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Effects of Xylanases from Different Sources and Combinations on Xylan Hydrolysis Product Composition, Bacterial Proliferation, and Escherichia coli Adhesion: Postprint

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Abstract

This study aimed to investigate the hydrolytic effects of xylanases from different sources and their combinations on xylan, and to examine the effects of different xylan hydrolysis products on bacterial proliferation and the adhesion of Escherichia coli to intestinal epithelial cells. Xylanase A and xylanase B were derived from Pichia pastoris and Aspergillus oryzae, respectively. Xylan was hydrolyzed using xylanase A, xylanase B, enzyme combination 1 (xylanase A: xylanase B = 3:7), enzyme combination 2 (xylanase A: xylanase B = 1:1), and enzyme combination 3 (xylanase A: xylanase B = 7:3), followed by determination of the effects of xylan hydrolysis products on the proliferation of Escherichia coli, Bacillus subtilis, and Lactobacillus, as well as the adhesion of Escherichia coli to intestinal epithelial cells. The results demonstrated that: 1) The two xylanases exhibited certain synergistic effects; the total contents of xylobiose and xylotriose in the xylanase A, xylanase B, enzyme combination 1, enzyme combination 2, and enzyme combination 3 groups were 95.70%, 86.79%, 93.11%, 94.55%, and 87.55%, respectively, with the xylanase A group showing the highest xylobiose content and the enzyme combination 2 group showing the highest xylotriose content. 2) At 20 h of culture, the five xylan hydrolysis products had no significant effect on Escherichia coli proliferation (as indicated by bacterial suspension absorbance) ($P > 0.05$); at 20 and 30 h of culture, the five xylan hydrolysis products significantly promoted Bacillus subtilis proliferation ($P < 0.05$); at 13 and 17 h of culture, the five xylan hydrolysis products significantly promoted Lactobacillus proliferation ($P < 0.05$). 3) All five xylan hydrolysis products significantly reduced the adhesion rate of Escherichia coli to intestinal epithelial cells ($P < 0.05$). These findings indicate that hydrolysis of xylan by xylanases from different sources and their combinations can produce hydrolysis

products primarily composed of xylobiose and xylotriose, thereby promoting the proliferation of Bacillus subtilis and Lactobacillus while reducing the adhesion of Escherichia coli to intestinal epithelial cells.

Full Text

Effects of Different Sources of Xylanase and Their Combinations on Xylan Hydrolysate Composition, Bacterial Proliferation, and *Escherichia coli* Adhesion

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Abstract

This experiment investigated the hydrolytic effects of different xylanase sources and their combinations on xylan, and examined how various xylan hydrolysates influence bacterial proliferation and the adhesion of *Escherichia coli* to intestinal epithelial cells (IEC). Xylanase A and xylanase B were derived from *Pichia pastoris* and *Aspergillus oryzae*, respectively. Xylan was hydrolyzed using xylanase A, xylanase B, combined enzyme 1 (xylanase A:xylanase B = 3:7), combined enzyme 2 (xylanase A:xylanase B = 1:1), and combined enzyme 3 (xylanase A:xylanase B = 7:3). The effects of these hydrolysates on the proliferation of *E. coli*, *Bacillus subtilis*, and lactobacilli, as well as on *E. coli* adhesion to IEC, were subsequently measured. The results demonstrated: (1) Synergistic effects were observed between the two xylanases. The total content of xylobiose and xylotriose in the xylanase A, xylanase B, combined enzyme 1, combined enzyme 2, and combined enzyme 3 groups were 95.70%, 86.79%, 93.11%, 94.55%, and 87.55%, respectively. Xylanase A produced the highest xylobiose content, while combined enzyme 2 yielded the highest xylotriose content. (2) After 20 hours of culture, the five xylan hydrolysates showed no significant effect on *E. coli* proliferation (expressed as optical density of bacterial suspension) ($P > 0.05$). However, they significantly promoted *B. subtilis* proliferation after 20 and 30 hours of culture ($P < 0.05$), and significantly enhanced lactobacilli proliferation after 13 and 17 hours ($P < 0.05$). (3) All five xylan hydrolysates significantly reduced the adhesion rate of *E. coli* to IEC ($P < 0.05$). These findings indicate that hydrolyzing xylan with different xylanase sources and their combinations can produce hydrolysates rich in xylobiose and xylotriose, thereby promoting

the proliferation of *B. subtilis* and lactobacilli while reducing *E. coli* adhesion to intestinal epithelial cells.

Keywords: xylanase; xylan hydrolysate; bacterial proliferation; intestinal epithelial cells; bacterial adhesion rate

Introduction

Xylan, a major component of hemicellulose, consists of -D-xylopyranose units linked by 1,4-glycosidic bonds. Dietary xylan can increase digesta viscosity in the gastrointestinal tract, impair nutrient digestion and absorption, and promote the proliferation of harmful gut microorganisms, consequently increasing animal morbidity [1]. Xylanase can degrade dietary xylan, thereby reducing digesta viscosity, promoting beneficial bacterial growth, decreasing colonization by harmful microorganisms, maintaining normal intestinal flora structure, and eliminating the adverse effects of xylan.

Xylanases from different sources exhibit substantial differences in physicochemical properties and enzymatic activities. Yu et al. [2] measured the enzymatic characteristics of six xylanases and found variations in their optimal temperature, optimal pH, and thermostability. To enhance the catalytic efficiency of enzyme preparations, Feng [3] proposed the concept of combined enzymes, which leverages synergistic catalytic effects by combining two or more enzymes with complementary properties to improve overall catalytic rates. Dai et al. [4] reported that adding 100 mg/kg bacterial xylanase + 100 mg/kg fungal xylanase to broiler diets improved production performance compared to single xylanase supplementation. Additionally, when a combined enzyme with a cellulase-to-xylanase ratio of 7:3 was used to hydrolyze rapeseed meal, the reducing sugar yield was significantly higher than with single enzymes [5].

The hydrolysis products of xylanase are primarily xylo-oligosaccharides (XOS), a class of functional oligosaccharides proven to selectively promote the proliferation of beneficial bacteria such as *Bifidobacterium* [6] and reduce the risk of colon cancer, with xylobiose demonstrating the most effective performance in these aspects [7-8]. XOS with a degree of polymerization below 4 can promote *Bifidobacterium* proliferation [9], with the main active components being xylobiose and xylotriose. Xylotriose shows the best proliferation effect for *Bifidobacterium*, followed by xylobiose and other XOS [10]. Zhang et al. [11] reported that both xylobiose and xylotriose at 5 g/L could promote the proliferation of *Bifidobacterium adolescentis* in vitro. Ma [12] demonstrated that xylobiose and xylotriose could promote the proliferation of lactobacilli and *B. subtilis* while reducing the adhesion rates of *E. coli* and *Salmonella* to cells.

Some oligosaccharides can directly inhibit pathogen adhesion to intestinal epithelial cells [13]. When xylo-oligosaccharides were co-incubated with *Listeria* and Caco-2 cells, the number of bacteria adhering to cells decreased by two-

thirds compared to the control group [14]. However, whether different sources of xylanase can be employed to decompose xylan, thereby altering hydrolysate composition and exerting enhanced prebiotic effects, has not been reported in the literature. This study analyzed xylan hydrolysates to investigate how different xylanase sources and their combinations affect hydrolysate composition. The resulting hydrolysates were then used to culture bacteria to examine their effects on harmful and beneficial bacterial proliferation. Furthermore, bacterial adhesion assays were conducted to study how xylan hydrolysates affect *E. coli* adhesion, providing a scientific basis for understanding the mechanism by which xylanase regulates intestinal flora structure.

Materials and Methods

1.1 Xylanase Preparation and Hydrolysis Xylanase A and xylanase B were derived from *Pichia pastoris* and *Aspergillus oryzae*, respectively. Following the method described in GB/T 23874-2009, the activities of xylanase A and xylanase B were measured as 14,232 U/g and 7,088 U/g, respectively. Five experimental groups were established, each maintaining a total enzyme activity of 12,088 U/g: xylanase A, xylanase B, combined enzyme 1 (xylanase A:xylanase B = 3:7), combined enzyme 2 (xylanase A:xylanase B = 1:1), and combined enzyme 3 (xylanase A:xylanase B = 7:3). For each group, 1 g of enzyme source was dissolved in 100 mL phosphate-buffered saline (PBS), followed by addition of 3 g xylan. After 2 hours of reaction, the hydrolysates were obtained. High-performance liquid chromatography (HPLC) was used to determine the composition of xylan hydrolysis products, employing an Ecosil amino column (250 mm × 4.6 mm) with acetonitrile:water (67:33) as the mobile phase. The detector temperature was 35 °C, flow rate was 1 mL/min, and injection volume was 10 L. External standard method was used for quantification. Xylan and xylose were purchased from Sigma, while xylobiose, xylotriose, and xylotetraose were obtained from Floko.

1.2 Xylan Hydrolysate Processing The xylan hydrolysates were filtered through a 0.22 m membrane for sterilization and stored at 4 °C. A specific volume (V, mL) of hydrolysate was taken to measure total reducing sugar content. An equal volume of 8% H SO solution was added, mixed thoroughly, and incubated at 121 °C for 1 hour. The solution was then neutralized with 15% NaOH to pH 6.5–7.0 and diluted to volume (V , mL) with distilled water to achieve a final reducing sugar concentration of 0.2–2.0 g/L. The DNS method [6] was used to measure reducing sugar concentration (C, g/L) in the neutralized solution. The content of each reducing sugar in the xylan hydrolysate (TC, g/L) was calculated using the formula:

TC = (V /V) × C × 0.9

where 0.9 is the conversion coefficient from monosaccharide to polysaccharide.

1.3 Bacterial Culture *E. coli* was purchased from the Guangdong Microbial Culture Collection Center, lactobacilli were provided by the Guangdong Academy of Agricultural Sciences Institute of Animal Husbandry, and *B. subtilis* was isolated in our laboratory. Frozen bacterial stocks were retrieved from -80 °C storage, streaked onto agar plates using sterile loops, and incubated overnight at 37 °C. *E. coli* and *B. subtilis* were cultured on LB agar, while lactobacilli were cultured on MRS agar. Well-isolated single colonies were selected and inoculated into liquid medium for expansion. *E. coli* and *B. subtilis* were cultured overnight at 37 °C with shaking at 170 rpm, while lactobacilli were cultured statically overnight at 37 °C.

To determine the effects of xylan hydrolysates on bacterial proliferation, bacterial cultures were inoculated into fresh liquid medium at 0.5% inoculum. The five experimental groups received corresponding xylan hydrolysates at a final concentration of 0.5% (m/V), while the control group received an equal volume of PBS. Cultures were incubated at 37 °C. The optimal wavelength for each bacterium was determined using a full-wavelength microplate reader, and optical density (OD) values were measured at different time points using medium as blank control.

1.4 Chicken Embryo Intestinal Epithelial Cell (IEC) Culture Intestinal tissue was isolated from 18-day-old chicken embryos, digested with 0.1% type II collagenase for 50 minutes, filtered, and centrifuged to collect cells, following the procedure described in reference [15]. Cryopreserved cells were revived in complete medium consisting of 10% serum, 0.5% penicillin-streptomycin, and low-glucose DMEM (1 g/mL glucose). Cells were seeded in 25 cm^2 flasks and cultured at 37 °C with 5% CO . Upon reaching 80% confluence, cells were passaged. For adhesion assays, cell suspensions were seeded in 6-well plates at 1 × 10 cells/mL (3 mL per well). After cells attached and formed a confluent monolayer, the medium was removed and cells were washed three times with DMEM before treatment.

1.5 Adhesion Assay *E. coli* was cultured in LB medium with shaking until reaching the desired concentration, then centrifuged at 6,000 rpm for 10 minutes. The supernatant was discarded, and bacterial pellets were washed three times with sterile PBS, followed by centrifugation. Finally, bacterial concentration was adjusted to 1 × 10 CFU/mL using sterile DMEM medium. Washed IEC monolayers in 6-well plates were treated with 1 mL of bacterial suspension mixed with corresponding xylan hydrolysates at a final concentration of 0.5% (m/V). The control group received bacterial suspension without hydrolysate. After 45 minutes of incubation, cells were washed three times with PBS to remove non-adherent bacteria. Cells were then lysed with 0.2 mL PBS containing 1% Triton X-100 for 10 minutes, followed by addition of 1.3 mL PBS and thorough mixing. The suspension from each well was collected, diluted 100-fold with PBS, plated, and incubated at 37 °C for 48 hours before colony counting. Each sample was analyzed in duplicate. The adhesion rate was calculated using the formula:

Adhesion rate (%) = 100 × N f / N f

where N f is the viable bacterial count after adhesion (CFU/mL) and N f is the viable bacterial count before adhesion (CFU/mL).

1.6 Statistical Analysis Data were analyzed using one-way ANOVA in SPSS 20.0 software, followed by Duncan' s multiple comparison test. Results are expressed as mean ± standard error (SE). Differences were considered significant at $P < 0.05$.

Results

2.1 Effects of Different Xylanase Sources and Combinations on Hydrolysate Composition As shown in Table 1 , all hydrolysates were predominantly composed of xylobiose and xylotriose. The total content of xylobiose and xylotriose in the xylanase A, xylanase B, combined enzyme 1, combined enzyme 2, and combined enzyme 3 groups were 95.70%, 86.79%, 93.11%, 94.55%, and 87.55%, respectively. Xylobiose contents were 50.08%, 42.64%, 44.60%, 44.21%, and 40.88%, while xylotriose contents were 45.62%, 44.15%, 48.51%, 50.34%, and 46.67%, respectively. Xylanase A produced the highest xylobiose content, whereas combined enzyme 2 yielded the highest xylotriose content. Notably, all combined enzyme groups showed higher xylotriose contents than single enzyme groups.

2.2 Effects of Xylan Hydrolysates on Bacterial Proliferation The effects of xylan hydrolysates on bacterial growth, measured by OD values, are presented in Table 2 . In the *E. coli* proliferation assay, OD values in all treatment groups except xylanase A were significantly higher than the control at 5 hours ($P < 0.05$). At 8 hours, all treatment groups showed significantly higher OD values than the control ($P < 0.05$). However, after 20 hours of culture, no significant differences were observed among groups ($P > 0.05$).

In the *B. subtilis* proliferation assay, all treatment groups exhibited significantly lower OD values than the control at 7 hours ($P < 0.05$). At 24 and 30 hours, all treatment groups showed significantly higher OD values than the control ($P < 0.05$), with combined enzyme 2 demonstrating significantly higher OD values than other treatment groups at 30 hours ($P < 0.05$).

In the lactobacilli proliferation assay, the control, xylanase A, xylanase B, and combined enzyme 2 groups showed significantly higher OD values than combined enzyme 1 and combined enzyme 3 groups at 3 hours ($P < 0.05$). At 13 and 17 hours, all treatment groups displayed significantly higher OD values than the control ($P < 0.05$).

2.3 Effects of Xylan Hydrolysates on *E. coli* Adhesion to IEC As shown in Table 3 , all treatment groups containing xylan hydrolysates exhibited

significantly reduced *E. coli* adhesion rates to IEC compared to the control group ($P < 0.05$). However, no significant differences were observed among the treatment groups themselves ($P > 0.05$).

Discussion

3.1 Effects on Hydrolysate Composition Xylanases expressed by different microbial sources possess distinct enzymatic properties, including differences in optimal pH, isoelectric point, and Michaelis constant [16-18], as well as variations in enzyme binding sites and cleavage sites. Meagher et al. [19] reported that endo-xylanase from *Aspergillus niger* has eight binding sites, while Vršanská et al. [20] found that acidic xylanase from *A. niger* has seven subsites. Biely et al. [21] discovered that endo-xylanase from *Cryptococcus* has four subsites related to substrate binding. The specificity of some enzyme cleavage sites is also associated with the branched structure of xylan, as these substituents may facilitate positioning of the catalytic groups [22]. For instance, two xylanases from *A. niger* did not produce arabinose in their reaction products and showed minimal or no activity on xylo-oligosaccharides with removed arabinose residues, indicating that their active sites require the presence of neighboring arabinose residues [23]. Consequently, xylanases from different sources produce hydrolysates with varying compositions.

This study investigated how xylanases from different microbial sources and their combinations affect xylan hydrolysate composition. The results revealed that both single enzymes produced hydrolysates predominantly composed of xylobiose and xylotriose, consistent with findings by Shi et al. [24]. Combined enzyme groups showed higher xylotriose contents than single enzyme groups, with combined enzyme 2 achieving the highest xylotriose content at 50.34%, representing increases of 5.27% and 6.09% compared to xylanase A and xylanase B groups, respectively. However, all three combined enzyme groups showed lower xylobiose contents than xylanase A alone. Xylanase A produced the highest total XOS yield, followed by combined enzyme 1. These results likely arise from different cleavage site specificities of the two single enzymes, which may mutually influence substrate transformation and final product composition when acting simultaneously. These findings provide guidance for producing XOS with specific component profiles: xylanase A is preferable for obtaining more xylobiose, while a 1:1 combination of xylanase A and xylanase B is optimal for maximizing xylotriose production.

3.2 Effects on Bacterial Proliferation Among various bifidogenic factors, xylo-oligosaccharides have attracted considerable attention due to their low caloric value, resistance to digestion, and high selectivity for *Bifidobacterium* proliferation. Current XOS production methods include microwave treatment, acid hydrolysis, and enzymatic hydrolysis, with enzymatic hydrolysis being most widely applied due to its low cost, safety, and high efficiency [25]. Previous

reports on enzymatic XOS production have utilized single enzymes, with no studies examining the effects of different microbial xylanases on hydrolysate composition.

Xylanase hydrolysis produces XOS with various degrees of polymerization, with only XOS ranging from 2 to 7 showing prebiotic effects [26]. However, few studies have investigated the biological activity of individual XOS components, primarily because their physicochemical properties are extremely similar except for minor differences in molecular weight, making effective separation challenging [6]. Current research indicates that XOS can effectively promote the proliferation of *Bifidobacterium adolescentis*, *B. infantis*, and *B. longum* [27], while harmful intestinal bacteria utilize XOS poorly.

This study found that xylan hydrolysates promoted *E. coli* proliferation at 5 and 8 hours, but showed no significant difference from the control after 20 hours. In contrast, Ma [12] reported that xylobiose and xylotriose did not promote *E. coli* or *Salmonella* proliferation. The hydrolysates significantly promoted lactobacilli and *B. subtilis* proliferation, with the extent of promotion varying according to hydrolysate composition. Xylanase A showed the best effect on lactobacilli proliferation, while the 1:1 combination of xylanase A and xylanase B was most effective for *B. subtilis*. Considering that xylanase A produced the most xylobiose and combined enzyme 2 produced the most xylotriose, xylobiose may be the optimal carbon source for the lactobacilli strain used in this study, while xylotriose may be optimal for *B. subtilis*, consistent with Ma's [12] findings.

3.3 Effects on *E. coli* Adhesion to IEC For bacteria to function in the intestine, they must successfully colonize the gut, which protects them from elimination by intestinal peristalsis. Microbial adhesion to host cells represents the first step of colonization [28]. Proteins on pathogen surfaces that bind to specific oligosaccharide ligands on host cells are often called adhesins, lectins, or hemagglutinins. Most adhesins can bind to specific oligosaccharide fragments containing 3–5 monosaccharides [29]. Studies have demonstrated that milk-derived oligosaccharides can inhibit pathogen colonization in the intestine [30]. The functional groups believed to be responsible include sialic acid, N-acetylglucosamine, L-fucose, and galactose [31], with acetylated mannose and glucosamine being primary substrates for sialic acid synthesis [32]. For example, chitosan oligosaccharides contain N-acetylglucosamine, a component of intestinal mucin, and can serve as adhesion receptors for pathogens [33-34], binding to bacterial adhesins and thereby inhibiting pathogen colonization and facilitating their elimination through intestinal peristalsis. Although most pathogens possess multiple adhesins capable of recognizing various oligosaccharide fragments, using single oligosaccharide receptor analogues in combination with the host' s clearance mechanisms can sufficiently affect pathogen colonization [28].

Ebersbach et al. [14] reported that xylo-oligosaccharides reduced *Listeria* adhesion to Caco-2 cells and significantly decreased expression of *Listeria* adhesins InlA and Lap, which may explain why guinea pigs fed xylo-oligosaccharides

showed reduced severity of *Listeria* infection [35]. Xylo-oligosaccharides may inhibit *Listeria* adhesion to intestinal epithelium and/or alter bacterial surface structures to reduce adhesive capacity. Additionally, *Bifidobacterium* proliferated by XOS can cooperate with other intestinal flora to promote intestinal peristalsis and reduce opportunities for pathogen attachment by competing for nutrients and adhesion sites on the intestinal epithelium [36]. This study found that xylan hydrolysates similarly reduced *E. coli* adhesion to cells by up to 27.27%, with no significant differences among treatment groups. Ma [12] reported that xylotriose showed significantly higher inhibition of *E. coli* adhesion to epithelial cells than xylobiose. This trend was also observed in our study, as all three combined enzyme groups had higher xylotriose contents than single enzyme groups and showed numerically lower *E. coli* adhesion rates to IEC.

Conclusion

1. The primary products of xylan hydrolysis by different xylanase sources and their combinations are xylobiose and xylotriose. Xylanase A produces the most xylobiose, while a 1:1 combination of xylanase A and xylanase B yields the most xylotriose.
2. All xylan hydrolysates obtained from different xylanase sources and their combinations promote the proliferation of *B. subtilis* and lactobacilli. The 1:1 combination of xylanase A and xylanase B shows the best promotion effect on *B. subtilis* proliferation, while xylanase A alone is most effective for lactobacilli proliferation. None of the hydrolysates promote *E. coli* proliferation at later culture stages.
3. All xylan hydrolysates significantly inhibit *E. coli* adhesion to IEC, though no significant differences exist among the various hydrolysates in this inhibitory effect.

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