Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

One-step fermentation for producing xylo-oligosaccharides from wheat bran by recombinant *Escherichia coli* containing an alkaline xylanase

Jiawen Liu
Jiangsu Normal University
Cong Liu
Jiangsu Normal University
Shilei Qiao
Jiangsu Normal University
Zhen Dong
Jiangsu Normal University
Di Sun
Jiangsu Normal University
Jingrong Zhu
Jiangsu Normal University
Weijie Liu (leonliu2013@126.com)
Jiangsu Normal University https://orcid.org/0000-0002-7152-6013

Research Article

Keywords: prebiotics, Bacillus agaradhaerens, single-step fermentation, xylanase, response surface optimization

Posted Date: September 7th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-871631/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Version of Record: A version of this preprint was published at BMC Biotechnology on February 5th, 2022.
See the published version at https://doi.org/10.1186/s12896-022-00736-8.

Abstract

One-step fermentation is a cheap way to produce xylo-oligosaccharides (XOS), where production of xylanases and XOS is integrated into a single process. In spite of having cost advantage, one-step fermentation is still short in yield so far due to the limited exploration. To cope with this issue, production of XOS from wheat bran by recombinant *Escherichia coli* through one-step fermentation was investigated here. A xylanase gene belonging to glycoside hydrolase family 11 of *Bacillus agaradhaerens* was employed to construct recombinant *E. coli*. This xylanase showed maximal activity at 60°C and pH 8.0. Its activity retained more than 60% after incubation at 70°C for 4 hours, showing a good stability. The recombinant *E. coli* successfully secreted xylanases that directly hydrolyzed wheat bran to XOS in fermentation medium. The generated XOS consisted of xylose, xylobiose and xylotriose accounting for 23.1%, 37.3% and 39.6%, respectively. Wheat bran concentration was found to be the most crucial factor affecting XOS production. The yield reached 5.3 mg/mL at 10% of wheat bran, which is higher than previous reports employing one-step fermentation. Nitrogen source type could also affect XOS yield by changing extracellular xylanase activity, and glycine was found to be the best one for fermentation. Optimal fermentation conditions were finally studied by response surface optimization. The maximal yield emerged at 44.3°C, pH 7.98, which is affected by characteristics of the xylanase and growth conditions of *E. coli*. This work indicates that the integrated fermentation using recombinant *E. coli* is highly competitive in cost and yield for production of XOS.

1. Introduction

Prebiotics, which are mainly various oligosaccharides, can specifically promote the activity of beneficial bacteria in gastrointestinal tract (Gibson et al., 2010). With insight into the effect of gut microbiota on human overall health, prebiotics have been the hotspot in consumption and research currently (Davani-Davari et al., 2019). Xylo-oligosaccharides (XOS) are emerging probiotics which consist of several β-1,4 linked xylose units(Aachary and Prapulla, 2011). They have been attached importance to recent years due to remarkable prospect of application in food, medicine, poultry and other fields (Cordero et al., 2019; Jia et al., 2020; Wu et al., 2020). Furthermore, XOS are more efficient than other prebiotics in enhancing growth of certain bifidobacteria and in protecting lactobacilli under stress environments (Pan et al., 2009; Makelainen et al., 2010; de Figueiredo et al., 2020). XOS also present good heat and pH stability, which is beneficial to retaining more nutritional properties in digestive tract (Courtin et al., 2009). Given these advantages, market demand for XOS is rising quickly and expected to reach 130 million U.S. dollar in 2023 at an annual growth of 5.3% (Amorim et al., 2019a).

Enzymatic hydrolysis is one of the major methods to produce XOS, which is more environmentally friendly and generates less undesired by-products than chemical hydrolysis (Santibanez et al., 2021). Xylanases are the critical factor for enzymatic production of XOS, which act on backbone of xylan and convert it into XOS as well as xylose. Xylanases belonging to glycoside hydrolase (GH) family 11 attack unsubstituted sites of xylan, whose hydrolysate mainly consists of xylobiose and xylotriose; GH10 xylanases can accommodate a decorated xylopyranosyl residue at -1 subsite, resulting in production of

both linear and substituted XOS with low degree of polymerization (DP); GH30 xylanases prefer branched xylan than the linear one so substituted XOS are their principal products (Karlsson et al., 2018; Madan and Lee, 2018). Hydrolyzing extracted xylan or raw lignocellulosic biomass using these xylanases to produce XOS has been widely reported, and XOS yields are very attractive in some works (Ma et al., 2017; Rajagopalana et al., 2017). However, preparation of these purified enzymes is unwieldy and costly. In addition, high temperature is commonly needed for an efficient enzymatic hydrolysis, which also prejudices the cost of production process (Kumar and Satyanarayana, 2015; Liu et al., 2018). To cope with these issues, some researches devote to integrating production of xylanases and XOS into a single process. In such one-step fermentation, microorganisms extracellularly secrete xylanases and meanwhile, these enzymes directly convert xylan or lignocellulosic biomass into XOS in medium. For example, a wild-type *Bacillus subtilis* was reported to produce XOS by direct fermentation utilizing brewers' spent grain, and XOS yield further increased when *B. subtilis* was genetically modified (Amorim et al., 2018). Some fungi, such as *Trichoderma reesei* and *Aspergillus nidulans*, exhibited XOS-production potential in one-step fermentation as well (Menezes et al., 2018; Amorim et al., 2019b). These integrated productions of XOS leave out separate process for enzyme expression as well as purification, and generally adopt mild conditions, which contribute to overcoming cost challenge limiting wide adoption of XOS (Amorim et al., 2019a). Nevertheless, XOS yields of one-step fermentation are commonly disadvantaged comparing with enzymatic hydrolysis. Indeed, yields can be improved by optimization of medium, substrates, fermentation microorganisms and conditions, but researches about these issues are scarce. For example, only a few bacillus and fungi are employed for one-step fermentation so far. These microorganisms, however, can all utilize XOS as carbon source, which would prejudice the accumulation of XOS. In addition, critical restriction limiting XOS yield remains unknown for such integrated fermentation, which leads to difficulty in effective optimization.

Escherichia coli has been used to produce food additives and drugs for decades, which has proved to be safe and reliable (Huang et al., 2012; Xu et al., 2020). *E. coli* is probably suited to fermentation for producing XOS because it cannot consume this kind of oligosaccharides (Falck et al., 2013). However, feasibility of one-step fermentation employing *E. coli* lacks sufficient study. Wheat bran is a xylan-rich by-product of white flour milling and has been used as cheap raw materials for XOS production previously (Bhardwaj et al., 2019; Wang et al., 2020). *Bacillus agaradhaerens* C9 is alkaliphilic strain with lignocellulose-degrading ability. Secretion of alkali-tolerant xylanases by *B. agaradhaerens* C9 was verified in our previous work (Liu et al., 2017). Bioinformatics analysis of its genome identified two xylanases, one of which belongs to GH 11 and was named *Ba*xyl11. In this study, *Ba*xyl11 was expressed using *E. coli* BL21 and enzymatic characteristics of recombinant *Ba*xyl11 (r*Ba*xyl11) were then investigated. Moreover, producing XOS from wheat bran by the recombinant *E. coli* BL21 containing r*Ba*xyl11 was carried out. Effects of wheat bran concentration, nitrogen source type and fermentation conditions on XOS yield were finally investigated. These results would contribute to overcoming yield and cost challenge in the production of XOS, and promote its wide adoption.

2. Results

2.1 Enzymatic characteristics of r*Ba*xyl11

The xylanase gene was cloned from genomic DNA of *B. agaradhaerens* C9 and ligated with plasmid pET22b(+). r*Ba*xyl11 was then expressed using *E. coli* BL21. The purified r*Ba*xyl11 showed electrophoretic homogeneity and its molecular weight corresponded to the calculated value of 28.9 kD (Fig. 1A). r*Ba*xyl11 presented hydrolytic activities to xylan but not to cellulose, mannan, starch and 4-nitrophenyl-beta-D-xylopyranoside (pNPX), which demonstrated that r*Ba*xyl11 is an endoxylanase.

To evaluate its catalytic activities, kinetic parameters of r*Ba*xyl11 against arabinoxylan and glucuronoxylan were measured (Table 1). V_{max} and K_{cat} against arabinoxylan were approximately two times as high as those against glucuronoxylan, showing higher activity for arabinoxylan. However, lower K_m against glucuronoxylan indicated the preference for such polysaccharide than arabinoxylan, suggesting that arabinofuranosyl side chains interfere with the interaction between r*Ba*xyl11 and substrate. As a result, the K_{cat}/K_m of r*Ba*xyl11 for glucuronoxylan was higher than that for arabinoxylan.

Table 1
Kinetic parameters of r*Ba*xyl11 for xylans

Substrate	V_{max} (μM/s)	K_{cat} (/s)	K_m (g/L)	K_{cat}/K_m (L/g/s)
Arabinoxylan	44.2 ± 3.7	599.0 ± 49.7	10.9 ± 0.9	55.0 ± 0.3
Glucuronoxylan	24. 3 ± 0.6	330.1 ± 7.7	4.1 ± 0.1	79.7 ± 1.2
Concentration of r*Ba*xyl11 was 220 nM for measurement. All data are presented as mean ± standard deviation (n = 3).				

To investigate the optimal conditions for catalysis, activities of r*Ba*xyl11 were measured at different temperatures and pH values (Fig. 2A and Fig. 2B). r*Ba*xyl11 showed highest activity at 60°C and its optimal pH ranged from 8.0 to 9.0, indicating it is an alkaline xylanase. Stability of r*Ba*xyl11 was then studied (Fig. 2C and Fig. 2D). Activity of r*Ba*xyl11 retained more than 80% after incubating at 70°C for 30 min, and even after incubation of 240 min, 60% of the enzyme activity could be maintained. Moreover, r*Ba*xyl11 showed good stability when incubated at the pH range of 5.0 to 9.0, which is commonly the appropriate pH environment for fermentation.

2.2 One-step fermentation for XOS production

To save cost and simplify process, direct fermentation by r*Ba*xyl11-transformation *E. coli* BL21 to produce XOS from wheat bran was carried out. Starch in wheat bran was removed in advance for a better XOS yield. Employ of the recombinant *E. coli* BL21, isopropyl-1-thio-β-D-galactopyranoside (IPTG) and wheat bran resulted in 1.41 mg/mL of reducing sugars at the 24th hour (Fig. 3A). By contrast, fermentation without wheat bran or using *E. coli* BL21 containing empty plasmid only produced negligible reducing sugars, demonstrating that r*Ba*xyl11 was able to hydrolyze wheat bran to XOS in such one-step fermentation. Interestingly, considerable XOS were also produced even without IPTG, which

could be attributed to induction of certain saccharides from wheat bran. Further study indicated that XOS yield induced by wheat bran alone reached 80% of the maximum, and adding only a small amount of IPTG (0.02 mM) could lead to the maximal XOS yield as well as xylanase activity in the fermentation medium (Figure S1).

Xylanase activity of fermentation containing r*Ba*xyl11 and wheat bran increased rapidly in the first 6 hours and slowly then (Fig. 3B). Activity of the fermentation without IPTG showed similar trend but at lower level. It is noteworthy that use of IPTG raised xylanase activity by 40% while only increased XOS yield by 18%, suggesting that xylanase activity is not the most important factor to yield (see Sect. 2.4 for details).

2.3 Product composition of r*Ba*xyl11 acting on wheat bran

To study the product composition of r*Ba*xyl11 acting on wheat bran, its hydrolysate was analyzed using high pressure ion chromatography (HPIC). Results demonstrated that xylose, xylobiose and xylotriose are the primary product (Fig. 4A). Further quantitative analysis basing on chromatogram showed that xylose, xylobiose and xylotriose respectively accounted for 23.1%, 37.3% and 39.6% (Fig. 4B). In other words, about 77% of the product of r*Ba*xyl11 is low-DP XOS when hydrolyzing wheat bran.

2.4 Effect of wheat bran concentration on one-step fermentation

The optimal wheat bran concentration was investigated here. As showed in Fig. 5A, XOS yield increased with wheat bran concentration at a range of 0%-10%, and further raise in substrate loading would lead to an excessive viscosity of medium. At 10% of wheat bran concentration, XOS yield reached 5.3 mg/mL (excluding xylose) that is very considerable.

To study how wheat bran affects XOS yield, xylanase activity and growth of *E. coli* BL21 were also measured. Results indicated a positive linear correlation between wheat bran concentration and xylanase activity (Fig. 5B). In addition, increase of xylanase activity did not result from proliferation of *E. coli* BL21 because bacteria biomass actually reduced with xylanase activity and wheat bran loading (Fig. 5A). Nevertheless, the rise of XOS yield was not exclusively due to the change of xylanase activity. Specifically, XOS yield increased nearly 16-fold when wheat bran concentration raised 10-fold from 1–10%, and meanwhile, xylanase activity only increased by 103%. It is obvious that augmentation of yield resulted from combined effect of the increase in both wheat bran concentration and xylanase activity, where the former contributed more. In other words, substrate concentration is the decisive factor to XOS yield in such fermentation process instead of enzymatic activity.

2.5 Effect of nitrogen source type on one-step fermentation

To investigate the optimal nitrogen source, NH_4SO_4 in initial medium was replaced with equal-mass tryptone, casein acids hydrolysate, yeast extract, urea, beef extract, NH_4NO_3, $NaNO_3$ or glycine for fermentation and the medium without additional nitrogen source was used as control (Fig. 6A).

Measurement of reducing sugar indicated that maximal XOS yield was obtained when using glycine as nitrogen source, which is slightly higher than that using yeast extract (p-value = 0.065). The lowest three yields showed when NH_4NO_3, $NaNO_3$ and NH_4SO_4 were used, indicating such inorganic salts are not suited to production of XOS. In addition, *E. coli* BL21 could still grow and secrete xylanases in control group, suggesting certain components of wheat bran could be utilized as nitrogen source.

Xylanase activity and biomass of *E. coli* BL21 were then measured to study how nitrogen source type affects XOS yield. To avoid the effect of different nitrogen sources on enzymatic activity assay, medium after dialysis was also used for measurement (Fig. 6B). Results demonstrated a positive linear correlation between XOS yield and xylanase activity regardless of whether medium was treated with dialysis. By comparison, no credible correlation between XOS yield and biomass was observed. Therefore, types of nitrogen source affected XOS yield mainly by changing xylanase activity.

2.6 Optimizing XOS yields by response surface methodology

To study effect of fermentation conditions, temperature, pH and glycine concentration were chosen as variables for optimization using Box-Behnken design. After 12-hour fermentation, XOS yields varied in the range of 1.629–1.895 mg/mL (Table 2 and Fig. 7). Glycine concentration is the most influential variable with p-value = 0.0006, followed by temperature with p-value = 0.0132. It was predicted that the optimal yield of 1.904 mg/mL would be obtained at 44.3°C, pH 7.98 with 3.36% of glycine, which corresponds approximately to the central-point condition of the design. The optimal pH of fermentation corresponded to that of catalysis by r*Ba*xyl11, and the optimal temperature is higher than 37°C which is suited to the growth of *E. coli* BL21, suggesting that best conditions for fermentation were highly affected by enzymatic characteristics of r*Ba*xyl11.

Table 2
Experimental design to study the effect of pH, fermentation temperature and glycine concentration on XOS yield

Run	pH	Temperature	Glycine	Yield (mg/mL)
1	8.1	40℃	0.2%	1.629 ± 0.008
2	8.1	44℃	2.6%	1.895 ± 0.009
3	7.6	48℃	2.6%	1.780 ± 0.009
4	7.6	44℃	0.2%	1.673 ± 0.014
5	8.1	48℃	5.0%	1.721 ± 0.014
6	8.6	44℃	5.0%	1.784 ± 0.039
7	8.1	40℃	5.0%	1.721 ± 0.013
8	8.1	44℃	2.6%	1.895 ± 0.009
9	8.1	48℃	0.2%	1.709 ± 0.018
10	8.1	44℃	2.6%	1.895 ± 0.009
11	7.6	44℃	5.0%	1.862 ± 0.044
12	8.6	44℃	0.2%	1.743 ± 0.003
13	7.6	40℃	2.6%	1.703 ± 0.011
14	8.1	44℃	2.6%	1.895 ± 0.009
15	8.6	48℃	2.6%	1.714 ± 0.008
16	8.1	44℃	2.6%	1.895 ± 0.009
17	8.6	40℃	2.6%	1.685 ± 0.055
All data are presented as mean ± standard deviation (n = 3).				

3. Discussion

High cost is a challenge limiting the enzymatic production of XOS. One-step fermentation is a cost-efficient way to produce XOS, but its yield is commonly modest comparing with enzymatic hydrolysis (Table 3). For example, hydrolyzing mahogany employing a xylanase belonging to *Clostridium* resulted in a XOS yield of 4.5 mg/mL (Rajagopalana et al., 2017). The yield could even exceed 8 mg/mL when using extracted xylan as substrate (Ma et al., 2017). By comparison, only 0.8–1.1 mg/mL of XOS were obtained employing *B. subtilis* or *Trichoderma* species in one-step fermentation despite optimization (Amorim et al., 2018; Amorim et al., 2019b). A higher yield of 3.2 mg/mL showed when using wheat middlings and *B. subtilis*, but the fermentation time, 48 hours, was less competitive (Reque et al., 2019). According to this study, substrate concentration is actually the most influential factor to XOS yield while XOS producing

was prejudiced by a large loading of substrates using *B. subtilis* and fungi (Amorim et al., 2018; Amorim et al., 2019b). Being different from current researches, XOS yield using *E. coli* BL21 is positively associated with loading of wheat bran, leading to a remarkable XOS concentration of 5.3 mg/mL at 10% of wheat bran concentration after 12 hours of fermentation. Therefore, using *E. coli* BL21 is promising to eliminate the disadvantage in XOS yield of one-step fermentation. Indeed, conversion ratios of substrate could be more outstanding with smaller loading, but higher final concentration of XOS is more valuable to the industrialized application in consideration of the low cost of such substrate. In addition, wheat bran not only acted as substrate for XOS producing, but also played an important role in stimulating the secretion of r*Ba*xyl11, which is conducive to economical use of extra inducer and to saving cost.

Table 3
XOS production by enzymatic hydrolysis and one-step fermentation

Substrate	Enzyme or strain	Reaction time[a]	XOS yield (mg/mL)[b]	XOS production method	Reference
Wheat bran	Engineering *E. coli* BL21	12 hours	0.8-5.3[c]	One-step fermentation (37°C)	This study
Wheat middlings	*Bacillus subtilis*	48 hours	3.2[c]	One-step fermentation (37°C)	Reque et al., 2019
Brewers' spent grain	Engineering *Bacillus subtilis*	12 hours	1.1	One-step fermentation (45°C)	Amorim et al., 2018
Brewers' spent grain	*Trichoderma reesei*	72 hours	0.8	One-step fermentation (30°C)	Amorim et al., 2019b
Pistachio shell	Commercial xylanase	10 hours	2.7[c]	Enzymatic hydrolysis (45°C)	Hesam et al., 2021
Mahogany	Xylanase from *Clostridium* strain BOH3	24 hours	4.5[c]	Enzymatic hydrolysis (50°C)	Rajagopalana et al., 2017
Sugarcane bagasse	Xylanase from *Bacillus subtilis*	15 hours	3.6	Enzymatic hydrolysis (50°C)	Bragatto et al., 2013
Rice straw	Commercial xylanase	24 hours	0.1[c]	Enzymatic hydrolysis (50°C)	Kumar et al., 2020
Rice straw	Xylosidase from *Weissella cibaria*	10 hours	2.6[c]	Enzymatic hydrolysis (37°C)	Le and Yang, 2019
Beechwood xylan	Xylanase from *Mycothermus thermophilus*	12 hours	8.0-8.8[c]	Enzymatic hydrolysis (65°C)	Ma et al., 2017
Xylan from corn cobs	Xylanase from *Thermomyces lanuginosus*	8 hours	6.9	Enzymatic hydrolysis (45°C)	Khangwal et al., 2020

[a] Reaction time indicates the hydrolysis or fermentation time when XOS yield reaches the presented value.

[b] Xylose is not included.

[c] These data are measured using liquid chromatogram and others are measured using DNS method.

Substrate	Enzyme or strain	Reaction time[a]	XOS yield (mg/mL)[b]	XOS production method	Reference
Xylan from data seed	Xylanase from *Aspergillus niger*	4 hours	4.1[c]	Enzymatic hydrolysis (38°C)	Ataei et al., 2020
Xylan from vetiver grass	Xylanase from *Aureobasidium melanogenum*	92 hours	4.7	Enzymatic hydrolysis (28°C)	Patipong et al., 2019

[a] Reaction time indicates the hydrolysis or fermentation time when XOS yield reaches the presented value.

[b] Xylose is not included.

[c] These data are measured using liquid chromatogram and others are measured using DNS method.

Xylanase activity is another factor influencing XOS production. For example, type of nitrogen source actually affected XOS yield mainly by changing xylanase activity, and the increase of activity also contributed to the yield when optimizing wheat bran concentration. However, a huge raise in xylanase activity commonly leading to a limited increase in yield (Fig. 3 and Fig. 6A), suggesting that the mere pursuit of high activity or large loading of xylanase could be less effective than expected in large-scale production of XOS. Interestingly, the biomass of *E. coli* BL21 was very low when the optimal nitrogen source or high wheat bran concentration was employed (Fig. 5A and Fig. 6A). It is also obvious that the optimal fermentation conditions (44.3℃, pH 7.98) were more in favour of the catalysis by xylanase than bacterial growth, especially in terms of pH value. It seems that ideal condition for fermentation prejudices bacteria growth but stimulates the accumulation of heterologous proteins.

The GH11 xylanase of *B. agaradhaerens* AC13, BadX, was previously reported to hydrolyze pNPX (Poon et al., 2003). However, r*Ba*xyl11 of *B. agaradhaerens* C9 was actually an endo-β-1,4-xylanase, and was not able to act on pNPX according to our results. This difference could be attributed to the change from lysine to glutamic acid of amino acid sequence (Fig. 1B). Xylobiose and xylotriose were the main products by r*Ba*xyl11 acting on wheat bran. Such low-DP XOS commonly present better prebiotic efficacy (Ho et al., 2018; Iliev et al., 2020). Moreover, employ of r*Ba*xyl11 made alkaline environment the optimal condition for fermentation, which could prevent hemicellulose from autohydrolysis during heat sterilization thereby avoiding undesired saccharides (Yang et al., 2020). Therefore, r*Ba*xyl11 is promising to one-step fermentation for XOS production with various advantages. The best fermentation temperature is 44.3℃ that is between the optimal temperature for catalysis by r*Ba*xyl11 and growth of *E. coli*. Comparing with using r*Ba*xyl11 directly, one-step fermentation adopts mild conditions, which contributes to save energy and cost.

4. Conclusions

This work demonstrates that *E. coli* is appropriate for producing XOS with a competitive yield thereby overcoming the current weakness of one-step fermentation. The critical factor leading to the breakthrough in yield is efficient producing XOS by *E. coli* at high substrate concentration. The optimal conditions, especially pH, for fermentation are highly affected by enzymatic characteristics of the xylanase used. This work provides theoretical basis for overcoming yield and cost challenge, and contributes to the wide adoption of XOS.

5. Methods

5.1 Strains, plasmid, and substrates

B. agaradhaerens C9 was isolated from saline-alkali soil, and has been maintained in our laboratory since then (Liu et al., 2015). *E. coli* DH5α was used for gene cloning and plasmid maintenance. *E. coli* BL21(DE3) was used for gene expression as well as fermentation. pET22b(+) was used for construction of recombinant plasmid.

Arabinoxylan, glucuronoxylan and XOS with DP ranging from 2 to 6 were all purchased from Megazyme (Ireland). Wheat bran was purchased from a flour mill in Huainan city, China. Starch presenting in wheat bran was removed according to the reported method before fermentation (Xu et al., 2019). In brief, milled wheat bran was treated with amylase and papain successively. These enzymes were then denatured by boiling for 25 min. After that, wheat bran was washed three times to remove enzymes and starch. The de-starched wheat bran was finally dried and screened through 80 meshes sieve for fermentation and hydrolysis. Its xylan content increased from 28.3–59.4% after de-starched treatment, which was measured according to the method offered by National Renewable Energy Laboratory (Sluiter et al., 2010).

5.2 Heterologous expression and purification

*Ba*xyl11 gene was cloned with forward primer containing *Bam*HI restriction site (5'-CTAGGATCCGCAAATCGTCACCGACAATTCCA-3') and reverse primer containing *Xho*I restriction site (5'-CCGCTCGAGATTGTTTTTGTCCAAAGTTAT-3'). *Ba*xyl11 gene was ligated into pET22b(+) after digested by endonucleases, and then transferred into *E. coli* DH5α. The validated recombinant plasmid was finally transferred into *E. coli* BL21 for heterologous expression.

Gene expression was carried out in LB-ampicillin medium using 0.6 mM of IPTG at 37°C, 200 rpm for 6 hours. Bacterial cells were then harvested by centrifugation to remove medium, and were resuspended using Tris-HCl buffer (20 mM, pH8.0) for ultrasonication. After that, soluble cell extract containing r*Ba*xyl11 was collected by centrifuging at 4°C and was filtered through 0.45-μm filters. r*Ba*xyl11 was purified by affinity chromatography as follows: 5 mL of soluble cell extract was loaded into a Ni-NTA column that was previously equilibrated with binding buffer (20 mM Tri-HCl, 500 mM NaCl, pH 8.0). 12 mL of washing buffer (20 mM imidazole, 20 mM Tri-HCl, 500 mM NaCl, pH 8.0) and 6 mL of elution buffer (250 mM imidazole, 20 mM Tri-HCl, 500 mM NaCl, pH 8.0) were then loaded to remove undesired

proteins and to elute r*Ba*xyl11, respectively. Saline ions in eluent were removed by dialysis and r*Ba*xyl11 was finally freeze-dried for reserve.

5.3 Enzyme assay

The freeze-dried r*Ba*xyl11 was dissolved using deionized water, and protein concentration was determined according to the absorbancy at 280 nm and the extinction coefficient of r*Ba*xyl11. To measure enzymatic activity, 50 μL of diluted enzyme solution and 100 μL of substrate solution were mixed and incubated at 60°C, pH 8.0 for 20 min, and the reducing sugars were then measured with dinitrosalicylic acid (DNS) assay. Kinetic parameters were worked out using Lineweaver-Burk plot according to enzyme activities which were measured with xylan solution whose concentration ranged from 1 to 20 mg/mL. The optimal reaction conditions were investigated by determining enzymatic activities at different temperatures or pH values. To study stability, activities of r*Ba*xyl11 were measured after incubated at 70℃ for different time or incubated in buffers with different pH value for one hour.

Product composition of r*Ba*xyl11 acting on wheat bran was analyzed by HPIC with a Dionex ICS3000 system. Analytical CarboPac PA10 pellicular anion-exchange resin column (250 by 4 mm) was used for sugar separation. 25 μL of sample was eluted with 250 mM NaOH (1.0 mL/min) at 30℃ and detected by ED 3000 pulsed amperometric detector.

5.4 One-step fermentation

One-step fermentation was carried out as following method if not specifically indicated. Recombinant *E. coli* BL21 was inoculated into LB-ampicillin medium and grown at 37°C overnight. Then, appropriate amounts of cells were collected by centrifugation and further diluted to an initial OD_{600} = 1.0 into fermentation medium. After that, ampicillin and IPTG were respectively added to 50 μg/mL and 0.6 mM, and cells were cultured at 37°C and 200 rpm immediately. After 12 hours, supernatant of medium was diluted to measure extracellular xylanase activity and XOS concentration (equivalent xylose) as the method introduced in Sect. 5.3. Medium before fermentation was used as control group for measurement of reducing sugar. Each liter of fermentation medium contains: 20 g wheat bran, 4.8 g $Na_2HPO_4{\cdot}12H_2O$, 2.65 g KH_2PO_4, 4 g $(NH_4)_2SO_4$, 0.3 g $MgSO_4{\cdot}7H_2O$, 0.01 g $FeSO_4{\cdot}7H_2O$, 0.01 g $CaCl_2{\cdot}2H_2O$ and 1 mL trace element solution (0.3 g/L H_3BO_3, 0.2 g/L $CoCl_2{\cdot}6H_2O$, 30 mg/L $ZnSO_4{\cdot}7H_2O$, 30 mg/L $MnCl_2{\cdot}4H_2O$, 30 mg/L $NaMoO_4{\cdot}2H_2O$, 20 mg/L $NiCl_2{\cdot}6H_2O$ and 10 mg/L $CuSO_4{\cdot}5H_2O$). The pH value of fermentation medium was adjusted to 7.0 before sterilization.

5.5 Measurement of biomass

Growth of *E. coli* was measured by dilute plate method. 100 μL of medium after fermentation was sampled and diluted 10^5-10^7 times using NaCl solution (0.9%). 200 μL of diluted medium was spread onto a LB-ampicillin agar plate and cultured at 37°C overnight. Colony forming unit (CFU) was finally counted to evaluate the biomass of *E. coli* in medium after fermentation.

5.6 Optimization of XOS yields

Response surface methodology was employed to optimize XOS yield of one-step fermentation using Box-Behnken experimental design. Nitrogen source concentration, pH and fermentation temperature were chosen for optimization. Experimental design was provided in Table 2.

5.7 Bioinformatic and statistical analysis

Signal peptide was predicted using Signalp 4.0 server (http://www.cbs.dtu.dk/services/SignalP-4.0/). Glycoside hydrolase family was predicted using dbCAN meta server (http://bcb.unl.edu/dbCAN2/blast.php). Sequence alignment was carried out using DNAMAN v6 software package. Statistical analysis was carried out using t-test (least significant difference).

Abbreviations

CFU: colony forming unit; DNS: 3,5-dinitrosalicylic acid; DP: degree of polymerization; GH: glycoside hydrolase; HPIC: high pressure ion chromatography; IPTG: isopropyl-1-thio-β-D-galactopyranoside; pNPX: 4-nitrophenyl-beta-D-xylopyranoside; XOS: xylo-oligosaccharides.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This study is supported by The Natural Science Foundation of the Jiangsu Higher Education Institutions of China (20KJB180001; 20KJA180007); National Natural Science Foundation of China (31970036, 31900401 and 31800020), Natural Science Foundation of Jiangsu Province (BK20171163, BK20181009), Natural Science Foundation of Xuzhou city (KC19196), Six Talent Peaks Project of Jiangsu Province (JNHB-103).

Authors' contributions

JL designed the work. CL drafted the work. SQ, ZD and DS performed the experiments. JZ analyzed and interpreted the data; WL revised the manuscript. All authors read and approved the final manuscript

Acknowledgements

Not applicable

References

Aachary AA, Prapulla SG (2011) Xylooligosaccharides (XOS) as an emerging prebiotic: microbial synthesis, utilization, structural characterization, bioactive properties, and applications. *Compr Rev Food Sci Food Saf* 10:2-16.

Amorim C, Silverio SC, Prather KLJ, Rodrigues LR (2019a) From lignocellulosic residues to market: production and commercial potential of xylooligosaccharides. *Biotechnol Adv* 37:107397.

Amorim C, Silverio SC, Rodrigues LR (2019b) One-step process for producing prebiotic arabino-xylooligosaccharides from brewer's spent grain employing *Trichoderma species. Food Chem* 270:86-94.

Amorim C, Silverio SC, Silva SP, Coelho E, Coimbra MA, Prather KLJ, Rodrigues LR (2018) Single-step production of arabino-xylooligosaccharides by recombinant *Bacillus subtilis* 3610 cultivated in brewers' spent grain. *Carbohydr Polym* 199:546-554.

Ataei D, Hamidi-Esfahani Z, Ahmadi-Gavlighi H (2020) Enzymatic production of xylooligosaccharide from date (*Phoenix dactylifera* L.) seed. *Food Sci Nutr* 8:6699-6707.

Bhardwaj N, Kumar B, Agarwal K, Chaturvedi V, Verma P (2019) Purification and characterization of a thermo-acid/alkali stable xylanases from *Aspergillus oryzae* LC1 and its application in xylo-oligosaccharides production from lignocellulosic agricultural wastes. *Int J Biol Macromol* 122:1191-1202.

Bragatto J, Segato F, Squina FM (2013) Production of xylooligosaccharides (XOS) from delignified sugarcane bagasse by peroxide-HAc process using recombinant xylanase from *Bacillus subtilis. Ind Crops Prod* 51:123-129.

Cordero G, Kim JC, Whenham N, Masey-O'Neill H, Srinongkote S, Gonzalez-Ortiz G (2019) Xylanase and fermentable xylo-oligosaccharides improve performance in grower-finisher pigs fed a corn-soybean meal based diet. *J Anim Sci* 97:91-92.

Courtin CM, Swennen K, Verjans P, Delcour JA (2009) Heat and pH stability of prebiotic arabinoxylooligosaccharides, xylooligosaccharides and fructooligosaccharides. *Food Chem* 112:831-837.

Davani-Davari D, Negahdaripour M, Karimzadeh I, Seifan M, Mohkam M, Masoumi SJ, Berenjian A, Ghasemi Y (2019) Prebiotics: definition, types, sources, mechanisms, and clinical applications. *Foods* 8:92.

de Figueiredo FC, Ranke FFD, de Oliva-Neto P (2020) Evaluation of xylooligosaccharides and fructooligosaccharides on digestive enzymes hydrolysis and as a nutrient for different probiotics and *Salmonella typhimurium. LWT-Food Sci Technol* 118:108761.

Falck P, Precha-Atsawanan S, Grey C, Immerzeel P, Stalbrand H, Adlercreutz P, Karlsson EN (2013) Xylooligosaccharides from hardwood and cereal xylans produced by a thermostable xylanase as carbon sources for *Lactobacillus brevis* and *Bifidobacterium adolescentis. J Agric Food Chem* 61:7333-7340.

Gibson GR, Scott KP, Rastall RA, Tuohy KM, Hotchkiss A, Dubert-Ferrandon A, Gareau M, Murphy EF, Saulnier D, Loh G, et al (2010) Dietary prebiotics: current status and new definition. *Food Science and Technology Bulletin: Functional Foods* 7:1-19.

Hesam F, Tarzi BG, Honarvar M, Jahadi M (2021) Pistachio (*Pistacia vera*) shell as a new candidate for enzymatic production of xylooligosaccharides. *J Food Meas Charact* 15:33-45.

Ho AL, Kosik O, Lovegrove A, Charalampopoulos D, Rastall RA (2018) In vitro fermentability of xylo-oligosaccharide and xylo-polysaccharide fractions with different molecular weights by human faecal bacteria. *Carbohydr Polym* 179:50-58.

Huang CJ, Lin H, Yang XM (2012) Industrial production of recombinant therapeutics in *Escherichia coli* and its recent advancements. *J Ind Microbiol Biotechnol* 39:383-399.

Iliev I, Vasileva T, Bivolarski V, Momchilova A, Ivanova I (2020) Metabolic profiling of xylooligosaccharides by Lactobacilli. *Polymers* 12:2387.

Jia CS, Cao DD, Ji SP, Lin WT, Zhang XM, Muhoza B (2020) Whey protein isolate conjugated with xylo-oligosaccharides via maillard reaction: characterization, antioxidant capacity, and application for lycopene microencapsulation. *LWT-Food Sci Technol* 118:108837.

Karlsson EN, Schmitz E, Linares-Pasten JA, Adlercreutz P (2018) Endo-xylanases as tools for production of substituted xylooligosaccharides with prebiotic properties. *Appl Microbiol Biotechnol* 102:9081-9088.

Khangwal I, Nath S, Kango N, Shukla P (2020) Endo-xylanase induced xylooligosaccharide production from corn cobs, its structural features, and concentration-dependent antioxidant activities. *Biomass Convers Biorefin.* doi:101007/s13399-020-00997-3.

Kumar B, Bhardwaj N, Verma P (2020) Microwave assisted transition metal salt and orthophosphoric acid pretreatment systems: generation of bioethanol and xylo-oligosaccharides. *Renew Energ* 158:574-584.

Kumar V, Satyanarayana T (2015) Generation of xylooligosaccharides from microwave irradiated agroresidues using recombinant thermo-alkali-stable endoxylanase of the polyextremophilic bacterium *Bacillus halodurans* expressed in *Pichia pastoris. Bioresour Technol* 179:382-389.

Le B, Yang SH (2019) Production of prebiotic xylooligosaccharide from aqueous ammonia-pretreated rice straw by beta-xylosidase of *Weissella cibaria. J Appl Microbiol* 126:1861-1868.

Liu C, Hao Y, Jiang JH, Liu WJ (2017) Valorization of untreated rice bran towards bioflocculant using a lignocellulose-degrading strain and its use in microalgal biomass harvest. *Biotechnol Biofuels* 10:90.

Liu C, Wang K, Jiang JH, Liu WJ, Wang JY (2015) A novel bioflocculant produced by a salt-tolerant, alkaliphilic and biofilm-forming strain *Bacillus agaradhaerens* C9 and its application in harvesting *Chlorella minutissima* UTEX2341. *Biochem Eng J* 93:166-172.

Liu XQ, Liu Y, Jiang ZQ, Liu HJ, Yang SQ, Yan QJ (2018) Biochemical characterization of a novel xylanase from *Paenibacillus barengoltzii* and its application in xylooligosaccharides production from corncobs. *Food Chem* 264:310-318.

Ma R, Bai YG, Huang HQ, Luo HY, Chen SF, Fan YL, Cai L, Yao B (2017) Utility of Thermostable xylanases of *Mycothermus thermophilus* in generating prebiotic xylooligosaccharides. *J Agric Food Chem* 65:1139-1145.

Madan B, Lee SG (2018) Sequence and structural features of subsite residues in GH10 and GH11 xylanases. *Biotechnol Bioprocess Eng* 23:311-318.

Makelainen H, Forssten S, Saarinen M, Stowell J, Rautonen N, Ouwehand AC (2010) Xylo-oligosaccharides enhance the growth of bifidobacteria and Bifidobacterium lactis in a simulated colon model. *Benefic Microbes* 1:81-91.

Menezes BD, Rossi DM, Squina F, Ayub MAZ (2018) Comparative production of xylanase and the liberation of xylooligosaccharides from lignocellulosic biomass by *Aspergillus brasiliensis* BLf1 and recombinant *Aspergillus nidulans* XynC A773. *Int J Food Sci Technol* 53:2110-2118.

Pan X, Wu T, Zhang L, Cai L, Song Z (2009) Influence of oligosaccharides on the growth and tolerance capacity of Lactobacilli to simulated stress environment. *Lett Appl Microbiol* 48:362-367.

Patipong T, Lotrakul P, Padungros P, Punnapayak H, Bankeeree W, Prasongsuk S (2019) Enzymatic hydrolysis of tropical weed xylans using xylanase from *Aureobasidium melanogenum* PBUAP46 for xylooligosaccharide production. *3 Biotech* 9:56.

Poon DKY, Webster P, Withers SG, McIntosh LP (2003) Characterizing the pH-dependent stability and catalytic mechanism of the family 11 xylanase from the alkalophilic *Bacillus agaradhaerens. Carbohydr Res* 338:415-421.

Rajagopalana G, Shanmugavelu K, Yang KL (2017) Production of prebiotic-xylooligosaccharides from alkali pretreated mahogany and mango wood sawdust by using purified xylanase of *Clostridium* strain BOH3. *Carbohydr Polym* 167:158-166.

Reque PM, Pinilla CMB, Gauterio GV, Kalil SJ, Brandelli A (2019) Xylooligosaccharides production from wheat middlings bioprocessed with *Bacillus subtilis. Food Res Int* 126:108673.

Santibanez L, Henriquez C, Corro-Tejeda R, Bernal S, Armijo B, Salazar O (2021) Xylooligosaccharides from lignocellulosic biomass: a comprehensive review. *Carbohydr Polym* 251:117118.

Sluiter JB, Ruiz RO, Scarlata CJ, Sluiter AD, Templeton DW (2010) Compositional analysis of lignocellulosic feedstocks. 1. review and description of methods. *J Agric Food Chem* 58:9043-9053.

Wang RN, Yang JS, Jang JM, Liu JW, Zhang Y, Liu L, Yuan HL (2020) Efficient ferulic acid and xylo-oligosaccharides production by a novel multi-modular bifunctional xylanase/feruloyl esterase using agricultural residues as substrates. *Bioresour Technol* 297:122487.

Wu YL, Chen YN, Lu YF, Hao HL, Liu J, Huang RM (2020) Structural features, interaction with the gut microbiota and anti-tumor activity of oligosaccharides. *RSC Adv* 10:16339-16348.

Xu J, Yu H, Chen X, Liu L, Zhang W (2020) Accelerated green process of 2,5-dimethylpyrazine production from glucose by genetically modified *Escherichia coli. ACS Synth Biol* 9:2576-2587.

Xu ZS, Wang T, Zhang SS (2019) Extracellular secretion of feruloyl esterase derived from *Lactobacillus crispatus* in *Escherichia coli* and its application for ferulic acid production. *Bioresour Technol* 288:121526.

Yang ZG, Cao LM, Li Y, Zhang M, Zeng FY, Yao SQ (2020) Effect of pH on hemicellulose extraction and physicochemical characteristics of solids during hydrothermal pretreatment of eucalyptus. *Bioresources* 15:6627-6635.

Figures

Figure 1

Electrophoresis and sequence analysis of rBaxyl11. (A) Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of rBaxyl11. Line 1: soluble cell extract containing rBaxyl11; Line 2: rBaxyl11 after purification; Line 3: marker. (B) Sequence alignment of Baxyl11 and BadX. Amino acid residues belonging to signal peptide are marked with yellow background. Different amino acid residues between Baxyl11 and BadX are marked with green background.

Figure 2

Effect of temperature and pH on activity and stability of rBaxyl11. (A) Effect of temperature on activity of rBaxyl11. (B) Effect of pH on activity of rBaxyl11. (C) Effect of temperature on stability of rBaxyl11. (D) Effect of pH on stability of rBaxyl11. In Figure 2A and 2B, the maximal activity was designated as 100%. In Figure 2C and 2D, activity of enzyme without incubation was designated as 100%. Measurement at pH 5.0-8.0 and 8.0-10.5 was carried out in Na2HPO4-NaH2PO4 buffer and Na2CO3-NaHCO3 buffer, respectively. All data are presented as means ± standard deviations (n=3).

Figure 3

The time course of (A) XOS yield and (B) extracellular xylanase activity during one-step fermentation. “With IPTG”: employ of inducer (IPTG), wheat bran and recombinant E. coli containing rBaxyl11; “Without IPTG”: employ of wheat bran and recombinant E. coli containing rBaxyl11 without inducer; “Empty vector”: employ of inducer, wheat bran and recombinant E. coli containing unmodified pET22b(+); “Without wheat bran”: employ of inducer and recombinant E. coli containing rBaxyl11 without wheat bran. Wheat bran concentration: 2%. All data are presented as means ± standard deviations (n=3).

Figure 4

Product composition of rBaxyl11 acting on wheat bran analyzed by HPIC. (A) Product composition of the hydrolysis. “Standards”: mixture of xylose, xylobiose, xylotriose, xylotetraose, xylopentaose and xylohexaose with respective concentration of 2 mg/mL; “Hydrolysate”: product of rBaxyl11 hydrolyzing wheat bran; “Control”: sample of hydrolysate without employ of rBaxyl11. (B) Quantitative analysis of xylose and XOS produced by rBaxyl11.

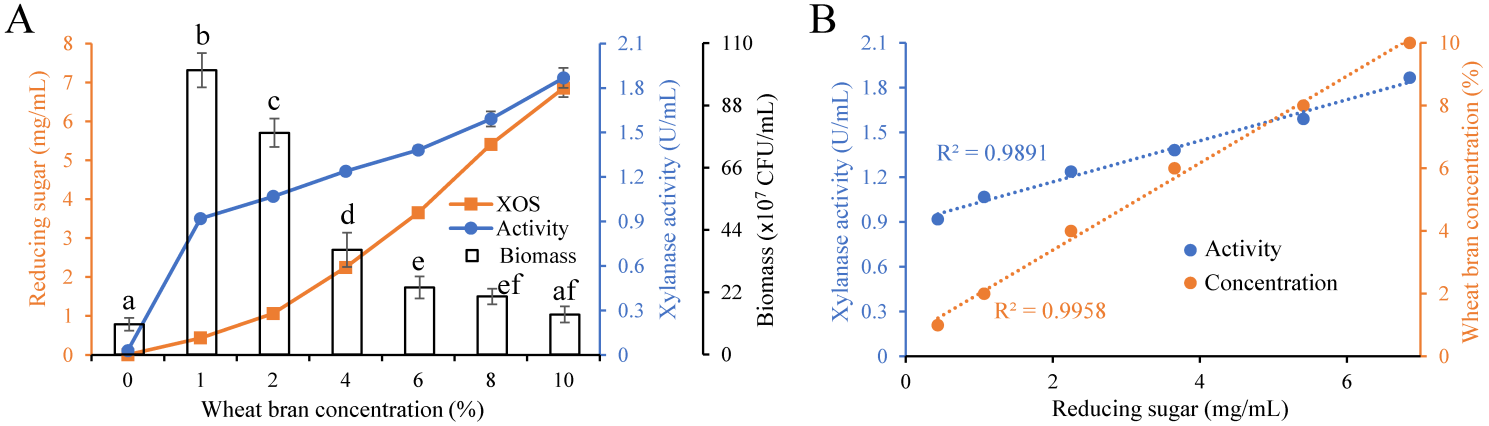

Figure 5

Effect of wheat bran concentration on one-step fermentation. (A) Effect of wheat bran concentration on XOS yield, xylanase activity and biomass of recombinant E. coli. (B) Correlation between XOS yield and xylanase activity as well as wheat bran concentration. Fermentation time: 12 hours. In Figure 5A, data are presented as means ± standard deviations (n=3).

Figure 6

Effect of nitrogen source type on one-step fermentation. (A) Effect of nitrogen source type on XOS yield, xylanase activity and biomass of recombinant E. coli. “Activity”: xylanase activity measured using supernatant of medium; “Activity after dialysis”: xylanase activity measured using dialysis-treated supernatant of medium. (B) Correlation between XOS yield and xylanase activity as well as wheat bran concentration. Wheat bran concentration: 2%. Fermentation time: 12 hours. In Figure 6A, data are presented as means ± standard deviations (n=3).

Figure 7

Response surface showing effect of temperature, pH and glycine concentration on XOS yield. (A) Effect of temperature and pH on XOS yield. (B) Effect of glycine concentration and pH on XOS yield. (C) Effect of glycine concentration and temperature on XOS yield. Wheat bran concentration: 2%. Fermentation time: 12 hours. All experiments were conducted in triplicate.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1.tif
- Graphicalabstract.jpg