Cloning, Expression and Characterization of Xylose Isomerase from the Marine Bacterium *Fulvimarina pelagi* in *Escherichia coli*

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Production of a xylose isomerase (XI) with high tolerance to the inhibitors xylitol and calcium, and high activity at the low pH and temperature conditions characteristic of yeast fermentations, is desirable for a simultaneous isomerization/fermentation process for cellulosic ethanol production. A putative XI gene (xylA) from the marine bacterium Fulvimarina pelagi was identified by sequence analysis of the F. pelagi genome, and was PCR amplified, cloned, and expressed in Escherichia coli. The rXI was produced in shake flask and fed-batch fermentations using glucose as the growth substrate. The optimum pH for rXI was approximately 7, although activity was evident at pH as low as 5.5. The purified rXI had a molecular weight in 160 kDA, a V_{max} *of 0.142 U/mg purified rXI, and a* K_M *for xylose in the range of 1.75–4.17 mM/L at pH 6.5 and a temperature of 35°C. The estimated calcium and xylitol* K_I *values for rXI in cell-free extracts were 2,500 mg/L and >50 mM, respectively. The low* K_M *of the F. pelagi xylose isomerase is consistent with the low nutrient conditions of the pelagic environment. These results indicate that* Ca^{2+} *and xylitol are not likely to be inhibitory in applications employing the rXI from F. pelagi to convert xylose to xylulose in fermentations of complex biomass hydrolysates. A higher* V_{max} *at low pH (<6) and temperature (30°C) would be preferable for use in biofuels production.* © 2016 American Institute of Chemical Engineers *Biotechnol. Prog.*, 000:000–000, 2016
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Introduction

Xylose isomerases are important commercial enzymes used in the high-fructose corn syrup industry, and are becoming of increasing importance in cellulosic ethanol production for use as fuels. Identifying new sources of xylose isomerases, and modifying known xylose isomerases to produce enzymes with biochemical characteristics compatible with industrial manufacturing processes, are research areas that have benefited from new methods for isolating and cultivating previously unculturable microorganisms and molecular methods for amplifying and cloning xylose isomerases directly from the environment. The marine environment is a potential source of novel enzymes of commercial interest, and these enzymes may be present in bacteria that are typically slow growing and difficult to culture.

Xylose isomerase (EC 5.3.1.5), also known as glucose isomerase, catalyzes the reversible isomerization of xylose and glucose to xylulose and fructose, respectively. Bacteria employ xylose isomerase to convert xylose to xylulose,[1] whereas fungi typically convert xylose to xylulose in a two-step processes using the enzymes xylose reductase (XR, EC

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1.1.1.21) and xylitol dehydrogenase (XDH, EC 1.1.1.9).[2] Xylose isomerases are used extensively in the high-fructose corn syrup industry. Xylose isomerase, referred to as glucose isomerase in this context, is a high value industrial enzyme used for the conversion of glucose to fructose to increase the sweetness of corn syrup. The most effective commercial enzymes have high glucose affinity and activity at high pH (7.5) and temperature (60°C). These conditions provide high rates of conversion of glucose to fructose, and yield high equilibrium fructose concentrations.[3] Commercially available xylose isomerases can be produced inexpensively and used as immobilized enzymes from crude cell lysates.

Xylose isomerases have also been investigated for use in biofuels production from cellulosic biomass. Cellulosic biomass is composed of three major components: cellulose, hemicellulose, and lignin. The cellulose fraction can be readily converted to glucose using cellulases, and then fermented to ethanol, but efficient use of the hemicellulose and lignin fractions remains elusive. The hemicellulose can be converted to xylose by xylanases, but this pentose sugar is not fermented by *Saccharomyces cerevisiae*, which has a high rate of glucose fermentation and ethanol tolerance. Although this yeast does not ferment xylose, it can utilize xylulose.[4] Xylose can be converted to ethanol in yeast fermentations when xylose isomerase is added to the medium.[5] This observation has resulted in numerous attempts to express bacterial xylose isomerase genes in yeast for the purpose of producing ethanol from xylose. Most of the known xylose isomerases have not been successfully expressed in yeast, probably due to misfolding of the recombinant bacterial protein,[6] but considerable progress has been made using more recently discovered xylose isomerases from anaerobic cellulolytic fungi and a rumen metagenomic library.[7–10]

An alternative approach to xylose utilization for ethanol production is to add extracellular xylose isomerases directly to the medium. The potential use of extracellular xylose isomerases to convert xylose to xylulose for yeast fermentations has been previously investigated using commercial preparations such as Sweetzyme IT Extra (*Streptomyces murinus*; Sigma-Aldrich, St. Louis, MO), as well as xylose isomerases derived from other sources.[11–17] Conversion processes can be designed to provide ideal conditions for xylose isomerase activity (70°C, pH > 7) separately from the yeast fermentation (30°C, pH 5). These processes result in more complete use of lignocellulosic biomass for ethanol production, but the required temperature and pH transitions add to the costs. Given the typical equilibrium ratio of xylose to xylulose (4:1) for xylose isomerases, addition of sodium tetraborate is required to achieve high equilibrium xylulose concentrations.[14,18] An alternative approach to drive the xylose to xylulose conversion forward is to remove xylulose as it is formed by metabolism to ethanol by yeast. To achieve the potential advantages of a simultaneous isomerization/fermentation system, extracellular xylose isomerases that operate effectively at the low pH and temperature conditions permissive for yeast fermentations are needed, including high activity and stability at pH < 6 in the temperature range of 30–35°C. Insensitivity to the inhibitor calcium, which is present in biomass hydrolysates, and xylitol, which is produced during fermentation in xylose/xylulose media, are also advantageous. Currently, there are no known xylose isomerases that completely meet these criteria. The effectiveness of this approach for xylose to ethanol conversion may be increased by the discovery of novel enzymes that possess specific attributes compatible with yeast fermentation conditions, and can serve as possible sources of information for rational enzyme design.

F. pelagi is a slow growing marine bacterium isolated from the Sargasso Sea using a high-throughput culture method developed to isolate previously uncultured microorganisms.[19] *F. pelagi* is most closely related to another marine bacteria, *Aurantimonas coralicida* (93% similarity), as determined by 16S rDNA sequence comparison. Together these organisms form a distinct and deep evolutionary lineage of descent within the order "*Rhizobiales*" of the *α-Proteobacteria*, and members of this order are not commonly found in marine environments.[20]

In this study, a putative xylose isomerase encoding gene (*xylA*) was identified in the genome of *F. pelagi*.[21] The encoded xylose isomerase was characterized biochemically, and enzyme properties relevant to biofuels production were investigated. The xylose isomerase gene was cloned into *Escherichia coli* to verify the gene sequence as a xylose isomerase, and production and purification methods were developed for this enzyme. Kinetic parameters were determined, and effects of pH, temperature, and Ca^{2+} and xylitol concentrations on xylose isomerase activity were investigated.

Materials and Methods

Chemicals and enzymes

All chemicals were reagent grade and were purchased from Sigma-Aldrich (St Louis, MO) and other major chemical suppliers (VWR, Radnor, PA). Restriction endonucleases, T4 DNA ligase and alkaline phosphatase were purchased from New England Biolabs (Ipswich, MA). Platinum *Pfx* DNA polymerase, nucleotides, and antibodies were purchased from Invitrogen (Carlsbad, CA). Dialysis cassettes, HisPur cobalt affinity columns, and chromatography gels were obtained from Thermo Fisher Scientific (Waltham, MA).

Bacterial strains and culture conditions

F. pelagi HTCC2506^T was obtained from the laboratory of Dr Stephen J. Giovannoni at Oregon State University. The draft genome sequence of HTCC2506^T is available at GenBank accession number AATP00000000 and Marine Microbial Genomics at Oregon State University (http://bioinfo.cgrb.oregonstate.edu/microbes/). *F. pelagi* was cultured on Fp medium (in g/L tap H_2O; Instant Ocean Sea Salt 30, K_2HPO_4 0.02, NH_4Cl 0.05, peptone 0.5, yeast extract 0.5, xylose 3) in shake flasks at 30°C. *E. coli* Mach1^{TM}-T1^R (Invitrogen, Carlsbad, CA) and Rosetta-gami 2 (DE3) (Novagen, Darmstadt, Germany) were cultured on Luria-Bertani, Miller medium (Amresco, Solon, OH) supplemented with 15 μg/mL kanamycin and 1.5% agar as needed.

DNA extraction, amplification, and cloning

Genomic DNA was extracted and purified from *F. pelagi* cultures using the DNeasy Blood and Tissue Kit (Qiagen GmbH, Hilden, Germany). The *F. pelagi* XI gene (*xylA*) (>gi/114537918/gb/EAU41041.1/xylose isomerase [*F. pelagi* HTCC2506]) was PCR amplified using Platinum *Pfx* DNA polymerase and the primers fXIpET (GTCCAAGGATCCAC GTCCCAGTTCTTCGG) and XIpETr (GCACGACCTCGAGG ACATACCTGTTGAGAATG) according to manufacturer's instructions (Invitrogen, Carlsbad, CA). PCR products and

restriction digested DNA products were purified using the Wizard DNA Clean-Up System (Promega, Madison, WI).

To construct the *E. coli* XI pET-24a(+) expression vector, the PCR fragments were *Dpn*I treated, purified, and cut with the restriction enzymes *Xho*I and *Bam*HIHF. The pET-24a(+) vector was also treated with *Xho*I and *Bam*HIHF, as well as CIP, and the PCR amplified XI gene was cloned in frame with the N-terminal T7-Tag and the C-terminal 6X His-Tag encoded by the vector.

After ligation, the DNA mixture was transformed into chemically competent *E. coli* (One Shot Mach1^{TM}-T1^{R}) cells, and plated on LB agar plates containing 50 μg/mL kanamycin. Several transformants were selected for cultivation on the same medium, plasmid extraction with the PureYield Plasmid Miniprep System (Promega, Madison, WI), and analysis by restriction digests for the correct plasmid insert size and orientation. These expression vectors were subsequently maintained in *E. coli* Mach1^{TM}-T1^{R} cells stored in 15% glycerol at −80°C.

Sequence and phylogenetic analysis

Plasmid constructs were sequenced to ensure DNA sequence fidelity and correct fusion with the flanking plasmid encoded DNA sequences. DNA sequencing was conducted by the Center for Genome Research and Biocomputing (CGRB, Oregon State University), and DNA sequences were compared, translated and analyzed using BLAST (National Center for Biotechnology Information, www.ncbi.nlm.nih.gov), ExPASy (Swiss Institute of Bioinformatics, www.isb-sib.ch), and a Rare Codon Calculator (NIH NBI Laboratory for Structural Genomics and Proteomics, www.nihserver.mbi.ucla.edu). The *F. pelagi* xylose isomerase amino acid sequence was compared with sequences in GenBank using the BLAST algorithm. Multiple xylose isomerase sequences were aligned using Clustal omega (EMBL-EBI).[22] Phylogenetic analysis was performed using the neighbor-joining method with MEGA 6.06 software.[23]

Expression in E. coli

Plasmid DNA was extracted and purified from *E. coli* Mach1^{TM}-T1^{R} cells containing the pET-24a(+) expression vector with the cloned *F. pelagi* XI gene, as well as the control expression vector pET-24a(+) with no insert, using the PureYield Plasmid Midiprep System (Promega, Madison, WI). The plasmids were then transformed into chemically competent Rosetta-gami 2 (DE3) cells, and plated on LB agar plates containing 15 μg/mL kanamycin.

Transformants were cultivated in shake-flasks containing LB medium supplemented with the following (per L): 28 g Na_2HPO_4, 10 g glucose, and 0.25 g $MgSO_4{\cdot}7H_2O$. The medium was pH adjusted to 7 with HCl, and amended with 15 μg/mL kanamycin before filter (0.2 μm) sterilization. This medium was inoculated from −80°C frozen stock cultures of *E. coli*. Shake flask cultures (100 mL) were incubated overnight at 25°C and 150 rpm, and then 25 mL was transferred to a 1 L flask containing 250 mL of the same medium. Cultivation was continued at 30°C, 200 rpm until an optical density at 600 nm (OD_{600}) of 0.3–0.4 was achieved. The cultivation temperature was decreased to 25°C and IPTG (0.218 g/L) added to induce rXI expression. After 7 h the cells were harvested by centrifugation (3,273*g*, 4°C, 30 min), washed with 100 mM Mes pH 6.5 buffer, and concentrated by centrifugation. The final cell pellets (~1.4 g wet weight cells per 250 mL culture) were frozen and stored at −20°C in 50 mL plastic centrifuge tubes.

Protein quantification, electrophoresis, and molecular mass determination

Frozen cell pellets were thawed, resuspended in 25 mL ice-cold 100 mM Mes pH 6.5, transferred to 15 mL glass scintillation vials, and sonicated in an ice-water bath using the large tip on a Qsonica sonicator (Qsonica, Newton, CT). The sonication parameters were as follows: amplitude 30, process time 1 min, pulse-on time 1 s, pulse-off time 1 s. Three cycles were performed with a 2 min delay on ice between each cycle for cooling. Cell debris was removed by centrifugation (16,100*g*, 4°C, 30 min) to yield the cell-free extract (CFE) containing the soluble rXI activity.

Protein concentration was determined using the Coomassie Plus Protein Assay Reagent (Thermo Fischer Scientific, Waltham, MA) and a bovine serum albumin standard curve. SDS-PAGE and western blots were performed using a Mini-PROTEAN 3 and a Mini Trans-Blot Electrophoretic transfer cell (Bio-Rad Laboratories, Hercules, CA), respectively. Precise Protein Gels (4–20%) (Thermo Fisher Scientific, Waltham, MA), GelCode Blue Stain Reagent, and pre-stained molecular weight markers were used for separation, detection, and identification of proteins, respectively. Anti-His antibodies (Invitrogen, Carlsbad, CA) were used to visualize the rXI (XylA). The molecular mass of the rXI was determined by size exclusion chromatography using a Sephacryl S-200 HR column (GE Healthcare, Chicago, IL) equilibrated and operated with 20 mM Mes, 150 mM NaCl, pH 6 at a volumetric flow rate of 4 mL/min and gel filtration markers (12–200 kDa, Sigma-Aldrich, St. Louis, MO).

Protein purification

The rXI was purified from crude *E. coli* cell lysates using HisPur Cobalt Spin Columns (Thermo Fisher Scientific, Waltham, MA). Imidazole was used to release the bound His-tagged protein from the cobalt columns. Dialysis (>10 kDa, Pierce Slide-a-Lyzer, 100 mM Mes buffer pH 6.5), protein concentration (Pierce 9K MWCO), and size exclusion chromatography (Sephacryl S-200 HR, GE Health Care, Chicago, IL, USA) were used for further purification and concentration of the rXI.

Enzyme assays

XI activity was determined as the rate of conversion of xylose to xylulose. The standard reaction mixture contained 67 mM xylose, 1 mM $MnCl_2$, 10 mM $MgCl_2$, and 270 μL lysed cell supernatant (CFE) in a final volume of 300 μL. The reaction mixture was incubated at 35°C for 1 h, and then was inactivated in a water bath at 95°C for 5 min before centrifugation (16,100*g*, 4°C, 30 min). The accumulated xylulose in the XI assay was routinely determined using the sorbitol dehydrogenase (SDH) assay[24] as follows: the XI assay supernatant (50 μL) was added to 200 μL 50 mM Tris pH 7.5 containing 0.2 g/L NADH (Abs_{340} of 1.5–2). Reactions were started by addition of 10 μL of a 1 g sorbitol dehydrogenase (Roche Diagnostics, Mannheim, Germany)/L 50 mM Tris pH 7.5 NADH solution to each

sample well in a 96-well microtiter plate. The absorbance decrease due to NADH consumption was monitored at 340 nm during the course of a 1 h incubation at 37°C using a Wallac Victor^{3}V plate reader (PerkinElmer, Shelton, CT). Assay controls consisted of heat treated and inactivated XI reactions, xylulose spiked, heat treated controls, distilled water amended SDH assays, as well as targeted experiments to determine if experimental variables, such as pH, Ca^{2+}, and xylitol in rXI assays affected subsequent SDH activity in assays of xylulose concentration. One unit (U) of XI activity was defined as the amount of enzyme required to produce 1 μmole of xylulose per min under these standard assay conditions. The amount of xylulose produced in XI reactions was also determined using the cysteine–carbazole–sulfuric acid method.[25]

Effects of pH, temperature, and calcium and xylitol concentrations on rXI activity

For pH, Ca^{2+}, and xylitol experiments, the cells were sonicated in 10 mM Mes pH 6.5. To determine the effect of pH on rXI activity, alternative pH adjusted buffer systems were added to a final concentration of 50 mM in rXI assays. The buffers and corresponding pH intervals were as follows: sodium citrate, 4.1–6.2; sodium acetate, 4.6–5.5; Mes, 5.8–6.3; Na_2HPO_4, 6.0–7.5; Mops, 6.7–7.0; and Tris, 6.7–7.8. For these assays, the volume of CFE was reduced to 240 μL, and 30 μL of each buffer was added in addition to the $MgCl_2$, $MnCl_2$, and xylose used in the standard rXI assay. The targeted pH was verified with a pH probe. Ca^{2+} ($CaCl_2$) and xylitol were also added as 10× solutions to CFEs, along with pH buffer (final concentration 50 mM Mes pH 6.5). The effect of temperature on rXI activity was determined using temperature controlled water baths and the standard cell lysis and rXI assay conditions. The results are reported as the relative rXI activity under each pH or temperature condition compared with the highest activity observed in the entire pH or temperature experiment, respectively. For the Ca^{2+} and xylitol inhibition experiments results are reported as the relative activity compared with the rXI activity in treatments containing no added Ca^{2+} or xylitol, respectively.

Determination of kinetic parameters

The kinetic parameters of rXI were determined in 100 mM Mes buffer (pH 6.5) containing 1 mM Mn^{2+}, 5 mM Mg^{2+}, and 3.3–133 mM xylose. The reaction mixtures were incubated for 1 h at 37°C. The data were fitted to the Michaelis-Menten equation and kinetic constants were calculated by nonlinear least-squares regression.

Production of rXI in fed-batch fermentation

Fermentation experiments with *E. coli* were conducted with a New Brunswick Scientific BioFlo 110 Modular Benchtop Fermentor (Edison, NJ) in a 3 L sterile bioreactor. The batch medium consisted of LB medium (25 g/L) supplemented with the following (per L): 28 g Na_2HPO_4, 4 g $(NH_4)_2SO_4$, 0.25 g $MgSO_4.7H_2O$, and 25 g glucose. The complete medium was filter (0.2 μm) sterilized after adjustment to pH 7 with 37% HCl and amendment with 15 μg/mL kanamycin, and then aseptically transferred to the fermentor vessel. The fed-batch medium was the same, except the glucose concentration was increased to 105 g/L.

The bioreactor, containing 900 mL batch medium, was inoculated with a 100 mL shake flask culture after overnight incubation in the same medium. The bioreactor was connected to the controller unit with temperature, pH and agitation control. A constant agitation speed of 800 rpm, a constant temperature of 30°C in batch mode and 25°C in fed-batch, and a constant pH 7 were maintained. The pH was controlled by addition of ammonium hydroxide (8%). The fed-batch phase commenced when the glucose was depleted. Cells were subsequently induced with 0.24 g IPTG/L. Air was sparged to the reactor at a flow rate of 4 L/min, and was supplemented with 1 L/min pure oxygen when high cell densities were achieved. An additional 0.24 g IPTG and 150 μL antifoam 204 (Sigma-Aldrich, St. Louis, MO, USA) were also added. The flow rate of the fed-batch medium was approximately 7 mL/h, and was adjusted based on glucose concentration measurements at each sampling point to maintain low glucose concentrations in the bioreactor. Glucose concentrations in the culture broth were monitored using an Ultima ReliOn glucose meter (Solartek Products, Alameda, CA). Samples were periodically taken from the bioreactor culture for measurements of cell density (OD_{600}), pH, and glucose concentration, and then centrifuged for cell storage at −20°C prior to cell lysis and protein and rXI activity determinations.

Results and Discussion

Sequence and phylogenetic analysis

The *F. pelagi* XI nucleotide sequence contains an open reading frame of 1,311 bp, encoding a polypeptide with 5 cysteine residues that may participate in subunit–subunit bridges in the final active protein configuration. The nucleotide sequence also contains 10 rare usage codons, including codons for Arg (AGG, CGA), Leu (CTA), and Pro (CCC)(NIH MBI Laboratory for Structural Genomics and Proteomics, Rare Codon Calculator). The *F. pelagi* xylose isomerase amino acid sequence showed 87%, 77%, and 74% identity to the xylose isomerases from *A. coralicida*, *Aureimonas* sp., and *Bradyrhizobium* sp., respectively. Multiple sequence alignments indicated that most amino acids involved in substrate, metal ion binding, and catalysis are conserved in the *F. pelagi* xylose isomerase (data not shown). The *F. pelagi* xylose isomerase contains the amino acids His-102, Asp-105, and Asp-340 that are directly involved in catalysis, as well as Lys-235, which is important for protein structure and catalysis.[9,26] Amino acid signature sequences characteristic of Family II xylose isomerases [ENYVFWG (183–189) and EPKP(N,K)EP (232–238)] were similar to those in the *F. pelagi* xylose isomerase [ANYVCWG (184–190) and EPKPKEP (233–239)].[27] The presence of 40–50 more amino acids at the N-terminal end of the *F. pelagi* xylose isomerase compared with Family I xylose isomerases indicates that the *F. pelagi* xylose isomerase is a Family II xylose isomerase. This is also indicated by the phylogenetic tree of the *F. pelagi* xylose isomerase and other Family II (*Thermoanaerobacterium* sp., *Anoxybacillus gonensis*, *Lactobacillus reuteri*, *E. coli*, *Piromyces* sp., *A. coralicida*) and Family I (*Actinoplanes missouriensis*, *Streptomyces rubiginosus*) xylose isomerase amino acid sequences (Figure 1).

Heterologous expression of the XI encoding gene in E. coli

The XI encoding gene from *F. pelagi* was successfully expressed in the *E. coli* strain Rosetta-gami 2 (DE3) using

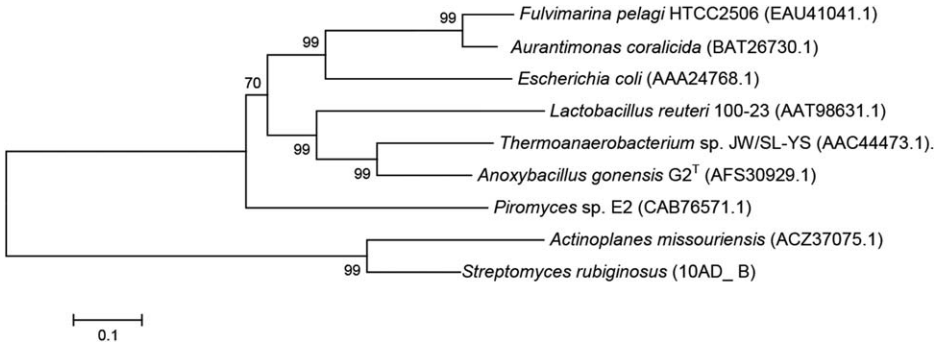

Figure 1. Phylogenetic tree of the *F. pelagi* xylose isomerase and other xylose isomerase amino acid sequences reported in the GenBank/EMBL Data Bank. Bar, 0.1 substitutions per nucleotide.

the pET-24a(+) expression vector. This expression host contains both a disulfide bond isomerase and rare codon tRNAs. A number of transformants produced a recombinant protein of approximately 55 kDa, as indicated by SDS-PAGE and western blots with anti-His antibodies and rXI enzyme activity assays. Control strains, either lacking the rXI gene or containing the rXI gene but not induced with IPTG, did not produce XI activity. At higher incubation temperatures (>30°C), antibody detection indicated that the rXI was present primarily in the insoluble fraction following cell lysis and centrifugation. This suggests that the rXI was present in insoluble inclusion bodies. One transformant, *E. coli* RgXI6 [*E. coli* Rosetta-gami (DE3) containing pET-24(a)+ with the cloned FpXI gene] was chosen for further studies.

Production of rXI by E. coli RgXI6

Combinations of induction temperatures and times (Protein Expression and Purification Core Facility—Protein Expression-*E. coli*-EMBL; www.embl.de) indicated that more active protein could be produced at 20–25°C than at 30–37°C (Figure 2). This appears to be the result of misfolding and formation of insoluble inclusion bodies at the higher temperatures. At 25°C, induction periods of 12 h or more yielded the highest amount of enzyme. Cell density after 12 h induction (OD_{600} 3.5) was approximately twice that after 5 h (OD_{600} 1.6), and activity per unit protein increased by a factor of approximately 2.5 (Figure 3). Hence, 12 h induction yielded roughly five times more active protein than 5 h induction. Western blots also demonstrated that increasing induction time yielded greater amounts of the His-tagged rXI protein (Figure 4). Approximately 1.3 U of rXI activity/L shake flask medium was produced under the standard induction conditions described above (12 h, 25°C) with a specific activity of 0.006 U/mg protein in the CFE. Production of rXI could be further increased using fed-batch fermentation (Figure 5). Cell densities of *E. coli* reached an OD_{600} of 7–8, with a maximum rXI activity of 24 U/L reactor volume and 0.016 U/mg protein in the CFE.

Effects of pH, temperature, and Ca and xylitol concentrations on rXI activity

Isomerization of xylose to xylulose was demonstrated over the course of 35 h by rXI in CFE of *E. coli* RgXI6 culture under standard reaction conditions (0.010 U rXI activity/mL in XI assay; Mes buffer, pH 6.5, 35°C) (Figure 6). The maximum rXI activity in CFE from *E. coli* RgXI6 was observed in a pH range of 6.3 to greater than 7.8. Mes appears to be a more effective buffer for maintaining rXI activity at low pH than phosphate buffer (Figure 7). Below pH 6.3, rXI activity decreased considerably, with only 30% of the maximum activity observed at pH 6 in either Mes, phosphate or citrate buffers.

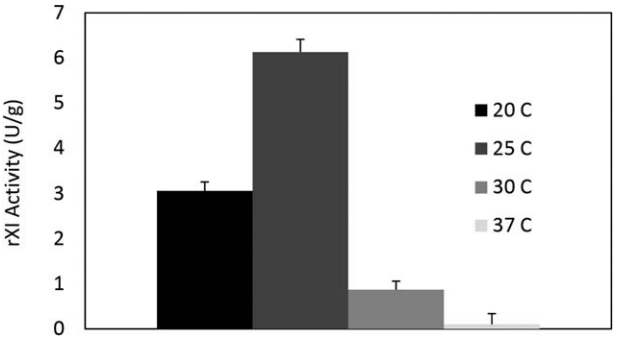

Figure 2. Effect of induction temperature on production of active rXI. Induction times at each temperature were as follows: 20°C, 12 h; 25°C, 7 h; 30°C, 4 h; 37°C, 2 h (rXI activity expressed as U/g protein in CFE).

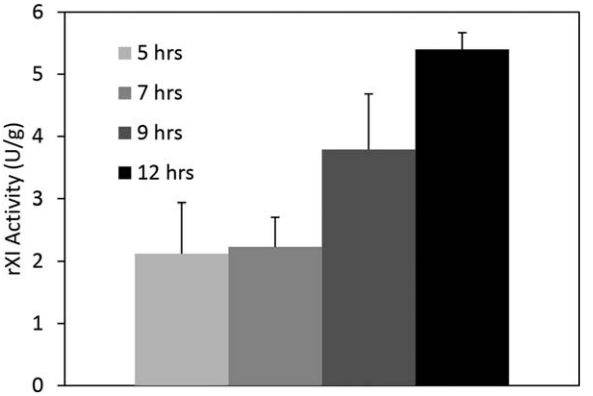

Figure 3. Effect of induction time duration on production of active rXI. *E. coli* RgXI6 was induced with IPTG at 25°C for 5, 7, 9, and 12 h (rXI activity expressed as U/g protein in CFE).

Yeast fermentations are commonly conducted at pH 4–5. At pH higher than 6, bacteria are more competitive with yeast than at lower pH, resulting in difficulties with bacterial contamination. At pH 5–5.5, the activity of the rXI from *F. pelagi* is very low. Most reported xylose isomerases have pH optimums greater than 7,[26,28–30] although XIs from

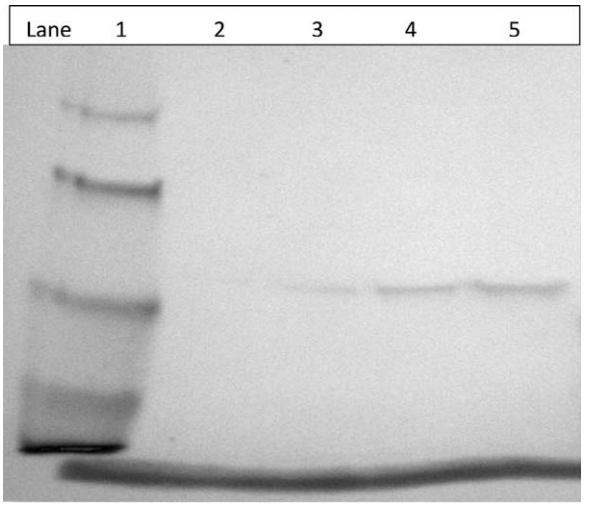

Figure 4. Anti-His western blot of cell-free extracts from *E. coli* RgXI6 cultures induced with IPTG at 25°C for 1, 2, 4, and 8 h (Lane 1, Molecular weight ladder, 120, 85, 50, 35, 25 kDa; Lanes 2–5, *E. coli* RgXI6 CFE from 1 to 8 h).

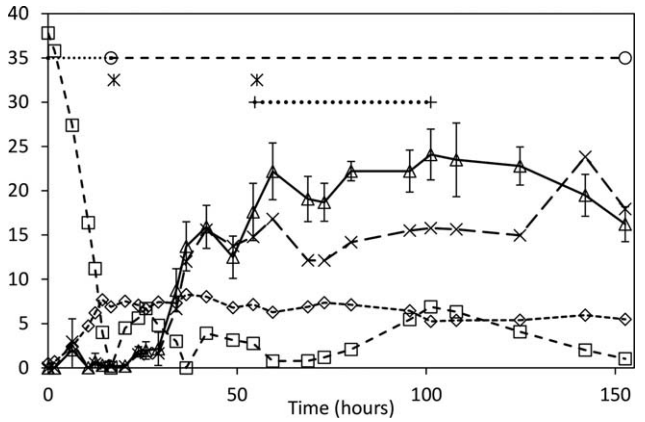

Figure 5. Production of rXI by *E. coli* RgXI6 during fed-batch fermentation. Symbols are as follows: ---◇--- —cell density (OD600), ---□--- —glucose concentration (g/L), —Δ— —rXI activity (U/L), −−x−− —rXI activity (U/g protein in CFE), ■·····■ —batch phase, ○----○ —fed-batch phase, +·····+—pure oxygen supplementation, ¥—IPTG induction.

Thermoanaerobacterium sp.[31] and *A. gonensis*[32] exhibit highest activity at pH 6.5. An XI from *L. reuteri* has been characterized that has a pH optimum of 5.[33] Although the activity at 37°C for the *L. reuteri* XI is only 20% of the activity at 65°C and the K_M is very high (177 mM), the high V_{max} (43 U/mg) and activity at low pH are advantageous for a simultaneous isomerization/fermentation process. The *E. coli* rXI activity, derived from expression of the *F. pelagi xylA*, increased with temperature up to approximately 55°C (data not shown).

The rXI exhibited high tolerance to calcium added with the CFE to the xylose isomerization assay (Figure 8), with an estimated K_I of approximately 2,500 mg/L. Ca^{2+} inhibits xylose isomerase activity via competition with Mg^{2+}, Co^{2+} and Mn^{2+} for the enzyme active site.[1] In purified enzyme preparations, Ca^{2+} concentrations as low as 5–10 mM (200–400 mg/L) are inhibitory.[30,34] In contrast, xylose isomerization was not inhibited in $Ca(OH)_2$ over-limed hydrolysate

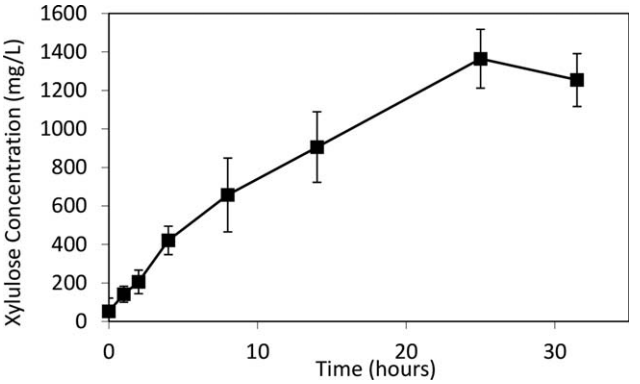

Figure 6. Isomerization of xylose to xylulose.

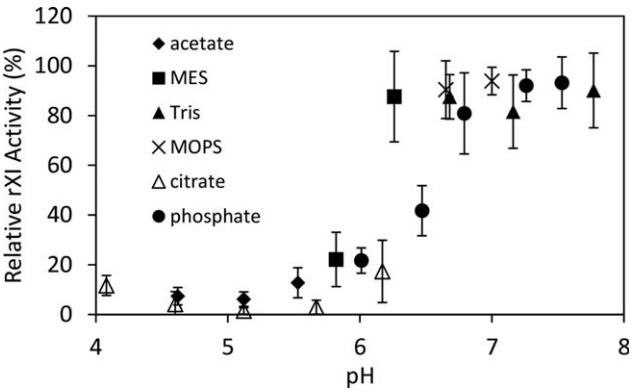

Figure 7. Effect of pH and buffer composition on rXI activity.

from acid-catalyzed steam pretreated corn stover containing 1,900 mg/L Ca^{2+}.[11] The higher resistance of XIs to Ca^{2+} in complex mixes may be due to precipitation or complexation of Ca^{2+} by other organic compounds in the reaction solution. These results suggest that Ca^{2+} inhibition is not likely to be of concern in applications employing the rXI from *F. pelagi* in complex biomass hydrolysates.

Inhibition of rXI activity was not significant in reaction solutions containing either 6.7 or 67 mM xylose in the presence of xylitol concentrations up to 50 mM (7.6 g/L) (Figure 9). Xylitol is a side-product of xylulose fermentation by yeast that decreases process efficiency. It is also a competitive inhibitor of xylose isomerases.[35–37] Xylitol accumulation can be reduced by deletion of the aldose reductase gene,[38] but this gene is beneficial for detoxification of inhibitors in biomass hydrolysates, and elimination of this activity can reduce the robustness of resulting yeast strains.

Xylitol concentrations in yeast cultures fermenting xylulose range from 1 g/L (6.6 mM) to 12 g/L (79 mM).[4,11,15,39,40] K_I values determined for inhibition of extracellular XIs by xylitol are in the range of 0.3 mM to 14.5 mM, including 0.3 mM for *Arthrobacter* sp.,[30] 2.7 mM for *Lactobacillus brevis*,[37] 14.5 and 4.6 mM for *S. cerevisiae* harboring the XI genes from *Clostridium phytofermentans* and *Piromyces* sp., respectively,[39] and 5.1 mM for *S. cerevisiae* harboring the XI gene from *Bacteroides stercoris*.[40] The relatively high K_I for the *F. pelag*i XI produced in *E. coli* suggests that this enzyme would remain effective for extracellular xylose isomerization during xylulose fermentation and xylitol by-product formation.

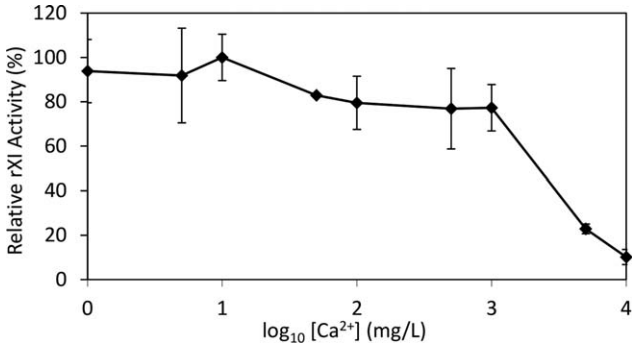

Figure 8. Inhibition of rXI by calcium.

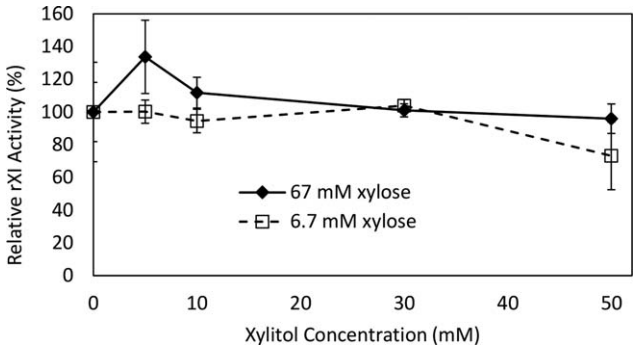

Figure 9. Inhibition of rXI activity by xylitol in the presence of low [(6.7 mM (1 g/L)] and high [(67 mM (10 g/L)] xylose concentrations.

Enzyme purification and kinetics

Purification of the rXI from *E. coli* RgXI6 was performed on cell-free extract (0.018 U/mg protein) using HisPur cobalt affinity columns, followed by protein concentration and dialysis, and size exclusion chromatography. The purified enzyme had a specific activity of 0.142 U/mg protein. Although the HisPur cobalt affinity columns increased the purity and concentration of the rXI, the yield of rXI activity was less than 20% of the total rXI activity applied to the column. This suggests the occurrence of some irreversible binding, proteolytic degradation, or enzyme inactivation. Based on calibration of the size exclusion column using molecular weight standards, the rXI has a molecular weight of ~160 kDa. This suggests that the rXI is a trimer, since the rXI monomer has consistently been observed to have a molecular weight of ~50 to 55 kDa.

A Lineweaver–Burk plot was determined for reactions conducted at 35°C using co-affinity column purified rXI and xylose concentrations between 3.3 and 133 mM. The V_{max} and K_M for rXI were determined to be 0.142 U/mg and 1.75 mM, respectively. Excluding data for substrate concentrations below 65 mM xylose yields roughly the same V_{max}, but a slightly higher K_M (4.17 mM). Enzyme kinetic parameters have been determined at high temperatures (60–95°C) on XIs from a number of species, and have yielded K_M and V_{max} values ranging from 16 to 300 mM xylose and 5 to 70 U/mg purified protein, respectively.[26,28,30,31] Kinetic parameters determined at lower temperatures (30–40°C) for XIs from other species have yielded values of K_M and V_{max} in the range of 1–4 mM xylose and 7–12 U/mg.[30] The specific activities of xylose isomerases from *E. coli*, *Piromyces* sp. *E2*, and *Sorangium cellulosum* expressed in *E. coli* were 2.77, 3.36, and 0.12 U/mg protein, respectively.[10] The K_M for the rXI from *F. pelagi* is in the range of values reported for other XIs investigated at mesophilic temperatures, but the V_{max} (0.142 U/mg purified enzyme) is very low. The low K_M and V_{max} of the *F. pelagi* XI may be related to the low nutrient conditions of the pelagic environment and the low growth rate of this species.[19,20,41] Although the *F. pelagi* xylose isomerase has a relatively low K_M and pH range, and a higher calcium and xylitol tolerance than many previously investigated xylose isomerases, a higher V_{max} at low pH (<6) and temperature (30°C) would be required for commercial use in yeast fermentations for biofuels production.

Conclusions

A xylose isomerase gene (*xylA*) from the marine bacterium, *F. pelagi*, could be successfully expressed in *E. coli*, and the rXI was produced in high cell density fed-batch fermentation. The *F. pelagi* xylose isomerase was most closely related to a xylose isomerase from the marine bacterium *A. coralicida*. The *F. pelagi* xylose isomerase exhibited a low K_M for xylose, which appears consistent with the low substrate concentrations commonly observed in the open ocean. The rXI converted xylose to xylulose at pH 6.5, and exhibited high tolerance to the xylose isomerase inhibitors calcium and xylitol. These attributes are of potential interest in biofuels production, as calcium and xylitol are commonly present at high concentrations in biomass hydrolysate and yeast fermentations.

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