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Recombinant Production of Bifidobacterial Endoglycosidases for N-glycan Release

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TITLE:

Recombinant Production of Bifidobacterial Endoglycosidases for *N*-glycan Release

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SUMMARY:

Bifidobacteria possess a unique genomic capability for *N*-glycan cleavage. Recombinantly producing these enzymes would be a promising novel tool to release bioactive *N*-glycans from glycoprotein-rich substrates such as colostrum.

ABSTRACT:

Protein glycosylation is a diverse and common post-translational modification that has been associated with many important roles such as protein function, including protein folding,

stability, enzymatic protection, and biological recognition. *N*-glycans attached to glycoproteins (such as lactoferrin, lactadherin, and immunoglobulins) cannot be digested by the host and reach the large intestine, where they are consumed by certain beneficial microbes. Therefore, they are considered next-generation prebiotic compounds that can selectively stimulate the gut microbiome's beneficial microorganisms. However, the isolation of these new classes of prebiotics requires novel enzymes. Here, we describe the recombinant production of novel glycosidases from different Bifidobacteria strains (isolated from infants, rabbits, chicken, and bumblebee) for improved *N*-glycan isolation from glycoproteins. The method presented in this study includes the following steps: molecular cloning of Bifidobacterial genes by an *in vivo* recombinational cloning strategy, control of transformation success, protein induction, and protein purification.

INTRODUCTION:

Glycosylation is a very crucial post-translational modification observed in proteins. Approximately more than 50% of proteins are found in their glycosylated forms in eukaryotes. *N*- and *O*-glycosylation are the two major types of glycosylation[1,2]. *O*-linked glycans (*O*-glycans) are covalently attached to proteins via *N*-acetylgalactosamine to the hydroxyl group of a serine (Ser) or threonine (Thr) amino acid residues. *N*-linked glycans (*N*-glycans) are complex oligosaccharides, which are covalently attached to asparagine (Asn) amino acid residue of the proteins through *N*-acetylglucosamine (GlcNAc) in a particular amino acid sequence As*N*-X-Ser/Thr and a less common one, As*N*-X-Cys (cysteine) (where X might be any amino acid except proline)[3,4]. The basic *N*-glycan core consists of two HexNAc and three mannose residues. Further elongation of this common core with other monosaccharides via glycosyltransferase and glycosidase enzymes determines the type of *N*-glycans based on the degree of branching and the type of linkage[5]. *N*-glycans are generally grouped into three main classes: high mannose (HM), complex type (CT), and hybrid (HY)[6].

N-glycans are indigestible compounds by the host organisms due to the lack of glycoside hydrolase enzymes. These compounds reach the small/large intestine in an undigested form where thousands of different bacterial species utilize them, and they can act as prebiotics by promoting specialized gut microbes, especially *Bifidobacterium* species[7]. Recent findings showed that *N*-glycans selectively stimulate the growth of certain bacterial species[8,9]. *N*-glycans released from bovine milk glycoproteins selectively stimulated the growth of *Bifidobacterium longum* subspecies *infantis* (*B. infantis*), which is a crucial Bifidobacterial species in the infant's gut, but other bifidobacterial species such as *Bifidobacterium animalis* (*B. animalis*) did not utilize these compounds[9]. In addition, a recent *in vivo* study demonstrated that 19 unique *N*-glycans from milk lactoferrin and immunoglobulins selectively stimulate the growth of *B. infantis*[8]. Especially, *B. infantis* possess a genomic capability for glycan cleavage and metabolism. An Endo-*β*-*N*-acetylglucosaminidase (EndoBI-1), which belongs to glycosyl hydrolase family 18, recombinantly produced from *B. infantis* ATCC 15697 showed a high activity on milk glycoproteins in *in vitro* conditions[9,10]. This novel glycoside hydrolase enzyme can cleave the *N*-*N*'-diacetylchitobiose parts found in the *N*-glycans[10,11]. The activity of EndoBI-1 is not affected by core fucosylation and different reaction conditions such as high temperature, pH, reaction time, etc[3,11,12]. This unique characteristic of Bifidobacterial glycoside hydrolases provides a promising tool for producing *N*-

glycans from glycoprotein-rich substrates such as bovine colostrum[13,14].

Several chemically and enzymatically developed deglycosylation methods have been widely used to obtain *N*-glycans and *O*-glycans from glycoproteins[2,15]. Chemical methods are widely used in glycobiology for deglycosylation of glycoproteins because of their ease of use, low cost, and high substrate specificity[16]. The most common chemical deglycosylation methods are β-elimination and hydrazination[17]. Among these methods, β-elimination is based on the principle of cleavage of glycans from glycoproteins by exposure of glycoproteins to alkaline conditions. The released glycans can be degraded during the process due to the β-elimination reactions, but this problem can be prevented using reducing agents such as sodium borohydride ($NaBH_4$)[18–20]. There are different limitations in the β-elimination method. The reductive agents convert glycans to alditols, prevent them from binding a fluorophore or chromophore. Thus, challenging to monitor glycan release becomes difficult[19,20]. Because of the high salt content in the cleaning step of the method, elution might result in sample losses[20]. Another method for releasing glycan from glycoproteins is the hydrazine method based on the principle of the hydrolysis reaction following the addition of anhydrous hydrazine to the glycoprotein. Since it allows for controlling the isolation of glycans by changing reaction conditions such as temperature, the hydrazination method has been widely used in glycobiology[21]. Chemical deglycosylation can also be carried out using the anhydrous formulation of hydrogen fluoride and trifluoroacetic acid, in addition to other chemical deglycosylation methods[16,22,23]. The enzymatic release of *N*-glycans from glycoproteins is commonly performed by peptidyl-*N*-glycosidases (PNGases) that generally release *N*-glycans, regardless of their size and charge[24–27]. Similar to the chemical deglycosylation methods, the enzymatic deglycosylation process has different challenges. PNGases show activity in the presence of several detergents used, which increase the enzyme accessibility to the glycans. However, these harsh treatments might disrupt the native glycans and the remaining polypeptide structures[28]. PNGases may not cleave the glycans when there is a fucose linked to *N*-acetylglucosamine[29]. Various endoglycosidases such as F1, F2, and F3 show more activity on the native proteins than PNGases. These endoglycosidases have low activity on the multiple-antennary glycans, whereas heat-resistant novel EndoBI-1 is effective in all types of *N*-glycans[10,11,28]. Regarding the limitations of the current methods, it is obvious that novel enzymes are still required for an effective glycan release without any restrictions. For this purpose, Bifidobacterial species, which have a large genomic island encoding various glycoside hydrolases enzymes, enable cleaving *N*-glycans from glycoproteins[30,31]. Within the scope of this context, the overall aim of this study is to discover new glycosidases from the various Bifidobacterial species. To recombinantly produce these enzymes, different fusion tags are intended to enhance their production as well as their activity.

PROTOCOL:

1. Molecular cloning of Bifidobacterial genes

1.1. PCR amplification of targeted genes by three vector primer sets (N-His, C-His, and N-His SUMO)

1.1.1. Make 100 µM stock primer (oligomers) solutions by adding sterile water in the amounts determined by the company. Prepare 10 µM new stocks from these stocks to be used in PCR amplification of the target genes.

1.1.2. Prepare the PCR mixture (total volume 50 µL) with 25 µL of master mix, 1 µL of forward and reverse primer at 0.2 µM, 21 µL of DNase/RNase-free distilled water and 2 µL of template DNA (bacterial cells) in the PCR tubes. Gently stir the mixture by pipetting up and down.

NOTE: The master mix (Lucigen) used for PCR contains Taq DNA Polymerase with high purity and high activity and can work at higher temperatures for reliable amplification of templates up to 5 kb.

1.1.3. Set the PCR program as follows: initial denaturation step at 95 °C for 5 min for the release of genomic DNA, then 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and elongation at 72 °C for 1 min, and the final extension at 72 °C for 10 min.

1.1.4. Check the PCR products by agarose gel electrophoresis method with a gel documentation system after being run at 100 V for 60 min on the 1% agarose gel. Mix the PCR products and DNA ladder with the loading dye by mixing at a ratio of 1:5 (5 µL of PCR product + 1 µL of loading dye and 5 µL of 1 kb DNA ladder + 1 µL of loading dye) to load on the gel (**Figure 1**).

1.1.5. Measure the DNA concentrations of PCR products using a fluorometer before the molecular cloning step.

NOTE: Concentrations of PCR products should be in the range required for molecular cloning (25–100 ng/µL).

1.2. **Preparation of Lysogeny Broth (LB) agar medium for molecular cloning**

1.2.1. Dissolve 12.5 g of LB and 6 g of agarose in 500 mL of dH_2O and autoclave the LB agar medium (sterilization at 121 °C for 20 min).

1.2.2. Dissolve 15 mg of kanamycin with 1 mL of dH_2O and store at -20 °C.

1.2.3. After autoclaving, add 1 mL of kanamycin into the bottle containing the sterile 500 mL of LB agar medium. The final concentration of kanamycin is 30 µg/mL. Pour ~25 mL of LB agar kanamycin media into each plate in the laboratory cabinet.

1.3. **Heat shock transformation of chemically competent *E. coli* cells**

1.3.1. Add 1–3 µL (25 to 100 ng) of the PCR products for each strain into the tube, including 40 µL of chemically competent *E. coli* cells. Then, add 2 µL of the vector DNA to the same tube. Stir gently with the pipette tip and transfer the mixtures to 15 mL centrifuge tubes.

NOTE: Perform this step on ice. Do not pipette up and down to mix to avoid air bubbles and inadvertently warming cells.

1.3.2. Incubate the tubes containing the competent cells and DNA on ice for 30 min. Apply heat shock to the mixture in a 42 °C water bath for 45 s. Put these tubes on ice immediately and incubate for 2 min.

1.3.3. Add 960 µL of the Recovery Medium, used for the rapid recovery of cells after molecular cloning, to the cells in the tubes and incubate the tubes at 250 rpm for 1 h at 37 °C in a shaking incubator.

1.3.4. Plate 100 µL of transformed cells on LB agar plates containing 30 µg/mL of kanamycin. Use only chemically competent *E. coli* cells as a negative control.

NOTE: Put LB agar plates containing 30 µg/mL of kanamycin prepared in step 1.2.2 to the incubator at 37 °C before using.

1.3.5. Incubate all plates overnight at 37 °C under ambient atmosphere (**Figure 2**).

1.4. **Preparation of LB medium for colony PCR**

1.4.1. Dissolve 7.5 g of LB with 300 mL of the dH_2O in a bottle and autoclave the LB medium (sterilization at 121 °C for 20 min).

1.4.2. Dissolve 9 mg of kanamycin (30 µg/mL) with 1 mL of dH_2O and put at -20 °C. After autoclaving, add 1 mL of kanamycin into the bottle containing the sterile 300 mL of LB medium. Store the liquid culture medium at +4 °C until using it.

1.5. **Screening of transformants by colony PCR**

1.5.1. To confirm all transformants carry the recombinant genes, select colonies randomly and amplify the target genes by PCR using the sequencing primers supplied with the cloning kit.

1.5.2. Perform all the steps on ice and pre-chill all PCR tubes and 15 mL tubes before use.

1.5.3. Using a pipette tip, transfer half of a selected colony to the PCR tube for each sample. Take another half of the colony with the pipette tip and put it into the 15 mL tube containing 5 mL of LB+kanamycin liquid medium (prepared in step 1.4). Vortex the 15 mL tubes and incubate the liquid cultures at 250 rpm at 37 °C overnight in a shaking incubator.

1.5.4. Put 50 µL of PCR reaction mixture (25 µL of master mix, 1 µL of forward primer, 1 µL of reverse primer, 23 µL of DNase/RNase-free distilled water) into all PCR tubes and disperse the cells by pipetting up and down gently.

1.5.5. Set the PCR program at 95 °C for 5 min for the release of genomic DNA by lysing bacterial cells, a total of 40 cycles of 95 °C for 30 s for initial denaturation, 60 °C for 30 s for annealing, 72 °C for 1 min for elongation, and 72 °C for 10 min for the final extension.

1.5.6. Check the PCR products by gel electrophoresis method after being run at 100 V for 60 min on the 1% agarose gel (**Figure 3**). The details of DNA gel electrophoresis are described in step 1.1.3.

1.5.7. Prepare 15% glycerol stocks of the successful transformants. Put 500 µL of 60% glycerol stock in the cryotubes and add 1,500 µL of the liquid culture of the successful transformants. Store prepared stocks at -80 °C.

2. L-rhamnose induction of protein expression

2.1. Prepare a preculture with 1 L of LB liquid medium containing 30 µg/mL of kanamycin.

2.2. Put 8 mL of the LB medium in 50 mL centrifuge tubes. Use one of the tubes as a negative control containing only the liquid medium. For 20% L-rhamnose as stock, dissolve 0.5 g of L-rhamnose with 2.5 mL of dH_2O and store at -20 °C until using it.

2.3. Put 10 µL of the bacterial stocks into the centrifuge tubes containing 8 mL of liquid media. Vortex them gently and incubate at 37 °C for overnight in the shaking incubator.

2.4. Pour 250 mL of LB liquid medium into a sterilized 2 L Erlenmeyer flask.

2.5. Inoculate 2.5 mL of the overnight liquid culture into a 2 L flask containing LB liquid medium at a ratio of 1:100 between the flask and the medium, and incubate at 37 °C and 150 rpm for 4 h in the shaking incubator.

2.6. Measure the optical density at 600 nm (OD_{600}) for the bacterial cells by a spectrophotometer. When the cells reach the optical density of 0.5–0.6, add 2.5 mL of 20% rhamnose (final concentration is 0.2%) to the 250 mL of LB culture and incubate at 37 °C overnight at 250 rpm in the shaking incubator.

2.7. Transfer the liquid culture into the 5 x 50 mL tubes, centrifuge samples 3724 x *g* for 15 min at +4 °C and discard the supernatant. Store the pellets at -20 °C until the purification step.

NOTE: To evaluate protein expression with SDS-PAGE, collect 1 mL of uninduced (when cultures at an optical density 600 nm of 0.5–0.6, without L-rhamnose) culture as control and 1 mL of induced culture (after overnight incubation) as induced sample. Microcentrifuge all samples at 12,000 x *g* for 1 min and resuspend uninduced and induced samples with 50 µL and 100 µL of SDS-PAGE loading buffer, respectively.

3. Cell lysis of chemically competent *E. coli* cells containing His-tagged enzymes

3.1. Prepare lysis buffer pH 8.0 (50 mM Tris-HCl, 200 mM NaCl, 1 mM imidazole, 1% SDS), equilibration buffer pH 7.4 (20 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole), wash buffer pH 7.4 (20 mM NaH_2PO_4, 300 mM NaCl, 25 mM imidazole), and elution buffer pH 7.4 (20 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole).

3.2. Place the 50 mL tubes containing the cell pellets at -80 °C for 15 min to freeze. Then, remove the pellets from -80 °C and thaw at room temperature.

3.3. Add 5 mL of dH_2O to the pellets and dissolve by pipetting up and down. Centrifuge at 3724 x *g* for 15 min at +4 °C and discard the supernatant.

3.4. For 50 mL culture pellets, add 6,300 µL of lysis buffer and 63 µL of EDTA-free halt protease inhibitor cocktail (1:100 ratio) into the pellets and dissolve by pipetting up and down. Incubate them on ice for 30 min, vortex every 10 min.

3.5. Set the pulse mode of the sonicator as 10 s ON and 59 s OFF, and the amplitude as 37%. Place the tube in a beaker containing ice and immerse the probe of the sonicator in the tube.

NOTE: The probe should be immersed completely without touching any side of the tube.

3.6. After the sonication process (6 pulses for 10 s with 1 min cooling), centrifuge the samples at 3,724 x *g* for 45 min at +4 °C. Next, collect all the supernatant parts in a tube and centrifuge at 3,724 x *g* for 5 min at +4 °C.

3.7. Collect the supernatant into a tube and take 100 µL of the sample for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in step 4.7. Measure the protein concentrations of the samples using a fluorometer.

4. Purification of His-tagged enzymes by batch method

4.1. Add 1 mL of Ni-NTA resin to a centrifuge tube and centrifuge for 2 min at 700 x *g*. Carefully remove the tube and discard the supernatant formed.

4.2. Add 2 mL (twice the resin volume) of the equilibration buffer into the tube and mix well until the resin is fully suspended. Centrifuge the tube for 2 min at 700 x *g* and carefully remove and discard the buffer.

4.3. Mix the protein extract and equilibration buffer in a ratio of 1:1 in a centrifuge tube. Add the mixture to the tube containing resin and put it on a shaker at 150 rpm for 30 min. Centrifuge the tube for 2 min at 700 x *g* and discard the supernatant.

4.4. Wash the resin with 5 mL of wash buffer and centrifuge the tube for 2 min at 700 x *g*. Repeat the washing step until the concentration of the supernatant decreases to the baseline.

4.5. Add 1 mL of elution buffer into the tube to elute bound His-tagged proteins. Centrifuge the tube for 2 min at 700 x *g*. Save the supernatant and take 100 µL for SDS-PAGE analysis in step 4.7. Repeat the elution step three times. Measure the concentrations of each supernatant by using a fluorometer.

4.6. Collect all the supernatants in the 10 kDa cut-off tube and centrifuge it for 2 min at 700 x *g*. Repeat centrifugation until the volume of the supernatant decreases to 200 µL by pipetting up and down occasionally. Store the purified proteins at -20 °C and take 100 µL for SDS-PAGE analysis in step 4.7.

NOTE: Protein concentration should be measured and, if the concentration is low, centrifuge until it increases.

4.7. **SDS-PAGE analysis of the purified proteins**

4.7.1. Prepare a 4% stacking gel (40% acrylamide/bisacrylamide, 1 M Tris pH 6.8, 10% SDS, 10% ammonium persulfate, TEMED, dH_2O) and 12% resolving gel (40% acrylamide/bisacrylamide, 1 M Tris pH 8.8, 10% SDS, 10% ammonium persulfate, TEMED, dH_2O).

4.7.2. Mix the sample with 2x Laemmli sample buffer in a ratio of 1:1 and incubate at 95 °C for 5 min to denature the proteins.

NOTE: Protein concentration of samples should be measured before loading, and the loaded volume will be based on their concentration for equal loading.

4.7.3. Add 1x running buffer into the tank and load the samples and the protein ladder into the wells. Run the proteins firstly at 80 V, and raise the current to 120 V when the proteins move from the stacking gel to the resolving gel.

4.7.4. Put the gel in coomassie blue staining dye and put it in a shaker for 30 min. Wash the gel with a destaining solution (250 mL dH_2O + 50 mL acetic acid (HOAc) + 200 mL methanol), and take the image (**Figure 4**).

REPRESENTATIVE RESULTS:

Glycosyl hydrolase member enzymes selected from different origins were targeted in this study. It was assumed that the co-application of different enzymes with different structures could provide a better glycan release since they are evolved to be active in different glycoproteins. The list of target genes and their origin is listed in **Table 1**. Bacterial strains were obtained from Belgium Co-ordinated Collections of Micro-organisms. Primer sets were designed based on the manufacturer's guidelines (**Supplementary Table 1**). Forward and reverse primers for N-His Kan Vector were designed as; F: 5'-CAT CAT CAC CAC CAT CAC XXX_2 XXX_3 XXX_4 XXX_5 XXX_6 XXX_7 XXX_8 (XXX_2-XXX_8 represents codons 2 through 8 of the target coding region) and R: 5'-GTG GCG GCC GCT CTA TTA XXX_n XXX_{n-1} XXX_{n-2} XXX_{n-3} XXX_{n-4} XXX_{n-5} XXX_{n-6} (XXX_n - XXX_{n-6} represents the sequence

complementary to the last 7 codons of the target coding region), respectively. Forward and reverse primers for C-His Kan Vector were designed as F: 5'-GAA GGA GAT ATA CAT ATG XXX_2 XXX_3 XXX_4 XXX_5 XXX_6 XXX_7 XXX_8 (XXX_2-XXX_8 represents codons 2 through 8 of the target coding region) and R: 5'-GTG ATG GTG GTG ATG ATG XXX_n XXX_{n-1} XXX_{n-2} XXX_{n-3} XXX_{n-4} XXX_{n-5} XXX_{n-6} (XXX_n - XXX_{n-6} represents the sequence complementary to the last 7 codons of the target coding region), respectively. Forward and reverse primers for N-His SUMO Kan Vector were designed as; F: 5'-CGC GAA CAG ATT GGA GGT XXX_2 XXX_3 XXX_4 XXX_5 XXX_6 XXX_7 XXX_8 (XXX_2-XXX_8 represents codons 2 through 8 of the target coding region) and R: 5'-GTG GCG GCC GCT CTA TTA XXX_n XXX_{n-1} XXX_{n-2} XXX_{n-3} XXX_{n-4} XXX_{n-5} XXX_{n-6} XXX_n - XXX_{n-6} (XXX_n - XXX_{n-6} represents the sequence complementary to the last 7 codons of the target coding region), respectively. Targeted genes were amplified by PCR. Each reaction mixture was a total of 50 µL containing 25 µL of master mix, 2 µL of a template (Bifidobacterial cells), 1 µL of Forward Primer, 1 µL of Reverse Primer, 21 µL of DNase/RNase-free water. PCR reaction was initiated with 95 °C for 5 min to release genomic DNA by cell lysis. Then, 40 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min as well as a final extension step at 72 °C for 10 min. PCR products were visualized by DNA gel electrophoresis to control the concentration and the purity of the inserts (**Figure 1**). The gel images showed that each amplification was successfully completed as there was only one clear band for each insert. Each PCR amplicon was measured by a fluorometer to determine the concentration of the PCR product (**Table 2**).

[place Table 1 here]

[place Figure 1 here]

[place Table 2 here]

The transformation of His-tagged PCR products and vectors (N-His Kan, C-His Kan, and N-His SUMO Kan) into chemically competent *E. coli* cells were performed by the heat shock method. Firstly, 1-3 µL (25 to 100 ng) of the PCR products and 2 µL of vectors were added into 40 µL of chemically competent *E. coli*. The mixtures were stirred gently with a pipette tip, transferred into 15 mL centrifuge tubes, and incubated on ice for 30 min. Heat shock was performed in a 42 °C water bath for 45 s for the transformation of the PCR products and the vectors into the cells. The heat-shocked cells were then returned quickly to ice for 2 min. 960 µL of recovery medium, which allows the cells to repair, was added to each tube and incubated at 37 °C for 1 h at 250 rpm. 100 µL of the cells were plated on LB + agar plates containing 30 µg/mL of kanamycin. In this experiment, chemically competent *E. coli* cells were only used as a negative control. All plates were incubated overnight at 37 °C. After the transformations, 20–60 colonies were obtained as expected. The growth of these cells on a medium containing LB + agar + 30 µg/mL of kanamycin was indicated that the kanamycin-resistant vectors were successfully transferred to the cells. Successful transformants were confirmed by the colony PCR method. Firstly, one part of each colony was transferred into a PCR tube for PCR amplification, and the other part was inoculated into 5 mL of LB + kanamycin medium in a 15 mL falcon tube to be used in case of positive results. PCR amplification was performed using 25 µL of master mix, 1 µL of forward primer, 1 µL of reverse primer (sequencing primers provided by the manufacturer), 23 µL of dH_2O at 95 °C 5 min

for the release of genomic DNA by lysing cells, a total of 40 cycles of 95 °C 30 s for initial denaturation, 60 °C 30 s for annealing and 72 °C 1 min for elongation, and 72 °C 10 min for the final extension. 5 µL of PCR products mixed with a loading dye at a ratio of 1:1, were run on a 1% agarose gel at 100 V for 70 min and visualized with a gel documentation system. Consequently, positive transformants for all target genes were separated from the unsuccessful colonies. When the gel image was examined, it was observed that almost all colonies were successful in transformation of the target genes (**Figure 2**). Glycerol stocks were prepared from the liquid cultures of the successful colonies.

[place Figure 2 here]

Protein production was initially carried out on a small scale (8 mL), then it was produced in large quantities using 250 mL of medium. Firstly, protein production was initiated with 1% inoculation of LB medium containing 30 µg/mL of kanamycin, allowing the chemically competent *E. coli* cells optical density to reach approximately 0.5–0.6. Protein expression was induced by the addition of L-rhamnose with a final concentration of 0.2%. After overnight incubation, the bacteria pellets were collected by centrifugation. The cell lysis method was used to break the cell membranes to release DNA, RNA, or protein from the induced cells. The homogenization of the cells was performed by adding lysis buffer and protease inhibitor. The sonication method was also used for secondary homogenization. Then, the cell debris was removed from the lysed cells by centrifugation.

Protein purification was performed using the batch method. Firstly, an equilibration buffer was added into both the Ni-NTA resin and the proteins to ensure that the 6xHis-tagged proteins interact effectively with the resin. Ni-NTA resin was added to 6xHis-tagged proteins and incubated to attach the proteins to the resin. 6xHis tagged proteins bound to nickel resins, while other natural proteins flowed through the system. The mixture containing the proteins attached to the resin was washed with the Wash Buffer for the removal of potential pollution by repeated centrifugation processes. The proteins were separated from the resin with Elution Buffer containing high imidazole. The purified protein was concentrated using a 15 mL, 10 kDa cut-off centrifugal filter. The list of purified enzyme codes is listed in **Table 3**. Consequently, the targeted proteins were successfully purified with a high yield by the batch purification method (**Figure 3**).

[place Table 3 here]

[place Figure 3 here]

FIGURE AND TABLE LEGENDS:

Figure 1: PCR amplification of targeted genes by three different vector primer sets. (**A**) N-His (**B**) C-His (**C**) N-His SUMO. Lanes 1–5 represent OU11_RS07620, BSAE_0444, BBKW_1881, BBPC_1683, and BBOH_0438 amplification, respectively.

Figure 2: Gel image obtained after Colony PCR. Lanes 1–5, 6–10, 11–15, and 16–18 represent

OU11_RS07620, BBKW_1881, BBPC_1683, and BBOH_0438 amplification, respectively.

Figure 3: SDS-PAGE analysis of purified enzymes. N-His tagged, C-His tagged, and N-His-SUMO tagged enzymes were successfully purified with high yield by the batch purification method.

Table 1: Information about target genes and their origin.

Table 2: Measurement of DNA concentrations by a fluorometer (ng/µL).

Table 3: Codes of N-His tagged, N-His SUMO tagged and C-His tagged purified enzymes.

Supplementary Table 1: List of primers used for gene amplification and sequencing. Primer sets for the target genes were designed based on the manufacturer's guidelines.

DISCUSSION:

The *in vivo* recombinational cloning strategy used for the molecular cloning of the target genes provides fast and reliable results compared to other traditional cloning protocols. Even though there are many convenient methods for molecular cloning, the method described in this article has more advantages. *In vivo* cloning system, unlike other cloning systems, does not need any enzymatic treatment or purification of the PCR products. Also, there is no limitation related to sequence junctions or the requirement of restriction enzymes. In this system, cloning can be performed by mixing the amplified gene with the cell and the vector (provided with the kit) without the need for enzymatic ligation. That's why it is called *in vivo* cloning; recombination between the ends of the PCR product and the vector occurs in the cell. The linear vector in this system contains 18 nucleotide-long sticky ends at both ends and these sequences are not complementary to each other. Owing to this feature, the vector completely prevents the risk of self-ligation or inverted insertion of sequences. Primers required for the amplification of the target gene are designed according to these ends for the strong binding. The N-His, C-His, and N-His SUMO vectors provided in the cloning kit facilitate instant cloning of target genes with amino or carboxyl-terminal 6xHis affinity tags that provide fast and easy affinity purification of proteins under native or denaturing conditions.

Although the transformation success efficiency of this cloning system is above 95%, one of the few disadvantages is the competent cells, which cannot be produced in typical laboratory conditions. The linearized vector containing competent cells is also only provided by the manufacturer. Thus, the storage and their application should be performed carefully. We have observed that heat shock transformation should be performed in 15 mL falcon tubes compared to 0.2 mL PCR tubes, which are commonly preferred by conventional molecular cloning methods.

After molecular cloning, protein production is increased by using L-rhamnose and its purification is performed by binding to nickel columns with 6xHistidine tag in three different vectors. To improve the purification efficiency, an N-His SUMO tag has been tested in this study. SUMO protein is known to improve the solubility of proteins and this enhances the recovery of purified protein[32]. However, we have not observed significant increases in recovery when comparing the

N-His SUMO tag and N- and C-Histidine tags.

Protein purification is a critical step for obtaining enzymes with high purity. Impurities recovered with the purified protein might mislead downstream experiments as well as efficiency calculations or activity assays in the case of enzymes. Successful purification is often considered to be 95% or more based on visual observation of the DNA gel electrophoresis result. Although a similar process can be applied for structurally similar proteins, each step of the purification process should be optimized for higher protein production efficiency and purity. One of the important steps in this process is the application of washing buffer to remove unwanted proteins and other compounds. The concentration of the washing buffer can contain 10 to 50 mM imidazole. While a low concentration of imidazole can cause impurities in enzyme solution, a high concentration of imidazole might diminish the recovery of proteins from the column. Therefore, at least three different concentrations of imidazole should be tested for the highest efficiency of protein production with the highest purity (optimized based on SDS-PAGE gel profiling).

With this fast and efficient molecular cloning-protein purification technique, we would enable to clone the enzymes from different sources and use them in various applications in several studies related to microbiology, food applications, and microbiome. One possible future direction with these techniques is the integration of microbiome-based enzymes into *in vitro* digestion models or using them in some products such as infant formulas and/or milk.

ACKNOWLEDGMENTS:
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DISCLOSURES:
The authors have nothing to disclose.

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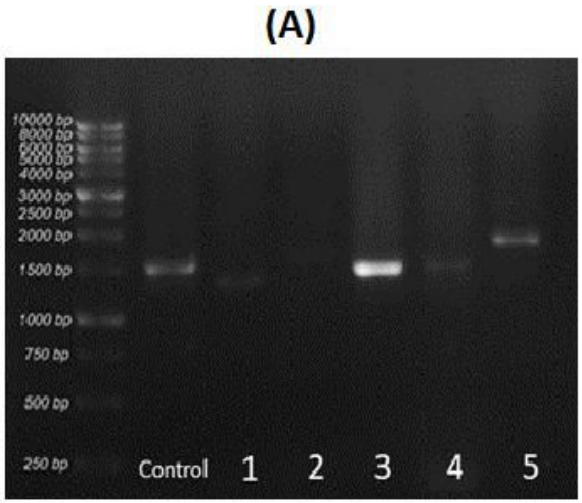
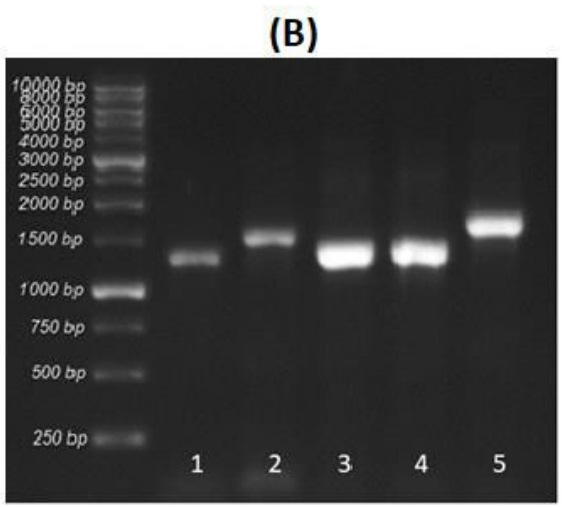
Figure 1. PCR amplification of targeted genes by three different vector primer sets. (A) pRham N-His (B) pRham C-His (C) pRham N-His SUMO. Lanes 1-5 represents





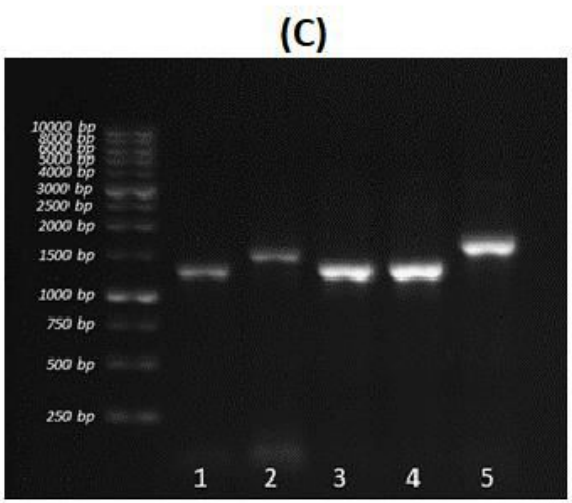


Figure 2. Gel image obtained after Colony PCR. Lanes 1-5, [illegible]10, 11-15, 16-18 represents OU11_RS07620,

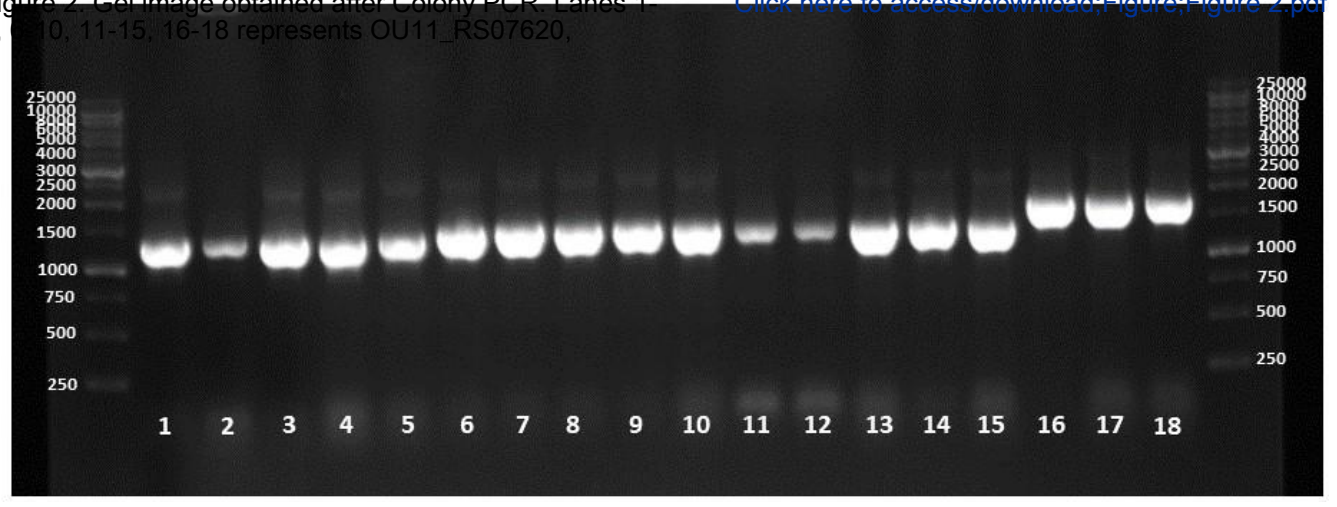


Figure 3. SDS-PAGE analysis of purified enzymes. N-His tagged, C-His tagged and N-His-SUMO tagged enzymes were successfully purified with high yield by the batch purification method.

Click here to access/download;Figure;Figure 3.pdf

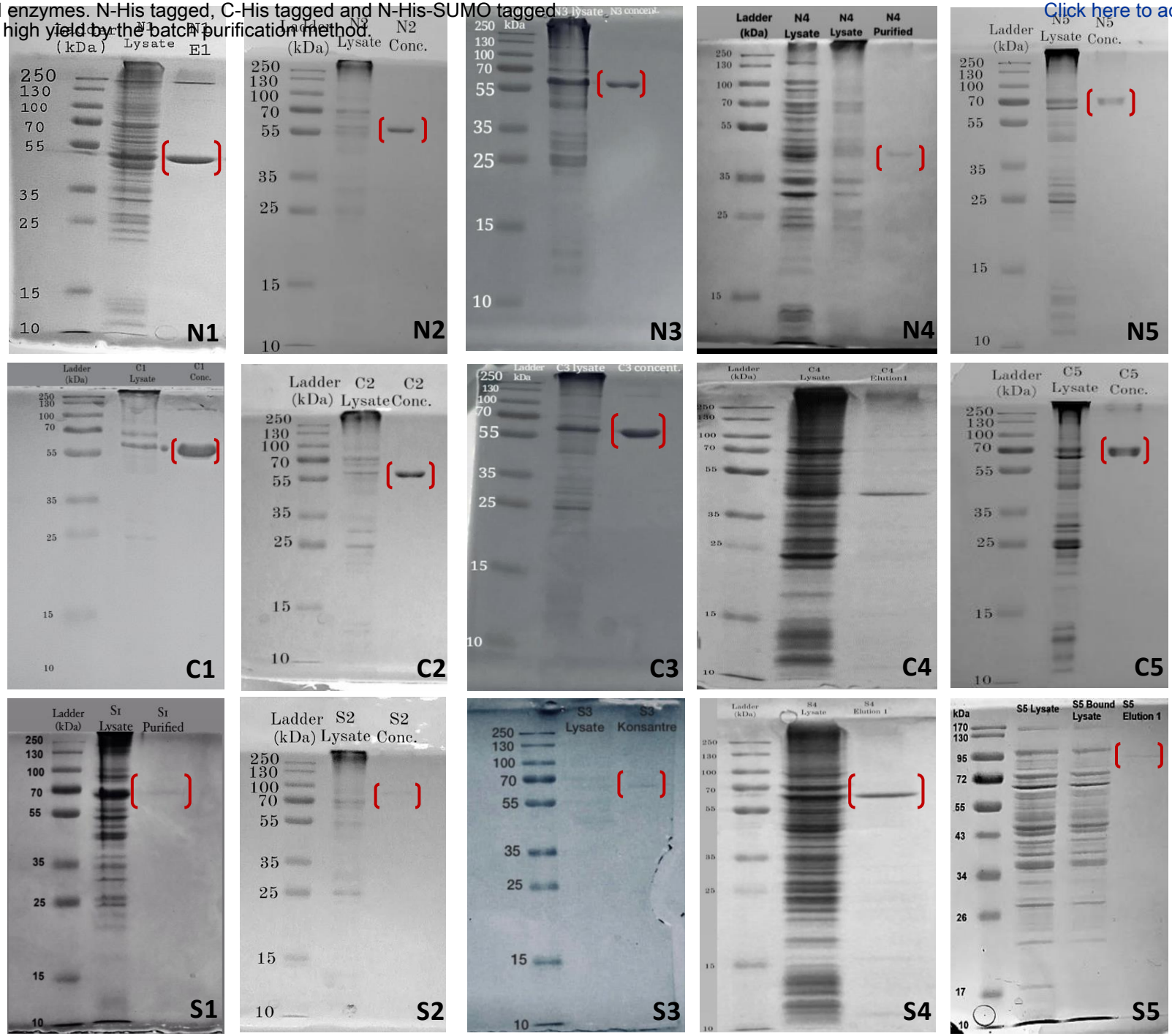


Table 1. Information about target genes and their origin.

Bacterial Strains	Origin	Accession Number	Locus Tag	Number of Base Pair
Bifidobacterium pullorum subsp. pullorum	Chicken feces	WP052122780.1	OU11_RS07620	1226
Bifidobacterium pullorum subsp. saeculare	Rabbit feces	KFI88990.1	BSAE_0444	2811
Bifidobacterium kashiwanohense	Child feces	BAQ30016.1	BBKW_1881	1302
Bifidobacterium pseudocatenulatum	Infant feces	BAR04361.1	BBPC_1683	1314
Bifidobacterium bohemicum	Bumblebee digestive tract	KFI46963.1	BBOH_0438	1650

Table 2: Measurement of DNA concentrations by a fluorometer (ng/µL).

Samples	N-His	N-His SUMO	C-His	Final Volume
B. pullorum subsp. pullorum (OU11_RS07620)	9	14.2	10.76	50 µL
B. pullorum subsp. saeculare (BSAE_0444*)*	24	12.66	11.74	50 µL
B. kashiwanohense (BBKW_1881)	14.6	35.9	20.6	50 µL
B. pseudocatenulatum (BBPC_1683*)*	20	35.3	21.9	50 µL
B. bohemicum (BBOH_0438)	30.8	32.5	10.4	50 µL

Table 3. Codes of N-His tagged, N-His SUMO tagged and C-His tagged purified enzymes.

Enzymes	N-His	N-His SUMO	C-His
OU11_RS07620	N1	S1	C1
BSAE_0444	N2	S2	C2
BBKW_1881	N3	S3	C3
BBPC_1683	N4	S4	C4
BBOH_0438	N5	S5	C5

Editorial and production comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
2. Please provide an email address for each author in the text manuscript.
3. Please make the title concise (please do not use words like novel, easy etc.)
4. Please ensure that the long Abstract is within 150-300-word limit and clearly states the goal of the protocol.
5. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Vilber Lourmat Quantum ST4 1100 gel documentation system, SafeRed, falcon, Qubit 3, E. cloni" 10G cells, pRhamTM vectors, Expresso Rhamnose Cloning and Expression System (Lucigen) kit, etc.
6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."
7. The Protocol should contain only action items that direct the reader to do something.
8. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step.
9. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?
10. Please move all the primer sequences used in your protocol in a table format and upload it separately to your editorial manager account as .xlsx file.
11. 1.1.3, 1.5.6. Please reword: DO you control with PCR or do you check with PCR?
12. 1.3: Do you perform ligation before transformation?
13. 3, 4: How do you make the protein express in the cells?
14. Please include a single line space between each substep of the protocol as well.
15. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
16. Please ensure the results are described in the context of the presented technique. e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.
17. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."
18. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
19. Figure 4: How do you check for equal loading in this case?

-We appreciate the editorial and production comments for general changes needed in the article. We added an e-mail address to the text for each author. We have checked the whole text considering grammar, spelling, line spaces, and appropriate language. We have made the title more concise according to comments. We have restated many sentences to increase their clarity. All steps have been checked for appropriate details and longevity and the commercial language has been replaced by generic terms. We also have restated the primer sequences as a table format and uploaded them separately. We have specified each sections to be visualized (1.3., 1.5., 2., 3., and 4.) in the text.

We also have replaced control with PCR by check with PCR in 1.1.3 and 1.5.6 parts. Regarding the comment for ligation mentioned in 1.3., we would like to explain the technique; there is no ligation before transformation, ligation occurs in the cells which is an advantage of the molecular cloning kit we used in the protocol. The mechanism of the *in-vivo* cloning system is also explained in the introduction in detail. Also, we have added an explanation about protein expression in 3.4. Lastly, in the discussion part, we have added a new paragraph related to future applications of the technique considering comments.

Reviewer #1:
Manuscript Summary:
Sucu et al present a very thorough description of the requirements for the cloning, overproduction and purification of Bifidobacteria glycosyl hydrolases. The protocol is well described and the specific application for enzymes of Bifidobacterial origin is very well presented.

Minor Concerns:
1.2.2 please do not give the final concentration of Kanamycin in 1.2.2. Please place the details of the final concentration of antibiotic in 1.2.3
-Changed as recommended.

1.3.1 is the vector and PCR product mixed first prior to mixing with the cells? Please clarify.
-Clarified in the text.

-We thank the Reviewer for their comments and have replaced the final concentration of kanamycin from 1.2.2. to 1.2.3. Additionally, we have amended 1.3.1. to state more clearly. The vector and PCR product both are mixed in the cell tube, there is no mix first prior to mixing the cells.

Reviewer #2:

Manuscript Summary:

This paper describes that five bifidobacteria-derived endo-β-N-acetylglucosaminidases were expressed in Escherichia coli and purified for the purpose of proposing a new tool for releasing N-glycan. Using the Expresso Rhamnose Cloning and Expression System (Lucigen), the production efficiency was compared with respect to the three types of fusion tags. Overall, problem is that there is a gap between the content expected from the title and the main text / conclusion. In the text, only the expression and purification of proteins are described, but this is no different from the instruction manual of the kit, and as the title suggests, I do not feel the need for the five Endoglycosidases of Bifidobacterium. If it's a scientific treatise, it would be nice to have at least a final comparison about the yield or activity of the endoglycosidases. Furthermore, when comparing the expression level for each tag, not only the SDS-PAGE picture but also the specific numerical value for the yield should be given. Without these additional data, rather than changing the title, it would be difficult to publish even in terms of method papers. We also need to make a number of minor corrections. Listed for reference.

Major Concerns:

1. There are no materials and equipment listed.

-The table has been added to the text.

2. Title: "N-glycosidase" should be "endoglycosidase". People might think N-glycosidase as PNGase.

-Changed as recommended.

3. Line 72: β-elimination is a method of release of O-glycan, so I think it would be misleading to write here.

-Generalization was made by adding the word O-glycans to the beginning of the paragraph. Thus, the methods used for the release of *N*- and *O*-glycans are described and then specialized for *N*-glycans.

4. Line 89-92, PNGase F is normally active at 37 ºC without harsh condition.

- Incorrect sentence corrected.

5. Line 93, Indeed, PNGase F cannot cleave alpha1,3-core fucosylated N-glycans. However, I do not know any endoglycosidase that is able to cut alpha1,3-core fucosylated N-glycans. So, the sentence above may be misleading in this context.

-*N*-glycans containing a fucose a(1-3)-linked to the glycan core are resistant to the PNGase enzyme and other endoglycosidases. EndoBI-1 cleaves the *N*-*N*'-diacetyl chitobiose moiety found in the *N*-glycan core, even if fucosylated. Here, it is emphasized that, unlike PNGase F, EndoBI-1 is active on all *N*-glycan structures.

6. Line 110: Specify the type / manufacturer of the enzyme used and primer concentrations.
-The concentrations of the primers (oligomers) were obtained as 100 µM by adding sterile water in the amounts determined by the company. 10 µM new stocks were prepared from these stocks to be used in PCR amplification of target genes. With the amount used during PCR, the final concentration of the primers is 0.2 µM. The master mix used for DNA amplification in PCR contains Taq DNA polymerase enzyme that can operate at high temperatures and provides reliable amplification of templates up to 5 kb with its high purity and activity. Explanations have been added in the text.

7. Table1: Although the target gene and the strain of origin are specified, it is better to describe the domain structure of the gene and the cloned region.
- We thank the Reviewer for their comment, and we appreciate the Reviewer's suggestion, but novel glycosidases from different bifidobacterial strains were cloned recombinantly, and there is no structural information on these novel enzymes in Protein Data Bank (PDB). However, bioinformatic investigation together with comparative modeling studies is required to describe the domain structures of the genes. In addition to this, to investigate structural information together with the unique properties of these cloned enzymes, our comparative modeling studies are ongoing.

8. Table1: Do you confirm that the enzymes from these bacteria have activity? If so, specify the literature.
- At the beginning of our study, the enzymes from these bacteria were selected in terms of similarities and differences in the amino acid sequence of the EndoBI-1 enzyme belonging to the glycosyl hydrolase 18 family. There is no study in the literature on the activities of selected enzymes on N-glycans. It will be the first to study these enzymes, which show similar activity with EndoBI-1, which is in the same enzyme family.

9. Figure 2: I don't think it's necessary to put this figure in the first place, but if you put it, clearly indicate what is smeared on the petri dish (I can't read).
- Figure 2 (related to the petri dish) has been removed.

10. Figure 4: Clarify the details of each panel more clearly. At present, it is unknown which panel is derived from which target gene. In addition, it is easier to understand the thin band if it is indicated by an arrow or the like. The yield of each protein is summarized in the attached table.
-The details of each panel have been clarified more clearly. Each enzyme code is indicated in the lower parts. In addition, the bands of the enzymes on the gel were shown by taking them into strips.

11. Table3: No description about C5, S5, N5
- Table 3 was restated by adding descriptions about N5, C5, S5.

12. Line 418: Examination of the concentration of imidazole does not tell what result this consideration came from.
In the enzyme purification method, the concentration of wash buffer we use to remove unwanted proteins and other compounds can include 10 to 50 mM imidazole. In our

previous optimization studies, we tested different imidazole concentrations for the highest efficiency of protein production with the highest purity. Then we determined that 50 mM and above imidazole eluted our enzyme by SDS-Page analysis.

13. Line 236: "measure the concentrations of the cell lysates by using a Qubit 3 fluorometer" does not tell whether it was a "cell lysate" or a protein in the cell lysate.
-The specified sentence has been restated as "measure the protein concentration of the samples".

Minor Concerns:
14. Line 61: Please also describe the GH family of Endo-β-N-acetylglucosaminidases.
-Described as recommended.

15. Lines 44 and 46: "HexNAc" is "GlcNAc"
-Changed as recommended.

16. Line 216: I am curious to know why you placed at -80 ° C for 15 minutes.
-Explained in the text.

17. Line 220: Describe the protease inhibitor in detail.
-Described as recommended.

18. Is it better to unify whether it is SUMO-tag or N-His-SUMO-tag?
-N-His-SUMO tag is better to use, it is used in the whole text.

Reviewer #3:
Manuscript Summary:
This manuscript provides a detailed description of the cloning and production of a number of enzymes predicted to have activity against N-glycans. The plasmid system used relies on rhamonse for induction. This includes the production of several different constructs per gene, which vary according to whether the His-tag is at the N- or C-terminus and if SUMO is included.

Major Concerns:
-Make Figure 4 much clearer with better annotations/labels.
-Figure 4 has been made much clearer with better labels.

Minor Concerns:
None

Video Produced by Author: Less than 50 MB. If your video is greater than 50 MB, click "offline" as the delivery method and our

This piece of the submission is being sent via mail.

Supplementary Table 1. List of primers used for gene amplification and sequencing. Primer sets for the target genes were designed

Fusion Tag	Codes of Primers	Base Sequence (5'-3')
	OU11_RS07620F	CAT CAT CAC CAC CAT CAC GGC GCA AGC CAT GTG TTT CCC
	OU11_RS07620R	GTG GCG GCC GCT CTA TTA AGC ACG GCC TTG TTG ACC TCG
	BSAE_0444F	CAT CAT CAC CAC CAT CAC TCA GAC CTT CCT CCG CTG CCA
	BSAE_0444R	GTG GCG GCC GCT CTA TTA ATG GTG TAC TAC GCG CGT GGC
N-His	BBKW_1881F	CAT CAT CAC CAC CAT CAC TTT CCA GGA AGG CGT TTT TCC
	BBKW_1881R	GTG GCG GCC GCT CTA TTA TAG CAC CGG TCC GAT GAT GCG
	BBPC_1681F	CAT CAT CAC CAC CAT CAC ATG AAA CCC CTG TTT CCA GGA
	BBPC_1681R	GTG GCG GCC GCT CTA TTA TAG CAC CGG TCC GAT GAT GCG
	BBOH_0438F	CAT CAT CAC CAC CAT CAC TCA TCG ACT CAT GCG TCT TCG
	BBOH_0438R	GTG GCG GCC GCT CTA TTA AGC ACG GCC TTG TTG ACC TCG
Sequencing	pRham Forward	GCTTTTTAGACTGGTCGTAGGGAG
Primers	pETite Reverse	CTCAAGACCCGTTTAGAGGC
	OU11_RS07620F	CGC GAA CAG ATT GGA GGT GGC GCA AGC CAT GTG TTT CCC
	OU11_RS07620R	GTG GCG GCC GCT CTA TTA AGC ACG GCC TTG TTG ACC TCG
	BSAE_0444F	CGC GAA CAG ATT GGA GGT TCA GAC CTT CCT CCG CTG CCA
	BSAE_0444R	GTG GCG GCC GCT CTA TTA ATG GTG TAC TAC GCG CGT GGC
N-His SUMO	BBKW_1881F	CGC GAA CAG ATT GGA GGT TTT CCA GGA AGG CGT TTT TCC
	BBKW_1881R	GTG GCG GCC GCT CTA TTA TAG CAC CGG TCC GAT GAT GCG
	BBPC_1681F	CGC GAA CAG ATT GGA GGT ATG AAA CCC CTG TTT CCA GGA
	BBPC_1681R	GTG GCG GCC GCT CTA TTA TAG CAC CGG TCC GAT GAT GCG
	BBOH_0438F	CGC GAA CAG ATT GGA GGT TCA TCG ACT CAT GCG TCT TCG
	BBOH_0438R	GTG GCG GCC GCT CTA TTA ATG ATG AAT AGG AAC TTG CAT
Sequencing	SUMO Forward	ATTCAAGCTGATCAGACCCCTGAA
Primers	pETite Reverse	CTCAAGACCCGTTTAGAGGC
	OU11_RS07620F	GAA GGA GAT ATA CAT ATG GGC GCA AGC CAT GTG TTT CCC
	OU11_RS07620R	GTG ATG GTG GTG ATG ATG AGC ACG GCC TTG TTG ACC TCG
	BSAE_0444F	GAA GGA GAT ATA CAT ATG TCA GAC CTT CCT CCG CTG CCA
	BSAE_0444R	GTG ATG GTG GTG ATG ATG ATG GTG TAC TAC GCG CGT GGC
C-His	BBKW_1881F	GAA GGA GAT ATA CAT ATG TTT CCA GGA AGG CGT TTT TCC
	BBKW_1881R	GTG ATG GTG GTG ATG ATG TAG CAC CGG TCC GAT GAT GCG
	BBPC_1681F	GAA GGA GAT ATA CAT ATG ATG AAA CCC CTG TTT CCA GGA
	BBPC_1681R	GTG ATG GTG GTG ATG ATG TAG CAC CGG TCC GAT GAT GCG
	BBOH_0438F	GAA GGA GAT ATA CAT ATG TCA TCG ACT CAT GCG TCT TCG
	BBOH_0438R	GTG ATG GTG GTG ATG ATG ATG ATG AAT AGG AAC TTG CAT
Sequencing	pRham Forward	GCTTTTTAGACTGGTCGTAGGGAG
Primers	pETite Reverse	CTCAAGACCCGTTTAGAGGC