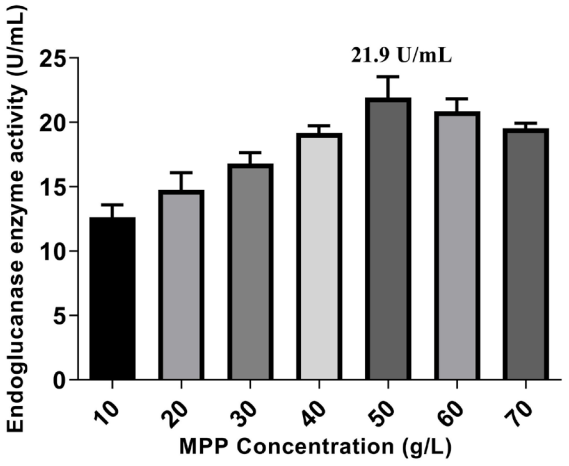

Figure 4. Effects of MPP concentration on the endoglucanase enzyme activity. Culture condions: 0.5 g/L yeast extract, Temperature 35°C, initial pH 8.0, and incubation time 48 h.

While the optimum pH for endoglucanase production was detected, a pH range from 5 to 10 at 40 °C was tested. Maximal endoglucanase activity was achived at pH 6.0 and the second-highest activity was 7.0. Also, the strain produces enzymes in high yields a wide pH range (5-9) (Fig. 7). These results indicate that the purifed enzymes of the OB24 may show high activity not only acidic pHs but also alkaline and neutral pHs.

The final experiment of optimization focused on determining the optimal incubation time for endoglucanase production in MPP medium. Endoglucanase production gradually increased from the 12th h to the 60th h and reached the maximum enzyme activity at 60th h (47.4 U/mL) (Fig. 8). Our results were contradictory to Nguyen et al. (2019) who investigated 96 h as an optimum incubation period for the production of endoglucanase (Nguyen et al. 2019).

As a result of the analysis optimal conditions were determined as 50 g/L MPP, 2g/L yeast extract, 60 h incubation time, pH: 6.0, and 40°C temperature. Under optimized conditions the enzyme activity increased approximately 3.8-fold (47.4 U/mL).

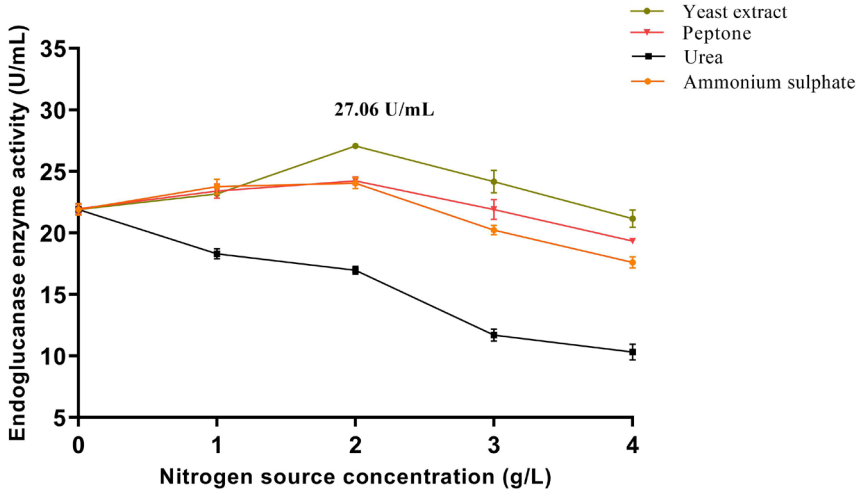

Figure 5. The effect of nitrogen source on the endoglucanase enzyme activity. Culture condions: MPP concentration 50 g/L, Temperature 35°C, initial pH 8.0, and incubation time 48 h.

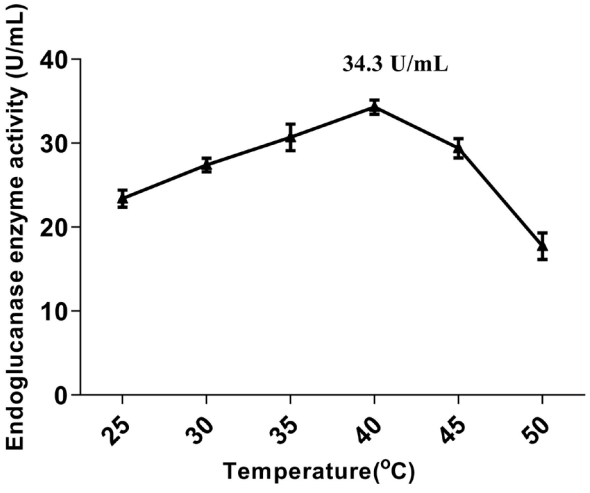

Figure 6. The effect of temperature on the endoglucanase enzyme activity. Culture condions: MPP concentration 50 g/L, 2 g/L yeast extract, initial pH 8.0, and incubation time 48 h.

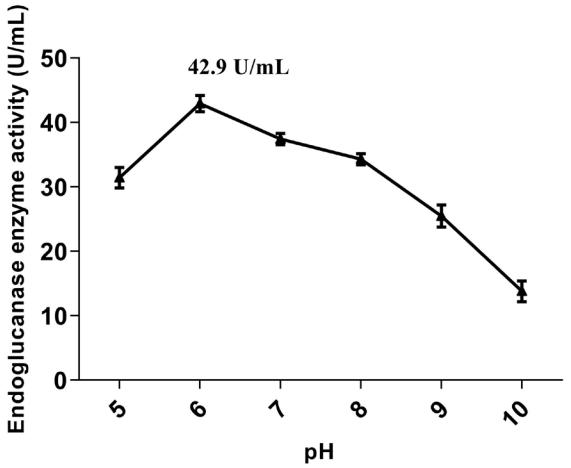

Figure 7. The effect of initial pH on the endoglucanase enzyme activity. Culture condions: MPP concentration 50 g/L, 2 g/L yeast extract, Temperature 40°C, and incubation time 48 h.

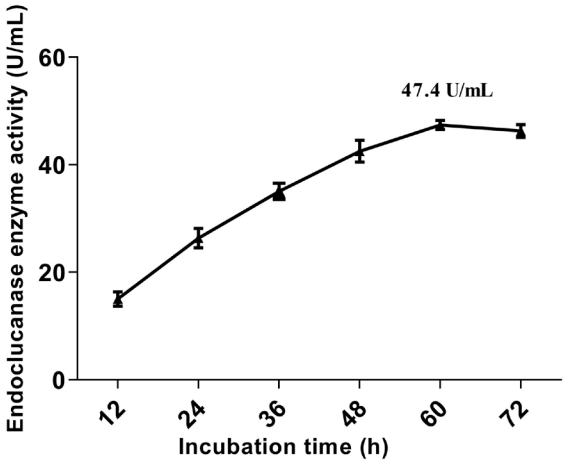

Figure 8. The effect of incubation period on the endoglucanase enzyme activity. Culture condions: MPP concentration 50 g/L, 2 g/L yeast extract, Temperature 40°C, and initial pH 6.0.

CONCLUSIONS

The present study revealed that *Exiguobacterium mexicanum* OB24 isolated from rumen samples could produce endoglucanase on waste melon peels. By means of the optimization of the culture conditions, the production of endoglucanase can be signifcantly increased. On the other hand, it has been shown that waste melon peels can be used as a substrate for endoglucanase production. Finally, this is the first report on the ability of *Exiguobacterium mexicanum* to produce endoglucanase from waste melon peels.

Acknowledgments

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Conceived and designed the experiments: AA (group leader). Performed the experiments: MOB, SA and MAO. Analyzed the data: MOB, SA, GA, and AA. Contributed reagents/materials/analysis tools: AA and GA. Wrote the paper: MOB and AA. All authors read and approved the final manuscript. The data that support the findings of this study are available from the corresponding author upon reasonable request.