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Expression of a functional cold active β-galactosidase from *Planococcus sp-L4* in *Pichia pastoris*

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ABSTRACT

Lactase deficiency problem discourages many adults from consuming milk as a major source of micro- and macronutrients. Enzymatic hydrolysis of lactose is an ideal solution for this problem but such processing adds significant costs. In this study, a cold active β-galactosidase from *Planococcus sp-L4* (*bgal*) was optimized for expression of recombinant "BGalP" in *Pichia pastoris*. As a result of codon optimization, the codon adaptation index was improved from 0.58 to 0.85 after replacing rare codons. After transformation of two *P. pastoris* strains (KM71H and GS115), the activity of BGalP enzyme was measured in the culture supernatants using ortho-Nitrophenyl-β-galactoside (ONPG). Maximal activity was recorded as 3.7 U/ml on day 11 in KM71H clone #2 which was 20% higher than the best GS115 clone. Activity measurements under different conditions indicated optimal activity at pH 6.5. It was active at temperatures ranging from 0 to 55 °C with deactivation occurring at or above 60 °C. Protein analysis of the crude ultrafiltrate showed the enzyme was ~75 kDa and was the major constituent (85%) of the sample. This enzyme have the potential to find utility for the breakdown of lactose in chilled milk and subsequently can be deactivated by pasteurization. The use of BGalP would minimize energy consumption thus decreasing cost and also help to preserve the nutritional elements of the milk.

1. Introduction

Milk and dairy products are major dietary sources of protein, calcium, phosphorous, magnesium and other micro- and macronutrients. However, lactase deficiency is a common problem around the globe with more than 70% prevalence in adults [1,2]. Importantly this condition discourages many adults from consuming milk and can predispose them to osteoporosis or malnutrition. The main approach used by the food industry is the enzymatic hydrolysis of lactose into glucose and galactose, to make dairy products consumable for people with lactase deficiency [3]. This process has the additional benefit of enhancing sweetness of the processed products. Additionally, it has been proposed to turn whey into sugar substitutes for cooking, confectionery and non-alcoholic beverages [4]. This would also make good use of the large quantities of waste that can be major environmental pollutants [5]. Additionally, lactose has low solubility and easily crystallizes at low temperatures causing unfavorable sandy texture of products such as ice-cream. The hydrolysis of lactose thus serves to overcome solubility issues and improve product quality.

The β-galactosidases (EC 3.2.1.23) belong to the class of hydrolytic enzymes capable of converting lactose to glucose and galactose [6]. These enzymes naturally occur in microorganisms (bacteria, fungi and yeasts), plants (almonds, peaches, apricots, and apples) as well as animal tissues [3]. Most of these enzymes display optimal activity at temperatures ranging from 25 to 40 °C. Lactozym (Novo Nordisk, Denmark), extracted from mesophilic yeasts *Kluyveromyces lactic*, is used commercially for lactose hydrolysis of dairy products at 35–45 °C. However, it has been proposed that performing hydrolysis at refrigerated temperatures could preserve nutrients and taste, increase process flexibility and reduce the cost of manufacturing lactose-free milk [7]. To achieve this it is necessary to identify cold-active β-galactosidases that are suitable for adaptation to the industrial processing of milk.

Cold adapted β-galactosidase enzymes have been isolated from several different organisms. An enzyme isolated from *Arthrobacter*

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sp. 20B was homotetrameric, each subunit being approximately a 116 kDa polypeptide, and optimally active at pH 6.0–8.0 and 25 °C [8]. The active enzyme cloned from an *Arthrobacter* isolate was also a homotetramer with high activity at below 20 °C, but dissociated into inactive monomers at 25 °C [9]. The enzyme β-D-galactosidases isolated from Antarctic bacterium *Arthrobacter* sp. 32c was active as a homotrimeric protein with 695 amino acid residues in each monomer. It had maximum activity at pH 6.5 and 50 °C and retained 60% and 15% of its maximum activity at 25 °C and 0 °C, respectively [10]. The enzyme cloned from Antarctic bacterium *Pseudoalteromonas* sp. 22b was also homotetrameric protein, with 1028 amino acid residues in each monomer. The enzyme had maximum activity close to 40 °C and retained 20% of its maximum activity at 10 °C [11]. Another cold active β-galactosidase has been reported from *Planococcus sp-L4* with catalytic efficiencies for lactose at 5 and 20 °C to be 14 and 47 times greater than that of *E. coli* β-galactosidase at 20 °C. It was heat liable losing its activity at 40 °C in 10 min [12]. Collectively this work has uncovered several promising leads but extraction from these natural sources is not economically viable due to low intracellular expression of the enzymes.

Cost-effective production of β-galactosidases is possible using genetic engineering and biotechnology in both prokaryotic and eukaryotic systems. Proteins expressed in prokaryotic systems occur as inclusion bodies that need to be solubilized, refolded and purified, increasing cost of recombinant protein production [13]. Therefore, the methylotrophic yeast *Pichia pastoris* has been adopted for the production of heterologous recombinant proteins as secretory, glycosylated products. Furthermore, *P. pastoris* secretes only very low amount of native proteins, facilitating the purification of produced recombinant protein [14]. Heat-stable and cold active β-galactosidases from *Alicyclobacillus acidocaldarius* and *Arthrobacter* sp.32c, respectively, have already been successfully expressed in *P. pastoris* [10]. Nevertheless, low efficiency is a known issue in the expression of heterologous products caused by codon bias. Here the cDNA encoding the protein of interest contain codons corresponding to low abundant tRNAs in the expression host [15]. Abundance of rare codons is thought to decrease translation speed and induce translational errors [16–18]. An effective strategy to improve recombinant expression involves optimizing codon usage towards the host organism [15,19–21].

In this study it was demonstrated that the codon-optimization of β-galactosidase (BGalP) was expressed with high-yield in *P. pastoris*. Herein the optimal expression conditions of BGalP, a promising candidate cold-active enzyme for lactose hydrolysis was reported.

2. Materials and methods

2.1. Enzyme selection

A literature review was conducted on publications describing the properties of 14 different β-galactosidases to select the most appropriate one for expression in *P. pastoris*. In addition to reported enzymatic activity, our primary selection criteria included low km for lactose, high activity at temperature below 10 °C, monomeric active structure, short protein sequence, optimum pH around 7 and structural stability at temperature 25–30 °C.

2.2. Gene optimization and synthesis

Codon optimization for *P. pastoris* and synthesis of BGalP coding sequence was carried out by GenScript USA (Piscataway, NJ). Quality of codon optimization was measured by two criteria of Codon Adaptation Indices (CAI) and Frequency of Optimal codons (FOP). CAI indicates the distribution of codon usage frequency along the length of the coding DNA sequence (CDS). FOP (also defined as Codon Frequency Distribution; CFD) is the percentage distribution of codons in computed codon quality groups.

2.3. Expression of BGalP

The synthetic construct for BGalP was subcloned into pPICZαA vector (Invitrogen, Carlsbad, CA, USA) and expressed as a fusion protein with c-Myc and His tags as previously described for expressing CSF-1 [22]. Transformed KM71H and GS115 cells were cultured on YPDS containing 100 μg/ml zeocin for three days. Single colonies from the YPDS plates were inoculated into 2 ml of BMGY. After 24 h incubation at 30 °C with 250 rpm shaking, the volume was increased up to 10 ml. When the OD_{600} reached 1.5, the BMGY medium was replaced with BMMY (substituting 2% methanol instead of 1% glycerol in BMGY). The cultures were incubated at 25 °C and samples were sequentially collected for analysis beginning the following day.

2.4. BGalP activity assay

BGalP enzyme activity was measured using ortho-Nitrophenyl-β-galactoside (ONPG) (4 mg/ml in 100 mM phosphate buffer, pH 7.0) in Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, pH 7.0). The reaction was stopped by 1 M sodium carbonate and the OD_{420} measured using a T60 UV–Vis spectrophotometer (PG Instruments, Leicestershire, UK). The enzyme activity in each ml of the culture supernatant was calculated using the equation: $\text{EnzymeActivity}(\text{U/ml}) = \frac{(\text{OD420}).\text{Vt}}{\varepsilon.\text{T}.\text{d}.\text{Vs}}$, where Vt was the total assay volume (ml), ε, the extinction coefficient of ONPG (4500 M^{-1} cm^{-1}), T, total incubation time (min), d, the cuvette diameter and Vs, the supernatant volume added to the assay mix (ml). Optimum temperature was determined by conducting enzyme activity assay at different temperatures ranging from 0 to 75 °C. The enzyme activity was also studied at pH values 3–9 (with 0.5 unit intervals) using citrate buffer (pH 3–5.5), phosphate buffer (pH 6–8) and Tris buffer (pH 8.5–9).

2.5. Partial protein purification and analysis

The supernatant of cultured *P. pastoris* strains was partially purified and concentrated (10-fold) using 5 MWCO Vivacell 100 membranes (Vivascience, Stonehouse, UK). This step removed molecules smaller than 5 kDa including water, remnant methanol and other components of the BMMY medium. Samples were resolved on 12% SDS–PAGE gels and stained with Coomassie Brilliant Blue R-250. Apparent molecular weight was determined in comparison with a protein ladder (PageRuler Plus Prestained; Thermo Scientific, Waltham, MA).The intensity of the protein bands on SDS–PAGE were compared using Multi Gauge V3.0 software (FujiFilm, Tokyo, Japan). Additionally, the resolved proteins were transferred to Polyvinylidene Difluoride (PVDF) membrane (Santa Cruz Biotech., Dallas, TX) and probed using the c-Myc Antibody (9E10) provided by Dr. Rick Thorne, Cancer Research Unit, the University of Newcastle, NSW, Australia. The membranes were washed three times in TBST buffer and incubated in home-made ECL reagent as previously described [23] for 15 s followed by detecting chemiluminescence signals using an Alliance Mini instrument (UVItec Limited, Cambridge, UK).

3. Results

3.1. Enzyme Selection

Functional and structural properties of 14 different β-galactosidases were assessed. Based on the criteria mentioned