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Heterologous Expression of a Bioactive β-Hexosyltransferase, an Enzyme Producer of Prebiotics, from *Sporobolomyces singularis*

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Galacto-oligosaccharides (GOS) are indigestible dietary fibers that are able to reach the lower gastrointestinal tract to be selectively fermented by health-promoting bacteria. In this report, we describe the heterologous expression of an optimized synthetically produced version of the β-hexosyltransferase gene (*Bht*) from *Sporobolomyces singularis*. The *Bht* gene encodes a glycosyl hydrolase (EC 3.2.1.21) that acts as galactosyltransferase, able to catalyze a one-step conversion of lactose to GOS. Expression of the enzyme in *Escherichia coli* yielded an inactive insoluble protein, while the methylotrophic yeast *Pichia pastoris* GS115 produced a bioactive β-hexosyltransferase (rBHT). The enzyme exhibited faster kinetics at pHs between 3.5 and 6 and at temperatures between 40 and 50°C. Enzyme stability improved at temperatures lower than 40°C, and glucose was found to be a competitive inhibitor of enzymatic activity. *P. pastoris* secreted a fraction of the bioactive rBHT into the fermentation broth, while the majority of the enzyme remained associated with the outer membrane. Both the secreted and the membrane-associated forms were able to efficiently convert lactose to GOS. Additionally, resting cells with membrane-bound enzyme converted 90% of the initial lactose into GOS at 68% yield (g/g) (the maximum theoretical is 75%) with no secondary residual (glucose or galactose) products. This is the first report of a bioactive BHT from *S. singularis* that has been heterologously expressed.

According to the latest definition by the International Scientific Association for Probiotics and Prebiotics (ISAPP), "a dietary prebiotic is a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health" (1). Galacto-oligosaccharides (GOS) are nondigestible owing to the conformation of their anomeric C atom (C_1 or C_2), which allows their glycosidic bonds to evade hydrolysis by digestive enzymes. Free oligosaccharides are found in the milk of all placental mammals, providing a natural example of prebiotic feeding during infancy. The composition of human milk oligosaccharides (HMO) is very complex, which makes it unlikely that alternative sources containing oligosaccharides of analogous composition can be found. Improved colonic health among breastfed infants has been attributed to the presence of GOS in the mother's milk (2). In fact, infant formula with added GOS replicated the bifidogenic effect of the human milk with respect to metabolic activity of colonic microbiota and bacterial numbers (3, 4). Among nonmilk oligosaccharides, GOS are of special interest, as their structure resembles the core molecules of HMOs (5). However, GOS concentration and composition vary with the method and the enzyme utilized for their generation, which in turn may influence their prebiotic effects and the proliferation of colonic probiotic strains (6). Traditionally, GOS have been produced using β-galactosidases from mesophilic microorganisms. Mesophilic β-galactosidases require high initial concentrations of lactose to evade lactose hydrolysis and promote GOS synthesis. Since lactose is more soluble at elevated temperatures, thermostable β-galactosidases exhibiting high initial velocities and increased half-lives have been utilized to reach a favorable equilibrium for the transgalactosylation reaction (7, 8). However, competitive inhibition by glucose and/or galactose remains an obstacle that may be overcome by incorporating cells into the reaction mixture (7, 9–12).

The basidiomycete yeast *Sporobolomyces singularis* (formerly *Bullera singularis*) cannot utilize galactose to grow but proliferates on lactose due to the activity of its β-hexosyltransferase (BHT; EC 3.2.1.21). Studies have shown that the BHT has transgalactosylation activity even at low lactose concentrations and very limited lactose hydrolysis. In addition, the enzyme does not appear to be inhibited by lactose concentrations above 20% and has the potential for conversions into GOS close the maximum theoretical of 75% (13–16). Unlike β-galactosidases, the BHT from *S. singularis* simultaneously carries out glycosylhydrolase and β-hexosyltransferase activities, converting lactose to GOS without extracellular accumulation of galactose. Two molecules of lactose are required during the transgalactosylation event: one molecule is hydrolyzed, and the second acts as a galactose acceptor, generating the trisaccharide galactosyllactose [β-D-Gal(1-4)-β-D-Gal(1-4)-β-D-Glc] and residual glucose. Galactosyl lactose can also act as an acceptor of a new galactose to generate the tetrasaccharide galactosylgalactosyl lactose [β-D-Gal(1-4)-β-D-Gal(1-4)-β-D-Gal(1-4)-β-D-Glc], and similar reactions take place for the tetrasaccharide and subsequent products. The tri-, tetra-, and pentasaccharides that accumulate in *S. singularis* have been collectively designated GOS (14, 15).

For practical applications, a recombinant secreted BHT could have several advantages over the native enzyme, including improved large-scale production and purification. Currently, purification of active enzyme from *S. singularis* requires cell lysis followed by multiple chromatography steps (9, 13, 17). Previous

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description or genotype	Source or reference
Strains		
E. coli		
BL21	F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3)	Novagen
BLR	F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm*Δ(*srl-recA*)*306*::Tn*10*(Tet^r)(DE3)	Novagen
CC118	F2 Δ(*ara-leu*)*7697 araD139* Δ(*lac*)*X74 phoAD20 galE galK thi rpsE rpoB argE*(*Am*) *recA1*	18
NovaBlue	*endA1 hsdR17*(r_{K12}^- m_{K12}^+) *supE44 thi-1 recA1 gyrA96 relA1 lac* F′ [*proA*$^+$*B*$^+$ *lacI*^{q} ZΔ*M15*::Tn*10*] (Tet^r) (DE3)	Novagen
Origami	Δ(*ara-leu*)*7697* Δ*lacX74* Δ*phoA PvuII phoR araD139 ahpC galE galK rpsL* F′ [*lac*$^+$ *lacI*^{q} *pro*] *gor522*::Tn*10 trxB* (Kan^r Str^r Tet^r) (DE3)	Novagen
Rosetta	F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* pRARE2 (Cam^r)(DE3)	Novagen
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}ZΔM15 Tn*10* (Tet^r)]	Stratagene
P. pastoris		
GS115	*his4* (His$^-$ Mut$^+$)	Invitrogen
GS115/*lacZ*	GS115 *his4*::*lacZ* (*E. coli* β-galactosidase 117 kDa) intracellularly [His$^+$ Mut$^+$])	Invitrogen
KM71	GS115 *arg4 his4 aox1*::*ARG4* (His$^-$ Mut^s)	Invitrogen
JB208 (GS115/*rBht*)	GS115 integrated with plasmid pJB108 (His$^+$ Mut$^+$)	This study
Plasmids		
E. coli		
pJB100	pGS21a-rBht	This study
pET24d	Optional C-terminal 6-His tag, T7*lac* promoter, Kan^r	Novagen
pJB101	pET24d-rBht-6His	This study
pJB102	pET24d-TET promoter-rBht-6His	This study
pET41a	GST tag, T7*lac* promoter, Kan^r	Novagen
pJB103	pET41a-GST-rBht	This study
pET32a	Trx tag, T7lac promoter, Amp^r	Novagen
pJB104	pET32a-Trx-rBht	This study
pET22b	PelB coding sequence, T7lac promoter, Amp^r	Novagen
pJB105	pET22b-pelB-rBht	This study
pET39b	DsbA tag coding sequence, T7lac promoter, Kan^r	Novagen
pJB106	pET39b-DsbA-rBht	This study
pJB107	pUC57-rBht	This study
P. pastoris		
pPIC9	*P. pastoris* expression plasmid carrying *AOX1* promoter and transcription terminator, *HIS4*, Amp^r in *E. coli*, PBR322 ori, α-factor secretion signal from *S. cerevisiae*	Invitrogen
pJB108	pPIC9-αMF-6-His-TEV-rBht[a]	This study

[a] αMF, *S. cerevisiae* α mating factor secretion signal.

attempts to express recombinant β-hexosyltransferase in *Escherichia coli* BL21 have resulted in high levels of production, but the enzyme was inactive and insoluble (9). This study reports expression of the *S. singularis* BHT using a codon-optimized, synthetic *Bht* gene (GenBank accession number JF29828) expressed in *Pichia pastoris*. We investigated the kinetics of GOS production from lactose by the secreted recombinant β-hexosyltransferase (rBHT) compared to *P. pastoris* resting cells harboring membrane-bound rBHT.

MATERIALS AND METHODS

Design of a codon-optimized β-hexosyltransferase gene. The DNA coding sequence for the *S. singularis* β-hexosyltransferase gene (9) (GenBank accession number AB126324; 1,782 bp) was assembled by joining exons using Clone Manager software (Scientific & Educational Software, Cary, NC). The coding sequence was redesigned based on *P. pastoris* and *E. coli* preferred codons and optimized for minimum free energy (−619.9), specific restriction sites (5′ NcoI and 3′ NotI), and GC content (48.89%). This redesigned version of the gene was labeled r*Bht* (GenBank accession number JF29828), synthesized, and inserted into pGS21a and pUC57 to generate pJB100 and pJB107, respectively (Table 1). The DNA sequence of r*Bht* was confirmed (GenScript, Piscataway, NJ).

Construction of plasmids containing r*Bht* for expression in *E. coli*. Cloning procedures were carried out as previously described (19). *E. coli* strains used for cloning and expression of r*Bht* are listed in Table 1. Restriction endonucleases and T4 DNA ligase were obtained from New England BioLabs (Beverly, MA). The plasmids pJB100 and pJB107 were digested with NcoI and NotI, and the fragment containing the r*Bht* gene was inserted into Novagen plasmids to generate the expression plasmids pJB101, pJB103, pJB104, pJB105, and pJB106 (see Table 1 for a description of the constructions).

Expression of rBHT fusion constructions in *E. coli* BLR. The expression was carried out as described in the pET system manual (TB055; Novagen). Briefly, the expression plasmids were transformed into *E. coli* BLR and, after IPTG (isopropyl-β-D-thiogalactopyranoside) induction, screened for rBHT activity. *In vivo* rBHT activity was assessed by incubating IPTG-induced BLR cells in 50 mM sodium phosphate buffer at pH 4 and pH 6, and 50 μg of the cell-penetrating chromogenic substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) ml^{-1} (Table 2).

TABLE 2 Primers, antibodies, and substrates used in this study

Primer, antibody, or substrate	Open reading frame or description	Sequence[a]	Source
Primer			
JBB1	6-His-TEV-r*Bht* forward primer	5′-ccgCTCGAGAAAAGAGAGGCTGAAG CTCACCACCACCACCACCACGAAA ACCTGTATTTTCAGATGATGCTGC ATGCTGCAC-3′	This study
JBB2	r*Bht* reverse primer	5′-aaggaaaaaaGCGGCCGCTTACAGATG ATTACGCCCAAATTG-3′	This study
JBB3	r*Bht* forward internal sequencing primer	5′-ATCACTATGCCAGCACGCAGTGTA-3′	This study
JBB4	r*Bht* reverse internal sequencing primer	5′-TTTAAAGCCGATTTCACCTGCCGC-3′	This study
5′ AOX1 sequencing primer	AOX1	5′-GACTGGTTCCAATTGACAAGC-3′	Invitrogen
3′ AOX1 sequencing primer	AOX1	5′-GCAAATGGCATTCTGACATCC-3′	Invitrogen
α-Factor sequencing primer	α-Factor	5′-TACTATTGCCAGCATTGCTGC-3′	Invitrogen
Antibody	Antigen		
Mouse anti-HIS	6-His tag		Qiagen
Rabbit anti-Bgal	*E. coli* β-galactosidase		Sigma
Rabbit anti-rBHT	β-Hexosyltransferase		This study
Substrate	Abbreviation		
*o*NP-β-D-glucopyranoside	ONP-Glu		Sigma
ONP-β-D-galactopyranoside	ONP-Gal		Sigma
PNP-β-D-glucopyranoside	PNP-Glu		Sigma
PNP-β-D-galactopyranoside	PNP-Gal		Sigma
5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside	X-Gal		Sigma

[a] Coding regions are capitalized, and restriction sites are underlined.

Cultures were incubated overnight at 37°C for visualization of the appearance of color. BL21 cells containing endogenous β-galactosidase activity were used as the positive control.

Production of anti-rBHT. Anti-rBHT antiserum was produced using 6-His-tagged rBHT expressed and purified from *E. coli* BLR cells harboring pJB101. Purification of 6-His-tagged rBHT present in the cell extract fraction was performed by using nickel agarose gel (Qiagen, Germany) chromatography according to the manufacturer's instructions (Qiagen Ni-NTA spin kit handbook 01/2008) and then further purified by electroelution from gels according to the manufacturer's instructions (Bio-Rad, Richmond, CA). The pure protein was used for rabbit immunization (Cocalico Biologics, Reamstown, PA). Additional antibodies used in the study are listed in Table 2.

Electrophoresis and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 4 to 12%) was routinely carried out in the Laemmli system (20). Proteins were visualized by Coomassie blue (Bio-Rad, Richmond, CA) or silver staining (Bio-Rad, Richmond, CA). SeeBlue Plus (Invitrogen, Carlsbad, CA) was used as a molecular mass marker. Immunoblot analysis on duplicate PAGE gels was carried out as previously described (21) except that detection was performed using alkaline phosphatase-conjugated goat anti-rabbit or goat anti-mouse antibodies (Rockland Immunochemicals, Gilbertsville, PA) and visualized using a premixed NBT (nitroblue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3′-indolylphosphate *p*-toluidine salt) solution (Sigma-Aldrich, St. Louis, MO).

Construction of a recombinant *P. pastoris* strain. The r*Bht* gene was PCR amplified from pJB107 using primers JBB1 and JBB2 to add XhoI and NotI restriction sites in frame with *Saccharomyces cerevisiae* pre-pro-alpha-mating factor signal sequence contained in pPIC9, followed by a 6-His tag, and a modified tobacco etch virus (TEV) protease cleavage site (Table 2). The PCR product was ligated into pPIC9 XhoI and NotI restriction sites to generate pJB108 (Table 1). Correct in-frame ligation was confirmed by sequencing (Sequatech, Mountain View, CA) using primers 5′ AOX1 and 3′ AOX1 (Table 2).

P. pastoris GS115 (Table 1) was electrotransformed with pJB108 linearized with SacI (Invitrogen's *Pichia* expression kit manual, version M) using a Bio-Rad gene pulser (Bio-Rad, Richmond, CA). Recombinants were selected on histidine-deficient regeneration dextrose (RDB-agar plates) at 30°C. His^+ colonies were randomly selected, and the genomic integration of the expression cassette was verified by PCR using primers 5′ AOX1, 3′ AOX1, and α-Factor (Table 2). The methanol utilization (Mut^+) phenotype of recombinant GS115/r*Bht* was determined according to the procedure outlined in Invitrogen's *Pichia* expression kit manual, version M.

rBHT production in *P. pastoris*. To select a high-level producer of the recombinant rBHT, six His^+ isolates were grown in yeast extract peptone dextrose medium (YPD) at 30°C and 250 rpm for 12 h and then used to inoculate 100 ml buffered glycerol complex medium (BMGY) to an initial optical density at 600 nm (OD_{600}) of 0.1. When the culture OD_{600} exceeded 10, methanol was added to a final concentration of 0.5% at 24-h intervals until the culture OD_{600} exceeded 50, after which methanol was added every 12 h. Medium samples were analyzed for presence of BHT activity and by Western blotting using rabbit anti-rBHT every 24 h to determine the optimal harvest time. The selected GS115/r*Bht* recombinant was routinely grown in 0.5 liter BMGY and induced with methanol for 6 days.

Purification of secreted rBHT. Culture supernatants (500 ml) were fractionated with ammonium sulfate. Precipitates between 60 to 80% ammonium sulfate were resuspended in 50 mM sodium phosphate buffer (pH 6). After desalting and concentrating with an Amicon molecular weight cutoff (MWCO) 15 membrane (Amicon Inc., Beverly, MA), the solution was applied to a 1/30 (5-ml) Mono Q pre-equilibrated column (quaternary amino ethyl) (Amersham Biosciences). The column was then washed with 50 ml of buffer and eluted with 3 column volumes of a linear gradient of sodium chloride from 0 to 0.2 M in 50 mM sodium phosphate buffer (pH 6.0) at a flow rate of 1 ml min^{-1}. The eluate was collected in 1-ml portions. The active fractions were pooled, concentrated, resuspended in 10 mM sodium phosphate (pH 6.8), and then applied to a Bio-Gel HT hydroxyapatite column (Bio-Rad, Richmond, CA) (1/10, 2 ml) pre-equilibrated with the same buffer, washed with 10 mM sodium phosphate (pH 6.8), and eluted with 50 mM sodium phosphate (pH 6.8).

The fractions with the highest specific activity contained pure rBHT with a specific activity of 8.2 U mg^{-1}. Enzymatic activity was assayed (described below) on all chromatography fractions, and purification steps were carried out at 25°C.

Determination of molecular mass. Culture medium concentrated 20-fold by ultrafiltration (0.5 ml) was applied to a 1/30 (18-ml) Superdex 200 size exclusion column (Amersham Biosciences) pre-equilibrated with 50 mM sodium phosphate buffer (pH 6.0), 0.1 M NaCl. Fractions of 0.5 ml were collected at a flow rate of 0.5 ml min^{-1} and assayed for rBHT activity using ONP (*o*-nitrophenol)-Glu as the substrate and by zymogram as described below. Elution of rBHT and molecular mass standards was monitored at 280 nm. The column was calibrated using the following molecules: thyroglobulin, 669 kDa; ferritin, 440 kDa; catalase, 232 kDa; lactate dehydrogenase, 140 kDa; bovine serum albumin, 67 kDa (GE Healthcare). The molecular mass of rBHT was extrapolated from a calibration plot of log molecular mass (*y* axis) versus elution volume (*x* axis). All chromatographic steps were carried out at 25°C.

Enzymatic activity assays. The initial reaction rate of rBHT was measured by a modification of the Kuby method (22, 23) under the established conditions. Briefly, the reactions were performed in a volume of 250 μl containing 1.3 mM ONP-Glu and 50 mM sodium phosphate buffer (pH 5). The assays were carried out for 10 min under the established conditions and stopped by adding 1 volume of 0.25 M Na_2CO_3. The reaction mixture containing boiled rBHT and substrate served as negative control. Assays were always performed in duplicate with a reliability of ±5%. Samples of cell-free broth and protein concentrates were obtained as described above. Resting cells (harboring membrane-bound rBHT) prewashed with 50 mM sodium phosphate buffer (pH 5.0) were assayed, under established conditions. When X-Gal was the substrate of the reaction, the concentration was 50 μg ml^{-1} in 50 mM sodium phosphate buffer (pH 4).

One unit of enzyme activity equals 1 μmol of *o*-nitrophenol released per min under the assay conditions. Specific activity is defined as units/mg protein. Molar extinction coefficients of *o*-nitrophenol were as follows: 0.033 mM^{-1} cm^{-1}, pH 4; 0.036 mM^{-1} cm^{-1}, pH 5; 0.038 mM^{-1} cm^{-1}, pH 6. The amount of *o*-nitrophenol released was extrapolated from a calibration plot of the *o*-nitrophenol absorbance at 405 nm (*y* axis) versus *o*-nitrophenol concentration (*x* axis).

Enzymatic activities were also visualized by zymograms. Native PAGE were performed using a modification of the protocol described by Gallagher (24). Proteins from *E. coli* lysates or *P. pastoris* supernatants were solubilized in 5% (wt/vol) sucrose–10 μg ml^{-1} bromophenol blue and separated in 6% native polyacrylamide gels, utilizing 50 mM sodium phosphate buffer as running buffer (pH 6). The gel was kept cool in a Mighty Small Hoefer electrophoresis apparatus where cold water was recirculated during electrophoresis at 60 mA for 5 h. After electrophoresis, the gel was rinsed twice in wash buffer (50 mM sodium phosphate buffer, pH 4.0) for 10 min. The zymograms were developed by laying filter paper soaked in wash buffer containing 50 μg ml^{-1} X-Gal on the gel for 24 h at 20°C. A blue precipitate defined the location of the enzyme.

Enzyme kinetics. Series of enzyme dilutions ranging from 0 to 1.2 U ml^{-1} were assayed in 50 mM sodium phosphate (pH 5) at 42°C. The enzymatic activity assay was initiated by adding 1.3 mM ONP-Glu, and absorbance at 405 nm was monitored for 1-min intervals for a total of 20 min. The experimental absorbance values were plotted against time, showing linear proportionality up to 0.6 U ml^{-1} for at least 20 min, while at enzyme concentrations above 1.0 U ml^{-1} the absorbance values plateaued prior to 5 min.

The Michaelis-Menten constants (K_m and V_{max}) of 0.2 U ml^{-1} rBHT (at 42°C) were determined by varying ONP-Glu from 0 to 10.4 mM in 50 mM sodium phosphate (pH 5) and measuring the initial reaction rate at 20°C, 30°C, 40°C, and 50°C. The kinetic constants at each temperature were determined with OriginPro 7.5 using nonlinear regression of the Hill equation with a Hill coefficient of 1. The values obtained under the established conditions were as follows (temperature, K_m, V_{max}): 20°C, 0.37 mM, 0.09 mM min^{-1}; 30°C, 0.48 mM, 0.12 mM min^{-1}; 40°C, 0.71 mM, 0.23 mM min^{-1}; and 50°C, 1.3 mM, 0.42 mM min^{-1}. The fitting coefficients of regression (R^2) were 0.9869, 0.99065, 0.99115, and 0.98996 at 20°C, 30°C, 40°C and 50°C, respectively.

Characterization of rBHT. Enzymatic activity assays were performed under the established conditions described above. The influence of pH on enzyme activity was tested in buffer solutions, including 50 mM sodium phosphate (pH 5.0 to 11.0), 50 mM citrate (pH 2.0 to 5.0), and 50 mM phosphate-citrate (pH 2 to 11) (see Fig. 2A). Competitive inhibition by monosaccharides (glucose and galactose) was examined by varying their concentrations in the reaction mixture (see Fig. 2C). Temperature and thermostability were determined by measuring residual activity at 20, 30, 40, 50, 60, 70 or 80°C (see Fig. 2B and D). Similarly, enzymatic activity assays were used to evaluate additives as potential inhibitors/activators. The following additives up to 10 mM were tested: chelating agent (EDTA), reducing agents (dithiothreitol [DTT], 2-mercaptoethanol [2-ME], and copper [Cu^{2+}]), and ions (monovalent cations, NH_4^+, Cs^+, K^+, Na^+, Li^+, and Rb^+; divalent cations, Ba^{2+}, Ca^{2+}, Co^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+}; trivalent cation, Ag^{3+}). Heavy metals (Co^{2+}, Fe^{2+}, and Zn^{2+}) were tested in 50 mM citrate buffer (pH 5.0) to avoid precipitation. Additionally, surfactants added to the reaction mixture at 1% (vol/vol) were: Triton X-100, Tween 20, Tween 80, and sodium dodecyl sulfate (SDS). Solvents tested at 10% (vol/vol) included ethanol, methanol, acetone, acetonitrile, PEG400, and glycerol.

GOS production and analysis. The standard transgalactosylation reactions, utilizing either purified rBHT or *P. pastoris* resting cells (harboring membrane-bound enzyme), were initiated by adding standardized amounts of enzyme (0.5 U g^{-1} lactose) or cells (μg g^{-1} lactose) in 5 mM sodium phosphate buffer (pH 5.0) to a similarly buffered solution containing lactose (22 g $liter^{-1}$ or 200 g $liter^{-1}$) at 30°C or 42°C.

Products and substrates of the reactions were analyzed by high-performance liquid chromatography (HPLC) (Shimadzu Corporation, Kyoto, Japan) under isocratic conditions at 65°C and at a 0.4-ml min^{-1} flow rate. The mobile phase was 5 mM sulfuric acid (H_2SO_4) using an Alltech IOA-1000 organic acids column (300 mm by 7.8 mm) (Alltech, IL) coupled to a refractive-index detector. The column was calibrated using galactosyl lactose (Carbosynth, Berkshire, United Kingdom), lactose, glucose, and galactose (Sigma-Aldrich, St. Louis, MO). The residual nonquantified GOS species (tetrasaccharide and pentasaccharide) are reported as signal intensity readings from the refractive index detector.

Nucleotide sequence accession number. The nucleotide sequence of the r*Bht* gene has been submitted to GenBank (accession number JF29828).

RESULTS

Expression of a recombinant β-hexosyltransferase (rBHT) in *E. coli*. *E. coli* BL21 is the most widely used host for heterologous protein production. Unfortunately, this host strain contains an active endogenous β-galactosidase that interferes with the evaluation of β-hexosyltransferase, designated rBHT (see Materials and Methods). After screening of different *E. coli* strains appropriate for pET-based expression systems, including BL21, BLR, NovaBlue, Origami, and Rosetta (Table 1), *E. coli* BLR was confirmed as lacking of endogenous β-galactosidase activity. *E. coli* CC118 (Δ*lacZ*) strain was used as a control (18).

The *rBht* gene was inserted into pET expression plasmids containing a C-terminal 6-His tag or one of the four solubility-enhancing coexpression partners (glutathione *S*-transferase [GST], thioredoxin [Trx], the PelB leader, and DsbA), resulting in pJB101, pJB103, pJB104, pJB105, and pJB106, respectively (Table 1). Transformation into *E. coli* BLR and induction with IPTG resulted in expression of inactive rBHT either in whole cells or in cell extracts. Immunoblotting analysis of the fusion proteins with rBHT antiserum detected all rBHT fusion proteins at their pre-

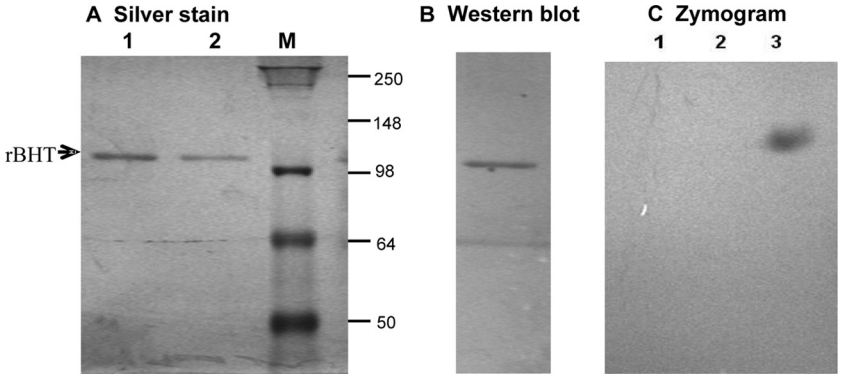

FIG 1 Gel electrophoresis of purified rBHT expressed in *P. pastoris*. (A) SDS-PAGE of purified, silver-stained rBHT. Lane 1, 0.5 μg rBHT; lane 2, 0.1 μg rBHT; lane M, marker proteins (molecular masses, in kilodaltons, are on the right). (B) Western blot analysis with anti-rBHT antiserum. (C) Zymogram of rBHT. Lane 1, purified rBHT-6XHis expressed in *E. coli* BLR cultures; lane 2, broth from untransformed methanol induced GS115; lane 3, broth from methanol induced recombinant GS115/*rBht*. Activity was visualized by the formation of a blue precipitate resulting from enzymatic cleavage of X-Gal.

dicted molecular masses, with the strongest reactivity observed in the insoluble fractions. To rule out possible host-dependent protein insolubility, rBHT expression was analyzed in *E. coli* CC118 harboring pJB102 (pJB101 with its T7 promoter replaced with a tetracycline [TET]-inducible promoter) but also proved to be unsuccessful (data not shown).

Expression and purification of rBHT from *P. pastoris*. *P. pastoris* is able to introduce posttranslational modifications and is well known for its ability to produce a number of active recombinant proteins (where *E. coli* fails to do so) (25). Thus, we inserted the codon-optimized *rBht* gene into pPIC9 under the control of the alcohol oxidase promoter (AOX1), in frame with the *S. cerevisiae* α-factor signal (sequence for protein secretion) and an N-terminal 6-His tag followed by a TEV protease cleavage site (pJB108, Table 1). *P. pastoris* GS115 was transformed with pJB108 (GS115/*rBht*), and the activity of rBHT was evaluated in six GS115/*rBht* recombinants. The recombinant strain secreting the highest concentration of bioactive protein was studied further. Zymograms confirmed the presence of an active rBHT: only GS115/*rBht* gave a positive signal, while cell extracts from *E. coli* BLR harboring pJB101 and culture supernatants from untransformed GS115 were negative (Fig. 1C). As expected, protein transmembrane regions in BHT also resulted in GS115/*rBht* cells displaying cell surface-associated rBHT activity, emulating the location of native BHT in *S. singularis* (9, 26).

Purification of rBHT was attempted using nickel affinity chromatography, but the His tag was not present, nor was the protein detected by Western blotting using anti-His antiserum, indicating possible processing of the N-terminal signal sequence at predicted cleavage sites (9). Subsequently, the rBHT enzyme was purified (specific activity of 8.2 U mg^{-1} at 20°C) using Mono Q and hydroxyapatite chromatography (Table 3).

Characterization of rBHT expressed in *P. pastoris*. (i) Apparent molecular mass of rBHT. The estimated molecular mass for a nonglycosylated, fully processed rBHT that included the 6-His and TEV protease site tag was 68 kDa. The enzyme has been previously purified as a dimer as well as a monomer with an apparent molecular mass ranging from 53 to 146 kDa, data that reflect variations in protein glycosylation (Table 3) (9, 13). Here, the enzyme activity eluted as one monomeric peak with an experimental apparent molecular mass of 110 kDa. We surmised that the dimeric form may predominate within the acidic range of the native enzyme's pH optimum (3.7 to 6) (Table 3). However, fractions from the Superdex 200 column at pH 4.0 depicted the same profile, confirming the stable monomeric form of the recombinant enzyme. No higher-molecular-mass aggregates were detected by enzyme activity assay, zymogram analysis, or Western blotting. Purification of the column fractions and immunoblot analysis using anti-rBHT verified that the enzyme migrated as a single band with an apparent molecular mass of 110 kDa (Fig. 1A and B).

(ii) Substrate specificity. ONP-Gal has traditionally been used as the substrate for β-galactosidases, and ONP-Gal, PNP-Gal, and PNP-Glu have all been used in previous studies for detection of native BHT activity (Table 3). The enzyme activities of rBHT (0.2 U ml^{-1}) were compared between the above substrates under the same experimental conditions. The recombinant enzyme was equally active in response to ONP-Glu and PNP-Glu. The substrates with a galactose in the glycan moiety were hydrolyzed at a rate of approximately 41% (ONP-Gal) and 23% (PNP-Gal) of that for ONP-Glu. These results indicate that rBHT has a narrow specificity with respect to the sugar and more flexibility for the configuration of the carbon linkage position at C-2 and C-4 when glucose sugar derivatives are used as substrates.

(iii) Optimum pH, temperature, and thermostability of rBHT. rBHT was active within a broad pH range (from pH 3.5 to 6), displaying the highest values at pH 4.0 (Fig. 2A). The enzymatic activity profile showed a steep decline to less than 50% maximal enzyme activity at pH values greater than 7 or less than 3.5. These results were consistent with reported pH optima (13; also, see references cited in Table 3), suggesting that alkaline conditions may have a detrimental effect on enzyme activity and stability.

The initial reaction rate measured at temperatures ranging from 20 to 80°C indicated that the enzyme was active over a temperature range from 20 to 50°C, with the optimum occurring between 40°C and 50°C (Fig. 2B). At temperatures below 30°C, a 50% reduction in the initial reaction rate was observed, and temperatures above 50°C quickly and irreversibly inactivated the enzyme. The optimum temperature when maximizing the enzyme reaction rate can also be obtained from the highest value of V_{max}/K_m (29). V_{max} increased at a higher rate than K_m at temperatures between 20 and 40°C; consequently, the V_{max}/K_m values

TABLE 3 Reports evaluating BHT from *Sporobolomyces singularis* for the production of galactooligosaccharides (GOS)[a]

Enzyme	Mol mass (kDa)	Sp act (U mg^{-1} enzyme)	Temp (°C)	pH	U g^{-1} Lac	L_{int}	L_{util}	C_{max} (Y)	Conditions	Reference
β-Transglycosylase			22	3.75–4.0		10		50	STR, growing cells	14
β-Hexosyltransferase			20	6.5		6	68	25 (36)	Cell extract	15
β-Hexosidase	140–145	41.2[b]	45–50	6.5		5			Purified enzyme	13
β-Galactosidase			45	3.7		30	73	54[f] (51)	Batch IE, partially purified enzyme	27
β-Galactosidase			45	4.8		10	70	55[f] (53)	Cont. IE, PBR, partially purified enzyme	27
β-Galactosidase			45	3.7	0.13	30	70	40 (57)	Partially purified enzyme	12
β-Galactosidase	53	56[c]	50	5.0	5.4	18	71	50 (70)	Purified enzyme	17
β-Galactosidase-like	146	8.69[d]	40	6.0	0.8	20			Purified enzyme	9
β-Galactosidase			55	5.0, 6.0		60	60	41.1 (68)	Batch, resting cells	28
β-Galactosidase			55	5.0, 6.0		60	60	40.4 (67)	RB IE Alginate, resting cells	28
β-Hexosyltransferase	110	8.2[e]	42	6.0	0.5	2	52	37[g] (71[g])	Purified enzyme	This study
β-Hexosyltransferase	110		42	6.0	0.5	20	52	36[g] (70[g])	Batch, recombinant resting cells	This study
β-Hexosyltransferase	110		30	6.0	0.5	20	69	51[g] (74[g])	Batch, recombinant resting cells	This study

[a] U g^{-1} Lac, units per gram initial lactose; L_{int}, initial lactose concentration (%); L_{util}, lactose utilized (%); C_{max}, maximum conversion of GOS (%) from initial lactose; Y, conversion % (total GOS formed from utilized lactose); STR, stirred tank reactor; IE, immobilized enzyme; PBR, packed bed reactor; Cont. IE, immobilized enzyme (continuous); RB IE, immobilized enzyme (repeated batch).
[b] Substrate was ONP-Gal.
[c] Substrate was PNP-Gal.
[d] Substrate was PNP-Glu.
[e] Substrate was ONP-Glu.
[f] GOS include disaccharides.
[g] GOS values reported were performed at the value of maximum accumulation of trisaccharide (galactosyl lactose).

($0.242\ min^{-1}$, 20°C; $0.255\ min^{-1}$, 30°C; $0.324\ min^{-1}$, 40°C; $0.322\ min^{-1}$, 50°C) increased over this range and were constant at temperatures between 40°C and 50°C. Thus, the optimum temperature as determined by V_{max}/K_m was within 40 to 50°C, confirming

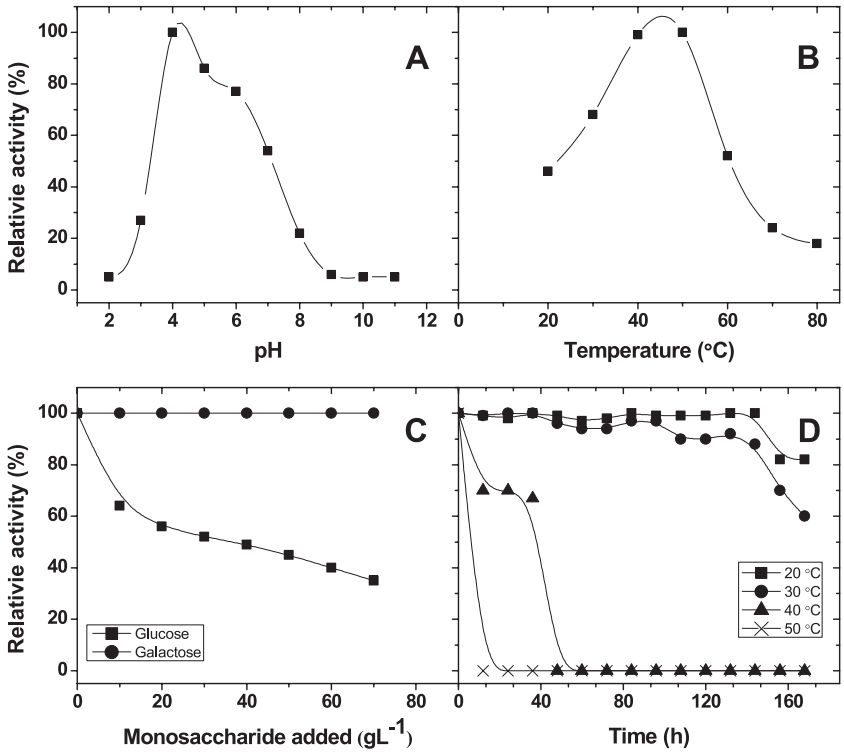

FIG 2 rBHT relative activity dependence on pH A) and (B) temperature (B). (C) Concentration of galactose and glucose. (D) Thermal stability at 20°C to 50°C. Samples were removed every 12 h and assayed for activity at 20°C. Enzyme activity assays were conducted in 50 mM sodium phosphate (pH 5.0) containing 1.3 mM ONP-Glu at 20°C, except for assays whose results are shown in panel A, which used sodium phosphate (pH 5 to 11) or citrate (pH 2to 5) buffers. Enzyme activities were calculated relative to the value taken at time zero (100%). The initial concentration of tested enzyme was 0.2 U ml^{-1} when assayed at 20°C (K_m = 0.37 mM and V_{max} = 0.09 mM min^{-1}). Data are means from two experiments, with a reliability of ±5%.

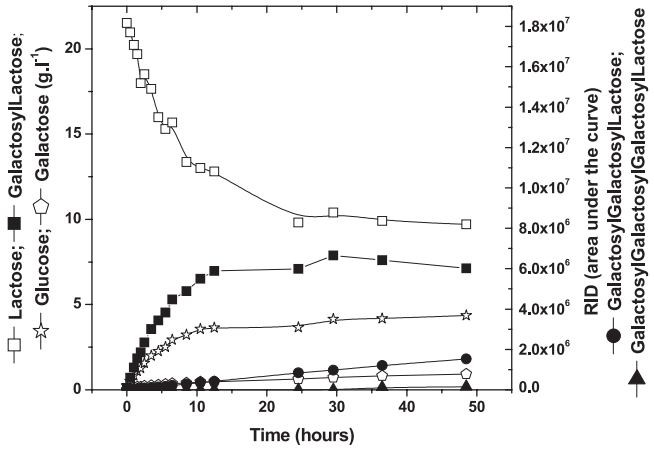

FIG 3 Synthesis of galactosyl lactose from lactose (2%) using partially purified rBHT (0.5 U g of lactose^{-1}) in 5 mM sodium phosphate buffer (pH 5.0) incubated at 42°C. Concentrations of lactose, glucose, galactose, and galactosyl lactose are in g liter^{-1}. The residual nonquantified GOS species are shown as signal intensity readings from the refractive-index detector. Data are means from two experiments, with a reliability of ±5%.

the optimal values established using the initial reaction rate values at each temperature.

As shown in Fig. 2D, the thermostability of rBHT was evaluated from 20 to 50°C. At 20°C and 30°C, the enzyme retained at least 90% of the original activity for 6 days, confirming previously reported results for the native enzyme (17). Five independent batches of rBHT stored for 6 months at 4°C retained 95% of the initial activity (data not shown). Although the optimal temperature was found in the 40 to 50°C range, incubation at temperatures above 40°C was deleterious to rBHT. At 40°C, the enzyme retained 70% of the initial activity at 12 h, and this level of activity persisted for only an additional 36 h. In contrast, enzyme activity decreased sharply at 50°C within the first 12 h of incubation.

(iv) Effects of metals, salts, surfactants, and solvents on rBHT activity. Enzyme inhibition/activation was tested within a broad range of additives. rBHT did not exhibit a requirement for any of the ions tested (NH_4^+, Ba^{2+}, Ca^{2+}, Cs^+, Co^{2+}, Cu^{2+}, Li^+, Rb^+, Mg^{2+}, Ni^{2+}, Na^+, and Zn^{2+}), even though magnesium dramatically increases the enzyme activity of some β-galactosidases (30). Moreover, the recombinant enzyme was fully active in the presence of 1 and 5 mM concentrations of the ion-chelating agent EDTA, confirming the above findings and a previous report (13). Additionally, compounds proven to disrupt disulfide bridges, such as Cu^{2+} and the reducing agents dithiothreitol (DTT) and 2-mercaptoethanol (2ME) (13, 31, 32), had no negative consequences on the activity. The solvents methanol, ethanol, acetone, and acetonitrile only partially inhibited the enzyme (retaining 66% to 81% relative activity). In contrast, the addition of 10% glycerol or 1% SDS (a nonionic surfactant) almost completely inhibited the enzyme.

GOS synthesis using purified rBHT. Once the enzyme was characterized, the secreted rBHT was tested for biotransformation of 2% lactose into GOS. The conditions of the reaction were 0.5 U rBHT g of lactose^{-1} at 42°C in 5 mM sodium phosphate buffer (pH 5.0). Figure 3 shows lactose consumption and GOS accumulation over time. The highest rate of production was observed during the first 12 h, and galactosyl lactose and glucose were the main products. Galactose was not detected, indicating that it was being completely incorporated in the transgalactosylation reaction to form GOS. After 30 h, 4.2 g liter^{-1} of glucose had accumulated and lactose utilization (54%) was at its maximum. Furthermore, at this time point, 7.8 g liter^{-1} of the trisaccharide had accumulated, reaching an average of 67% conversion of the utilized lactose. As the reaction proceeded, galactose began to escape the enzymatic reaction and accumulate at trace concentrations. Since competitive enzyme inhibition could reduce the efficiency of lactose biotransformation, we examined the effect of varying concentrations of glucose or galactose on enzyme activity in the reaction mixture. The presence of 5 g liter^{-1} glucose reduced rBHT activity up to 90%, whereas enzymatic activity was unaffected by up to 70 g liter^{-1} galactose, under the established conditions (Fig. 2C).

GOS synthesis by *P. pastoris* resting cells (harboring membrane-bound rBHT). To avoid competitive inhibition and confirm that conversion of lactose to GOS could be improved upon if glucose was simultaneously eliminated from the reaction mixture, we evaluated the biotransformation of 20% lactose by resting cells of *P. pastoris* GS115/r*Bht*. *P. pastoris* GS115/r*Bht* harboring membrane-bound rBHT were normalized to a cell density containing 0.5 U rBHT g^{-1} of lactose in 5 mM sodium phosphate buffer (pH 5). Reactions were conducted for 10 days at 30°C, the optimal temperature for growth of *P. pastoris*, and at 42°C, the temperature for which the initial reaction rate is at its maximum for the secreted rBHT. As expected, 90% of the initial lactose was converted to GOS, with no secondary products at 30°C, compared to 51% lactose utilization at 42°C. The results indicated that resting cells were physiologically active and able to consume the glucose by-product of the reaction, thereby avoiding competitive inhibition. However, the initial reaction rate of GOS formation at 42°C was double the rate at 30°C during the first 48 h. A final concentration of 80 g liter^{-1} galactosyl lactose was reached, corresponding to a productivity of 1.6 g liter^{-1} h^{-1} at 42°C (Fig. 4A). At 30°C, when the lactose utilization was at 63%, the concentration of galactosyl lactose was 100 g liter^{-1}, and a productivity of 0.8 g liter^{-1} h^{-1} was reached after 5 days (Fig. 4B).

DISCUSSION

In this study, we optimized the DNA sequence of the β-hexosyltransferase gene from *S. singularis* (accession number AB126324) for expression in *E. coli* and *P. pastoris*. The resulting *rBht* gene was synthetically generated (accession number JF29828) and expressed in *E. coli*. However, this bacterial host lacked the ability to incorporate posttranslational modifications essential for producing a soluble and active rBHT, as previously observed (9). Subsequently, the r*Bht* gene under the control of the *AOX1* promoter was successfully integrated into the *P. pastoris* chromosome, resulting in the expression of a fully active enzyme that was detected in the culture broth as well as associated with the cell surface. Secretion of rBHT by *P. pastoris* GS115 allowed us to avoid the complex purification processes that are required to obtain pure BHT from *S. singularis*. Furthermore, since *P. pastoris* naturally secretes only very low levels of native proteins, recovery of the extracellular rBHT was as simple as removal of whole cells from the medium by centrifugation or filtration (33).

The molecular mass of the recombinant enzyme corresponded to a single 110-kDa catalytically active polypeptide, and no smaller

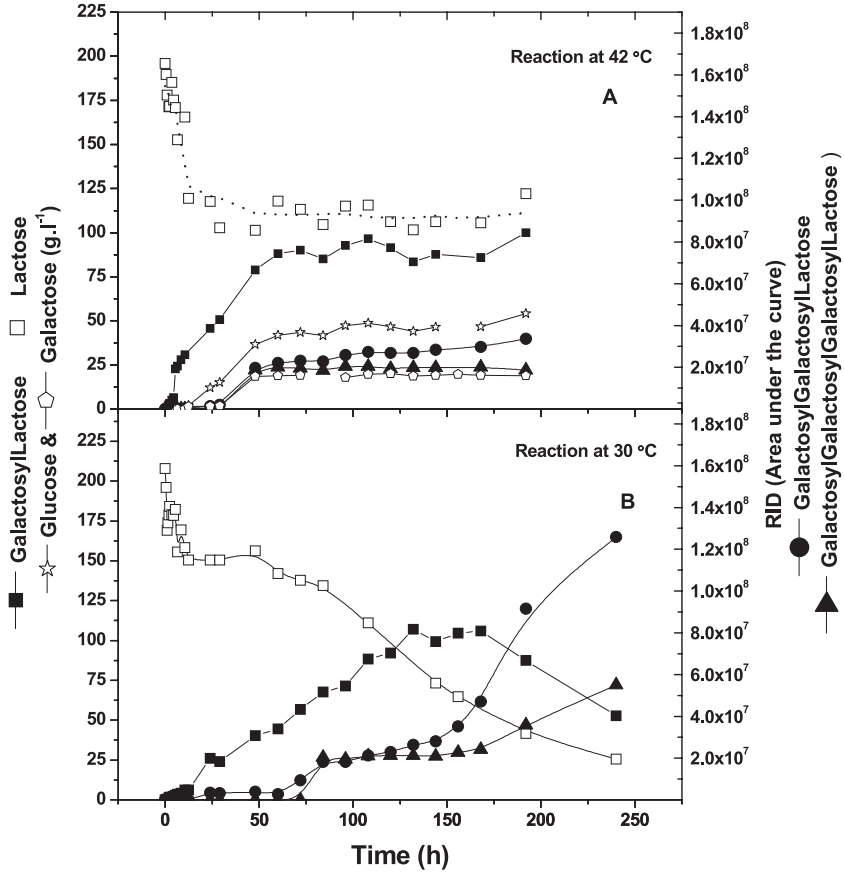

FIG 4 Synthesis of galacto-oligosaccharides from lactose (20%) using *P. pastoris* resting cells (harboring membrane-bound rBHT at 0.5 U g^{-1} lactose) in 5 mM sodium phosphate buffer (pH 5.0) incubated at 42°C (A) or 30°C (B). Concentrations of lactose, glucose, galactose, and galactosyl lactose are shown in g $liter^{-1}$. The residual nonquantified GOS species are shown as signal intensity readings from the refractive index detector. Data are means from two experiments, with a reliability of ±5%.

polypeptides or rBHT aggregates were detected. Posttranslational modifications play a critical role in protein folding, structural stability, oligomer formation, and substrate recognition (34, 35), so it was not surprising that the molecular mass was higher than the 68-kDa mass of the protein expressed in *E. coli* and predicted by the amino acid sequence. Posttranslational glycosylation of the native BHT by *S. singularis* has been previously reported, and a shift in the molecular mass of the purified protein from 73.9 to 66.3 kDa was observed after treatment with chitinase and EndoHf (9). Future mutagenesis of the predicted glycosylation sites should aid in determining whether glycosylation is also the cause for the shift in rBHT molecular mass.

The data reported here are part of a larger study that compared the utilization of rBHT to documented data for the native *S. singularis* BHT (Table 3). Our study confirmed that the recombinant enzyme does not require cofactors or reducing agents, which are often essential for β-galactosides. The enzyme showed better thermostability at lower temperatures (below 40°C) and optimal activities at temperatures from 40 to 50°C and pH 3.5 to 6. Additionally, the enzyme was controlled by glucose inhibition, though rBHT was not sensitive to galactose or Ag^{3+}, as previously reported for native BHT (9, 11, 12, 17).

Lactose utilization and initial lactose concentration are two key factors that contribute to maximizing the final GOS accumulation. Here, we demonstrated a process with improved lactose utilization (90%) employing physiologically active resting cells of *P. pastoris* GS115/r*Bht*. Under these conditions, the cells consume residual glucose at 30°C, circumventing glucose inhibition and ensuring a significant process improvement and a higher degree of final prebiotic purity (Fig. 4B). In contrast, temperatures higher than 25°C were reported as preventing resting *S. singularis* cells containing membrane-bound native BHT from consuming residual glucose, which in turn limits final GOS concentration and purity (reviewed by Gosling et al. [36]).

GS115/r*Bht* resting cells incubated at 42°C converted only 51% of the initial lactose into galactosyl lactose and residual glucose (Fig. 4A). These data closely resembled galactosyl lactose formation by secreted rBHT under the same conditions (Fig. 3). Furthermore, conversion and utilization values were comparable to those previously reported for processes by *S. singularis* (Table 3). Typical lactose utilization values of 30 to 40% of the initial lactose concentration have been reported (36), with one study reporting 50% lactose utilization, using 10.8 times more enzyme per gram of lactose than secreted rBHT (Table 3) (17).

The discovery of GOS synthesis by *S. singularis* in the mid-20th century has encouraged the exploration for superior β-galactosidases that more efficiently produce GOS (8, 14, 15). However, the enzymes studied showed lower lactose utilization values and

higher final concentrations of undesirable by-products compared to the BHT from *S. singularis* (7, 9, 12, 13, 17, 27, 37). Nevertheless, advances in the industrial utilization of *S. singularis* BHT have been slower than desired due to the challenging multistep purification processes required to obtain pure native BHT (9, 13, 17). The bioactive rBHT either secreted or membrane bound enzyme from *P. pastoris* signifies a clear process advantage for producing GOS. Future studies will explore protein modification strategies to improve protein expression yield, protein stability, and enzyme activity.

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