Biochemical Properties of an Alkaline Xylanase From
*Bacillus Agaradhaerens* C9 and Its Application in
Producing Xylo-oligosaccharides With High Degree of
Polymerization From Wheat Bran

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Research Article

**Keywords:** xylanase, xylo-oligosaccharide, Bacillus agaradhaerens, wheat bran, single-step fermentation

**Posted Date:** February 16th, 2021

**DOI:** [https://doi.org/10.21203/rs.3.rs-201686/v1](https://doi.org/10.21203/rs.3.rs-201686/v1)

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Abstract

A xylanase of *Bacillus agaradhaerens* C9 was heterologously expressed and was then investigated. The recombinant xylanase (r*Baxyl11*) showed maximal activity at 60°C and pH 8.0-9.0. Under optimal conditions, *K*_{cat} of *rBaxyl11* for arabinoxylan and glucuronoxylan were 599 s^{-1} and 330 s^{-1}, respectively. *rBaxyl11* showed a good stability at pH ranging from 5.0 to 9.0, and retained 50% of activity after 6-hour incubation at 70°C. However, it was markedly inactivated by transition elements including Fe^{3+}, Ni^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+} and Fe^{2+}. *rBaxyl11* generated xylo-oligosaccharides (XOS) whose degree of polymerization (DP) is greater than 3 when hydrolyzing arabinoxylan, while the DP of XOS ranged from 2 to 6 when acting on glucuronoxylan. Simultaneously producing xylanase and XOS by recombinant *E. coli* containing *rBaxyl11* were then carried out. Results showed that the engineering *E. coli* generated xylanase and high-DP XOS extracellularly using wheat bran as substrate, and concentration of XOS reached 73 mg/g substrate after 12-hour fermentation. This study indicates the feasibility of producing XOS by a single-step fermentation approach with low cost using *rBaxyl11*.

1. Introduction

Xylo-oligosaccharide (XOS), which is composed of several β-1,4 linked xylose units, presents increasing commercial value because of great potential for application in many fields. The role of XOS as prebiotic food is the most known, which supports growth of beneficial intestinal microorganisms and stimulates the production of metabolites with helpful effect on health (1). Moreover, XOS improves growth performance of livestock or poultry in breeding industry (2). Additionally, XOS can also be made into microcapsules to load drugs (3). With various functions, market demand for XOS has increased in recent years. For example, the annual growth rate of global prebiotics market is predicted to exceed 10%, and it is expected to reach 7.37 billion USD by 2023 (4).

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 (5). Xylanases are essential to enzymatic production of XOS and application of those from glycoside hydrolase (GH) family 10, 11 and 30 has been extensively reported (6). However, most xylanases generate XOS with wide range of degree of polymerization (DP) as well as xylose, resulting in the low content of target product and the difficulty to purification. Utilization of xylanases with higher specificities would improve yield or proportion of desired XOS, and benefit to the downstream process. Moreover, XOS with low DP such as xylobiose and xylotriose are principal product of most reported xylanases (7–9). Medium-chain and long-chain XOS, however, are of specific functions that short-chain XOS do not possess. For example, XOS mixture with high DP is regarded as distally fermentable substrates of caecal and colonic microbiota (10). Therefore, xylanases producing high-DP XOS deserve concerns.

High cost is a severe challenge to enzymatic production of XOS. To cope with this issue, many researchers seek for utilization of cheap raw materials, or employ of efficient enzymes to save fermentation time (11, 12). Another remarkable method is process simplification. For instance, a wild-type *Bacillus subtilis* was employed to produce XOS by direct fermentation of brewers’ spent grain, and yield of XOS further increased when *B. subtilis* was genetically modified (13). *Trichoderma reesei* also presented XOS production potential in one-
step process (14). A recombinant Escherichia coli containing bifunctional xylanase/feruloyl esterase was reported to produce XOS and ferulic acid simultaneously through direct fermentation of wheat bran (15). Although a few efforts have been devoted to develop single-step fermentation, reports are still limited. In addition, efficiency of XOS production in such integrated process need to be further improved.

Bacillus agaradhaerens C9 is alkaliophilic strain with lignocellulose-degrading ability. Secretion of alkali-tolerant xylanase by B. agaradhaerens C9 was verified in our previous work (16). Bioinformatics analysis of its genome identified two xylanases, one of which belongs to GH 11 and was named Baxyl11. Although the catalytic mechanism for 4-nitrophenyl-beta-D-xylopyranoside (pNPX) of Baxyl11 have been previously reported, its enzymatic characteristics of hydrolyzing xylan are still unknown (17). In this study, Baxyl11 was heterogeneously expressed in E. coli and enzymatic characteristics of recombinant Baxyl11 (rBaxyl11) were investigated. Also, employ of the recombinant E. coli to produce XOS from wheat bran though single-step fermentation was carried out to assess the potential of saving cost and process.

2. Materials And Methods

2.1 Strains, plasmid, and substrates

B. agaradhaerens C9 was isolated from saline-alkali soil, and has been maintained in our laboratory since then. E. coli DH5α was used for gene cloning and plasmid maintenance. E. coli Rosetta(DE3) was used for gene expression. pET22b(+) was used for construction of recombinant plasmid. Arabinoxylan, glucuronoxylan, linear xylan and XOS with DP ranging from 2 to 6 were used as substrates of xylanase, and 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 before hydrolysis as follows: 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 several times to remove enzyme and starch. The de-starched wheat bran was finally dried and screened through 80 meshes sieve for hydrolysis. The xylan content of wheat bran increased from 28.3–59.4% after de-starched treatment, which were determined based on the method offered by National Renewable Energy Laboratory (18).

2.2 Heterologous expression and purification

Baxyl11 gene was cloned with forward primer containing BamH I restriction site (5'-CTAGGATCCGCAAATCGTCACCGACAATTCCA-3') and reverse primer containing Xho I restriction site (5'-CCGCTCGAGATTGTTTTTGTCCAAAGTTAT-3'). After digested by endonucleases, Baxyl11 gene was ligated to pET22b(+), and then transformed into E. coli DH5α. The validated recombinant plasmid was finally transformed into E. coli Rosetta(DE3) for heterologous expression.

Gene expression was induced in LB-ampicillin medium using 0.6 mM of isopropyl-1-thio-β-D-galactopyranoside at 37°C, 200 rpm for 3 hours. Bacterial cells were then harvested by centrifuging to remove medium, and were resuspended using Tris-HCl buffer (20 mM, pH8.0) for ultrasonication. After that, soluble cell extracts containing rBaxyl11 were collected by centrifuging at 4°C and were filtered with 0.45-μm filters. rBaxyl11 was purified by affinity chromatography as follows: 5 mL of soluble cell extracts were loaded into a
Ni\textsuperscript{2+} His-tag 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 unobjective proteins and to elute \textit{rBa}xyl11, respectively. Saline ions in eluent was removed through dialysis and \textit{rBa}xyl11 was finally freeze-dried for reserve.

2.3 Enzyme assay

The freeze-dried \textit{rBa}xyl11 was dissolved with deionized water, and protein concentration was determined according to the absorbancy at 280 nm and the extinction coefficients of \textit{rBa}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 5 min except specifically indicated, and the reducing sugars were then determined by dinitrosalicylic acid assay. Kinetic parameters were worked out using Lineweaver-Burk plot based on enzyme activities 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 \textit{rBa}xyl11 was measured after incubated at 70°C for different time or incubated in buffer with different pH values for one hour. Fe\textsuperscript{3+}, K\textsuperscript{+}, Ni\textsuperscript{2+}, Mn\textsuperscript{2+}, Ca\textsuperscript{2+}, Mg\textsuperscript{2+}, Co\textsuperscript{2+}, Zn\textsuperscript{2+}, Cu\textsuperscript{2+}, Fe\textsuperscript{2+}, SDS and EDTA were respectively added to substrate solution in advance to determine the effect of metal ions and chemical reagents on enzymatic activity.

2.4 Thin layer chromatography

\textit{rBa}xyl11 and substrate were mixed and incubated. Subsequently, 8 µL of mixture was spotted onto a silica gel plate (Merk, Germany) and developed in a n-butanol-acetic acid-water (2:1:1, v/v/v) solvent system. The silica gel plate was then immersed in a solution containing methanol and sulfuric acid (4:1, v/v) for 20 s, followed by heating at 105°C for 10 min to detect XOS produced by \textit{rBa}xyl11.

2.5 Integrated fermentation of XOS

250 µL of recombinant \textit{E. coli} Rosetta(DE3) seeds was inoculated to 25 mL of LB-ampicillin medium containing 0.5 g of de-starched wheat bran, and was cultivated at 37°C for 2.5 hours in a shaker at 200 rpm. Expression of \textit{rBa}xyl11 was then induced by 0.6 mM of isopropyl-1-thio-β-D-galactopyranoside under the same condition for fermentation. 100 µL of medium was sampled and diluted to measure extracellular xylanase activity and XOS concentration during this process. \textit{E. coli} Rosetta(DE3) containing empty vector was used as control group.

2.6 Bioinformatic 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.

3. Results And Discussion

3.1 Biochemical properties of \textit{rBa}xyl11
The Baxyl11 gene was successfully expressed in E. coli. Purified rBaxyl11 showed electrophoretic homogeneity and the molecular weight is consistent with calculated value of 28.9 kDa (Fig. 1a). rBaxyl11 displayed hydrolytic activities for both linear and branched xylans but not for cellulose, mannan, starch and pNPX, which demonstrates that rBaxyl11 is an endo-xylanase. To evaluate its catalytic activities, kinetic parameters of rBaxyl11 against arabinoxylan and glucuronoxylan were determined (Table 1). $V_{\text{max}}$ and $K_{\text{cat}}$ against arabinoxylan were approximately two times as high as those against glucuronoxylan, showing higher activity for arabinoxylan. However, lower $K_{\text{m}}$ against glucuronoxylan indicated the preference for such polysaccharide than arabinoxylan, suggesting that arabinofuranosyl side chains interfere with the interaction between rBaxyl11 and substrate. As a result, the $K_{\text{cat}}/K_{\text{m}}$ of rBaxyl11 towards glucuronoxylan is higher than that towards arabinoxylan.

### Table 1

| Substrate        | $V_{\text{max}}$ (µM/s) | $K_{\text{cat}}$ (µ/s) | $K_{\text{m}}$ (g/L) | $K_{\text{cat}}/K_{\text{m}}$ (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 rBaxyl11 was 220 nM for determination. Data reflect the mean ± standard deviation (n = 3).

To investigate the optimal condition for catalysis, activities of rBaxyl11 were determined at different temperatures and pH values (Fig. 2a and Fig. 2b). rBaxyl11 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 rBaxyl11 was then studied (Fig. 2c and Fig. 2d). Activity of rBaxyl11 retained more than 80% when incubating at 70°C for 30 min, and more than 60% after incubation for 240 min. rBaxyl11 showed good stability when incubated at the pH range of 5.0 to 9.0, but was inactivated when pH value further increased.

The GH11 xylanase of *B. agaradhaerens* was reported to hydrolyze pNPX with optimal pH of 5.6 (17). rBaxyl11 of *B. agaradhaerens* C9, however, was not able to act on pNPX in this study, which can be attributed to subtle difference in amino acid sequence and catalytic sites (Fig. 1b). rBaxyl11 showed optimal activity in alkaline environment when hydrolyzing xylan. This characteristic gives rBaxyl11 unique advantages in treatment of biomass pretreated with alkali, because the substrate need not wash to neutral. The good stability is also beneficial to the application of rBaxyl11.

### 3.2 Effect of metal ions and chemical reagents on activity of rBaxyl11

Effects of common metal ions, ethylene diamine tetraacetic acid (EDTA) and sodium dodecyl sulfate (SDS) on the catalytic activity for arabinoxylan and glucuronoxylan were investigated (Fig. 3). All the tested transition elements (Fe$^{3+}$, Ni$^{2+}$, Mn$^{2+}$, Co$^{2+}$, Zn$^{2+}$, Cu$^{2+}$ and Fe$^{2+}$) markedly reduced activity of rBaxyl11. Inactivation of xylanases caused by metal ions was widely reported and the mechanism can be interpreted as occupying the binding or catalytic sites of enzymes (19–22). By contrast, alkaline-earth metal ions, including
K\(^+\), Ca\(^{2+}\) and Mg\(^{2+}\), had weaker influence on rBaxyl11, and their effects were related to substrate type. For instance, Mg\(^{2+}\) stimulated the hydrolysis of arabinoxylan, but did not increase the activity of rBaxyl11 for glucuronoxylan. Such substrate-dependent effect suggested that these alkaline-earth metal ions may influence the interaction between rBaxyl11 and substrates with specific structure. Effects of tested chemical reagents were similar to that of alkaline-earth metal ions. Specifically, both EDTA and SDS positively affected enzymatic activity for arabinoxylan while inhibited that for glucuronoxylan. Inactivation caused by EDTA was weak, indicating rBaxyl11 did not rely on metal ions to catalyze.

3.3 Hydrolytic modes of rBaxyl11

To investigate hydrolytic modes of rBaxyl11, its products were determined using thin layer chromatography. rBaxyl11 did not hydrolyze xylo-oligosaccharides whose DP is less than five, and could converted xylopentaose and xylohexaose into xylotriose and xylohexaose as primary products (Fig. 4a). This result indicated rBaxyl11 contains five xylose-binding subsites for catalysis. When hydrolyzing glucuronoxylan, rBaxyl11 generated XOS with DP ≥ 3 at initial stage, and xylobiose was later generated after further incubation (Fig. 4b). When arabinoxylan was used as substrate, rBaxyl11 produced XOS with DP ≥ 4 in the whole stage (Fig. 4c). Moreover, migration rates of certain oligosaccharide products ranged between two XOS standards, which were probably arabinoxylan-oligosaccharides (AXOS), namely XOS containing arabinosyl side chains. Despite the same backbone of substrates, XOS profiles produced by rBaxyl11 were different when hydrolyzing xylohexaose, glucuronoxylan and arabinoxylan, indicating structure and type of side chains would influence its hydrolytic mode.

Hydrolysis products of xylanases generally contained low-DP XOS such as xylobiose and xylotriose (Table 2). An exception is a xylanase isolated from bovine rumen, which converted birchwood xylan mainly into xylohexaose (23). This xylanase showed a good product specificity but was unsuitable for XOS production due to a low rate of xylan degradation. The other reported example is a commercial xylanase, which generated XOS with DP range of 3 to 5 from microwave-CrO\(_3\)-H\(_3\)PO\(_4\) pretreated rice straw (24). Nevertheless, numerous monosaccharides were also produced in that system. By contrast, rBaxyl11 specifically generated series of high-DP XOS and AXOS without xylose, xylobiose and xylotriose when hydrolyzing arabinoxylan. Being different from linear low-DP oligosaccharides, XOS with high molecular weight and AXOS with more complex structures were considered as slower fermenting prebiotics, thereby promoting health of distal intestinal tract (10, 25, 26). Therefore, rBaxyl11 showed promising potential for production of such prebiotics.
Table 2

XOS production by reported xylanases using enzymatic hydrolysis and single-step fermentation

| Substrate                        | Enzyme or strain                          | Reaction time<sup>a</sup> | XOS yield<sup>b</sup> | MainDP | XOS production mode          | Reference |
|----------------------------------|-------------------------------------------|---------------------------|-----------------------|--------|------------------------------|-----------|
| De-starched wheat bran           | Engineering *E. coli*                     | 12 hours                  | 7.3%                  | > 3    | Single-step fermentation (37°C) | This study |
| De-starched wheat bran           | Engineering *E. coli*                     | 36 hours                  | /                     | /      | Single-step fermentation (37°C) | (15)      |
| Wheat middlings                  | *Bacillus subtilis*                       | 48 hours                  | 6.5%                  | 3–4    | Single-step fermentation (37°C) | (29)      |
| Rice husk                        | Engineering *Aspergillus nidulans*        | 48 hours                  | 2.4%                  | 3–6    | Single-step fermentation (37°C) | (28)      |
| Brewers’ spent grain             | Engineering *Bacillus subtilis*           | 12 hours                  | 5.4%<sup>c</sup>      | 2–6    | Single-step fermentation (45°C) | (13)      |
| Brewers’ spent grain             | *Trichoderma reesei*                      | 72 hours                  | 3.8%<sup>c</sup>      | 2–5    | Single-step fermentation (30°C) | (14)      |
| Beechwood xylan                  | Xylanase from *Mycothermus thermophilus*  | 12 hours                  | 83%                   | 2–3    | Enzymatic hydrolysis (65°C)   | (31)      |
| Wheat bran                       | Xylanase from *Aspergillus oryzae*        | 24 hours                  | 0.4%                  | 2–4    | Enzymatic hydrolysis (50°C)   | (32)      |
| Auto-hydrolysis pretreated corncobs | Mutant xylanase from *Talaromyces thermophilus* | 2 hours                  | 7.1%                  | 2–3    | Enzymatic hydrolysis (50°C)   | (33)      |
| Delignified sugarcane bagasse    | Xylanase from *Bacillus subtilis*         | 15 hours                  | 12.0%                 | 2–11   | Enzymatic hydrolysis (50°C)   | (34)      |
| Wheat husk                       | Crude xylanase from *Aspergillus fumigatus* R1 | 12 hours                  | /                     | ≥ 2    | Enzymatic hydrolysis (37°C)   | (35)      |

<sup>a</sup> Reaction time indicates the hydrolysis or fermentation time when XOS yield reaches the one represented here.

<sup>b</sup> XOS yield is represented as amount of XOS per gram of substrates (mg/g). Xylose is not included.

<sup>c</sup> These yields are expressed in xylose equivalents.
| Substrate                                      | Enzyme or strain                                      | Reaction time | XOS yield | MainDP | XOS production mode                        | Reference |
|-----------------------------------------------|------------------------------------------------------|---------------|-----------|--------|--------------------------------------------|-----------|
| Extracted xylan from corn cobs                | Xylanase from *Thermomyces lanuginosus*              | 8 hours       | 34.5%     | 2–3    | Enzymatic hydrolysis (45°C)                | (36)      |
| Birchwood xylan                               | Xylanase isolated from bovine rumen                  | 0.5 hour      | /         | ≥ 6    | Enzymatic hydrolysis (35°C)                | (23)      |
| Extracted xylan from data seed                | Commercial xylanase from *Aspergillus niger*         | 6 hours       | 36%       | 2–3    | Enzymatic hydrolysis (38°C)                | (37)      |
| Alkali-microwave pretreated wheat bran        | Xylanase from *Bacillus halodurans*                  | 72 hours      | 26%       | ≥ 2    | Enzymatic hydrolysis (70°C)                | (38)      |
| Microwave pretreated rice straw               | Commercial xylanase                                   | 24 hours      | 0.4%      | 3–5    | Enzymatic hydrolysis (50°C)                | (24)      |
| Extracted arabinoxylan from wheat bran        | Xylanase from *Geobacillus stearothermophilus*        | 24 hours      | 53%       | 2–5    | Enzymatic hydrolysis (50°C)                | (39)      |
| Extracted xylan from vetiver grass            | Xylanase from *Aureobasidium melanogenum*            | 96 hours      | 23.6%     | 2–3    | Enzymatic hydrolysis (28°C)                | (40)      |
| Steam-explosion corncobs                      | Xylanase from *Paenibacillus barengoltzii*           | 4 hours       | 23.4%     | 2–5    | Enzymatic hydrolysis (50°C)                | (12)      |
| Extracted xylan from mahogany                 | Xylanase from *Clostridium* strain BOH3              | 24 hours      | 57.2%     | 2–5    | Enzymatic hydrolysis (50°C)                | (8)       |
| Alkali pretreated mahogany                    | Xylanase from *Clostridium* strain BOH3              | 24 hours      | 9.0%      | 2–5    | Enzymatic hydrolysis (50°C)                | (8)       |

\[a\] Reaction time indicates the hydrolysis or fermentation time when XOS yield reaches the one represented here.

\[b\] XOS yield is represented as amount of XOS per gram of substrates (mg/g). Xylose is not included.

\[c\] These yields are expressed in xylose equivalents.

### 3.4 Single-step fermentation for XOS production
Wheat bran is a cheap by-product from flour milling industry and consists of 29–42% of arabinoxylan (27). To save cost and simplify process, direct fermentation of de-starched wheat bran by engineering E. coli containing rBaxyl11 to produce XOS and AXOS was attempted (Fig. 5). Extracellular secretion of rBaxyl11 was detected after two-hour fermentation. The xylanase activity rapidly increased to 48% of the maximum in the next two hours, and the increment slowed down from the 8th hour. Concentration of XOS in medium showed similar trend comparing with xylanase activity, which increased rapidly from the 2nd hour to 8th hour. It reached 1.46 mg/mL at the 12th hour and only further increased by 8.9% in the next 12 hours. Therefore, 12 hours were optimal fermentation time for XOS production considering the cost.

These results demonstrated the feasibility of single-step fermentation for XOS production by recombinant E. coli containing rBaxyl11. Theoretically, rBaxyl11 was transported to the periplasm when using pET22b(+). According to our measurement, however, about 30% of rBaxyl11 was secreted to medium, which made the fermentation workable. Although some purified or commercial xylanases generated considerable XOS by hydrolysis, preparation of these enzyme prejudices economy of the production process. In addition, high temperature is commonly employed to maintain enzymatic activity and a large dose of xylanase is needed to cope inactivation, which are both adverse to cost (Table 2). By contrast, an integrated production of XOS can leave out separate process for enzyme expression as well as purification, and generally adopts mild conditions. Therefore, direct fermentation by microorganisms was believed to be a remarkable way to save cost and was beneficial to industrial production of XOS (4).

There are a few cases of single-step fermentation so far (Table 2). Some fungi such as Aspergillus nidulans and Trichoderma reesei were showed to simultaneously produce xylanase and XOS using cheap biomass (14, 28). Nevertheless, the yields of XOS were modest despite optimization, which could be attributed to lack of pretreatment to substrate. Bacillus subtilis was also reported to specifically produce xylotriose, xylotetraose or xylopentaose from wheat middlings in a single-step fermentation process, but the fermentation required at least 48 hours for a high purity of XOS without xylose (29). By comparison, an recombinant B. subtilis could generate AXOS with DP ranging from 2 to 6 after 12-hour fermentation, despite slight reduction in yield (13). It seems that engineering bacteria is more promising in XOS production with low cost. As model bacteria, E. coli is widely utilized in bioengineering and, in addition, it neither uses XOS as carbon source nor produces other undesired saccharides, thereby contributing to enhance yield and purity of XOS. (30). A recombinant E. coli was recently reported to produce XOS and xylose by single-step fermentation, but the concentration and DP of XOS were not investigated (15). In this study, the engineering E. coli. retained the specificity of purified rBaxyl11, which generated high-DP XOS and AXOS from wheat bran (Fig. 4d). Yield of XOS reached 73 mg/g substrate at 12th hour and exceed those of previous reports using single-step fermentation (Fig. 5 and Table 2). Such system can also be further optimized to improve the overall yields or reduce cost to meet requirements of industrialization.

4. Conclusions

Enzymatic characteristics of rBaxyl11 from B. agaradhaerens C9 was studied. The alkaline xylanase of GH11 has good stability and activity to various xylans. DP of XOS produced by rBaxyl11 was influenced by type of substrates. It was notable that rBaxyl11 converted arabinoxylan into high-DP XOS, which is different from most reported xylanases. Results of direct fermentation demonstrated the feasibility of simultaneously
producing xylanase and XOS by recombinant *E. coli* containing *rBaxyl11* in an integrated process. Thus, such single-step fermentation approach could be a promising strategy for XOS production at industrial scale.

**Declarations**

**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 3180020), Natural Science Foundation of Jiangsu Province (BK20171163, BK20181009), Natural Science Foundation of Xuzhou city (KC19196), Six Talent Peaks Project of Jiangsu Province (JNHB-103), Qing Lan Project of Jiangsu Province, Priority Academic Program Development of Jiangsu Higher Education Institutions.

**Conflicts of interest**

None

**Availability of data and material**

All data generated or analysed during this study are included in this published article. Original data are available from the corresponding author on reasonable request.

**Code availability**

Not applicable

**Authors' contributions**

JL and DS conceived and designed the experiments, and wrote the original draft. Experiments were implemented mainly by JL, DS and ZD. Data collection and analysis were performed by CL. JZ prepared material. WL supervised the overall work, discussed the results, and revised the manuscript. All authors read and approved the final manuscript.

**Ethics approval**

Not applicable

**Consent to participate**

Not applicable

**Consent for publication**

Not applicable

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Figures
Figure 1

Electrophoresis and sequence analysis of rBaxyl11 (a) Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of rBaxyl11. Line 1: soluble cell extracts 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. For figure 1A and 1B, the maximal activity was designated as 100%. For figure 1C and 1D, activity of enzyme without incubation was designated as 100%. Determination 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).
Effect of metal ions and chemical reagents on activity of rBaxyl11 Activity of rBaxyl11 was measured using 0.3% of xylan in the presence of metal ion or chemical reagent (5mM). Activity of control group without metal ion or chemical reagent was designated as 100%. Data between control and test groups differ statistically (p<0.05, t test) except the activity measured with K+ and glucuronoxylan (p=0.355, t test). All data are presented as means ± standard deviations (n=3).
Figure 4

Product of rBaxyl11 hydrolyzing (a)XOS, (b)glucuronoxylan, (c)arabinoxylan and of (d) single-step fermentation For figure 3A, 5 μL of rBaxyl11 (0.2 μM) and XOS (5 mg/mL) were mixed and then incubated at 40°C for one hour. XOS mixed with buffer was used as control. For figure 3B and 3C, 20 μL of rBaxyl11 (6.5 μM) and 40 μL of arabinoxylan/glucuronoxylan (10 mg/mL) were mixed and then incubated at 50°C. Reaction was stopped at 0.5, 2, 10, 30, 120 and 720 min using 3 μL of NaOH (1 M). For figure 3D, 8 μL of LB medium sampled at the 24th fermentation was analyzed. X2-6: xylobiose, xylotriose, xylotetraose,
xylopentaose and xylohexaose; S: standards composed of xylose and X2-6; rBaxyl11(+): fermentation using E. coli containing rBaxyl11; rBaxyl11(-): fermentation using E. coli containing empty vector.

Figure 5

Single-step fermentation by recombinant E. coli Circle and triangle represented xylanase activity and reducing sugar, respectively. Solid and dotted lines represented recombinant E. coli containing rBaxyl11 and empty vector, respectively. Fermentation started from the time of inducing. All data are presented as means ± standard deviations (n=3).