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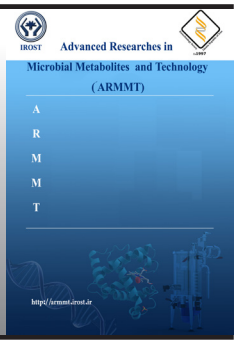

Heterologous expression and characterization of a recombinant thermostable amylopullulanases *Coh4159* from *Cohnella* sp. A01

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Abstract

Starch debranching enzymes that merely hydrolyze α-(1→6) glycosidic linkages are classified into isoamylases (EC 3.2.1.68) and pullulanases (EC 3.2.1.41). An exception to this definition is amylopullulanase, a type of pullulanase that is capable of cleaving both α-(1→4) and α-(1→6) linkages. Amylopullulanases are in demand in liquid sugar industries to generate glucose and some other starch derivatives. Pullulanases can be used in conjunction with amylases to improve sugar availability during sugar syrup production. Here, a thermophilic *Cohnella* sp. A01 amylopullulanase (EC 3.2.1.41) gene, namely *Coh4159*, was PCR amplified and cloned in pET-26b(+) and transformed into BL21(DE3). Recombinant *Coh4159* was heterologously expressed in the presence of 0.5 mM IPTG and purified via affinity chromatography, and further characterized. Enzyme activity was demonstrated via zymogram analysis in the presence of pullulan. The enzyme had a hydrolytic effect on pullulan with V_{max} = 2.85 µmol.min^{-1} and K_m = 0.5 mM. Temperature optima and pH were 60 ˚C and 6.0, in which the enzyme kept its activity at wide pH (4-9) and temperature (30-70 ˚C) ranges. The recombinant enzyme kept 50% of its activity for 60 min, 100 min and 120 min when incubated at 80, 70 and 60 ˚C, respectively. Amongst metal ions tested, Mn^{2+} and Ca^{2+} have improved the enzyme activity both at 5 and 10 mM. The results promise the capability of producing a commercial industrial enzyme well-suited to liquid sugar syrup industry specifications.

1. Introduction

The sugar industry is a thriving business that mainly relies on agriculture with a focus on sugarcane and sugar beet. These crop sources barely satisfy daily human consumption around the globe, a reason that justifies the increasing price of sugars in the last few years. The growing demand on sugar in other food and drug industries provides enough incentive to seek other natural sources. Starch, as a byproduct of industrial crops, such as cassava, potato and corn, has proven to be a valuable source to produce glucose. Acid hydrolysis of starch has been used for its conversion to

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smaller reduced sugars and glucose. However, use of chemicals in such refineries makes the final product expensive due to high energy demand, the factory sewage also contaminates the environment. In contrast, enzymatic bioconversion of starch can efficiently turn the biopolymer to sugar syrups containing glucose, dextrins and other oligosaccharides (Janeček *et al*., 2014) with minimal environmental effects and cost.

Starch is a polymer of α-(1→4) glucosyl residues, found both in amylose and amylopectin, that is substituted with chains of α-(1→6) glucosyl units in amylopectin alone. Starch debranching enzymes that merely hydrolyze α-(1→6) glycosidic linkages are classified into isoamylases (EC 3.2.1.68) and pullulanases (EC 3.2.1.41). An exception to this definition is amylopullulanase, a type of pullulanase that is capable of cleaving both α-(1→4) and α-(1→6) linkages (Wang *et al.*, 2012). The difference between these two lies in the fact that the pullulanases prefer pullulan as the substrate (Martin *et al.*, 1995; van der Veen *et al.*, 2000). Pullulan, α-(1→4), α-(1→6) glucan is a linear homopolysaccharide of 3 glucosyl residues, maltotriose, with α-(1→6) linkages that are connected to each other by an α-(1→4) bond. This water-soluble tasteless polymer has many food, drug and industrial applications (Leathers, 2003).

Plullulanases can be used in conjunction with amylases to improve sugar availability during sugar syrup production (Hii *et al.*, 2012). Likewise, in the baking industry bread hardening and taste change can be slowed down by using amylopullulanases instead of polyoxyethylene monosterate to produce short chain water soluble dextrins (Lord, 1950). These molecules can change the nature of starch gelatinization and therefore slow down bread staling (Prakash *et al*., 2012). For instance, supplementation of whole wheat dough with an amylopullulanase from Geobacillus improved the loaf volume, shelf-life, and bread texture. Additionally, the enzyme was capable of desizing cotton fabrics (Nisha and Satyanarayana, 2014). Pullulanases are also being used in cellodextrin formation which has application in pharmaceutical industries to improve solubility of water-insoluble drugs, to add to skin protectants against UV light, and in food industries to cover wrong flavor (Hassan *et al*., 2006; Prakash *et al*., 2012). Furthermore, pullulanases have a stake in the biofuel industry, allowing the release of more glucose from starch which can be harnessed during fermentation (KiliApar and Özbek, 2004; Prakash *et al*., 2012; Nahampun *et al*., 2013). For example, the ectopic expression of *Thermoanaerobacter* amylopullulanase in maize seeds under the control of a seed specific promoter improved the starch conversion efficiency by 90.5%, enhancing bioethanol production (Nahampun *et al*., 2013). Last but not least, amylopullulanases have found their ways into agricultural biotechnology through transgenesis of cereals to accelerate bioprocessing of starch and producing protein-enriched flour (Chiang *et al*., 2005). Despite better bioavailability of amino acids in transgenic rice, the plants grown in farm demonstrated higher susceptibility to brown spot caused by *Bipolaris oryzae* (Ting *et al.*, 2008).

Since more than 90% of industrial enzymes are mainly obtained from microorganisms, (Cho *et al*., 2007), looking for new enzyme sources with extraordinary features are always a blessing for the industry. Thermophilic bacteria bear such enzymes that are highly tolerant to extreme temperatures (Narang *et al*., 2001), something that is inevitable in technologies currently in use (Mrudula *et al*., 2011). We have previously reported the isolation of *Cohnella* A01, a Gram positive thermophilic bacterium, from a shrimp pond (Aliabadi *et al*., 2016), and heterologous expression of its thermostable lipase (Pooreydy Golaki *et al.*, 2015a), structural characterization of lipase (PooreydyGolaki *et al*., 2015b), cloning of β-glucanase (Rezaie *et al*., 2018) and two other amylopullulanases of this bacterium (Zebardast *et al*., 2017). Here an amylopullulanase sequence

(GenBank accession: KX013443), namely *Coh4159*, from its fully sequenced genome (data not shown) was found through in silico studies and used for heterologous expression and purification followed by enzymatic assays.

2. Materials and methods

2.1. Materials

All enzymes, pET-26b(+), IPTG and X-Gal were purchased from Fermentas (Burlington, Canada). DNA extraction kit was obtained from BioNEER (Anaheim, USA). High Pure PCR Product Purification and High Pure Plasmid Purification kits were obtained from Roche (Basel, Switzerland). *E. coli* host strains, DH5α and BL21 (DE3), were purchased from Invitrogen (Carlsbad, CA, USA). Ni-NTA resin was obtained from Qiagen (Netherlands). Other materials were from Merck (Darmstadt, Germany). *Cohnella* sp. A01 was obtained from sampled water from a shrimp pond at Choebdeh (Abadan, Iran; Aliabadi *et al.*, 2016).

2.2. Polymerase Chain Reaction

Single clone of *Cohnella* sp. A01 was cultured in nutrient broth for 72 h at 60 ˚C. The culture was centrifuged at 7000 ×g for 10 min. DNA was isolated from precipitated bacterial cells using the DNA extraction kit as instructed by the supplier. DNA (70 ng/µl) was used for PCR amplification of amylopullulanase in a 25 µl reaction containing 10 pmoles of each primers [(Forward:5'-CTTCCATGGTGAA CAAGCAACTGGGCAAAATCCTCGC-3', underline is *Nco*I restriction site and Reverse:5'-CTTCTCGAGTGGACGCTCCAGCAGCCG GAC-3', underlined is *Xho*I restriction site)], 0.5 µl of 100 U Pfu DNA polymerase, 2.5 µl of 10× PCR buffer, 0.5 µl of 10 mM dNTPs and 1.5 µl DMSO. The PCR was started at 94 ˚C for 5 min then followed by 35 cycles of 94 ˚C: 30 s; 61.5 ˚C: 30 s; 72 ˚C: 2 min with a final extension at 72 ˚C for 10 min. The PCR product (2439 bp) was separated on 1% agarose gel and isolated with a High Pure PCR Product Purification kit according to the manufacturer. The PCR product was restricted with 10 U/µl of *Nco*I and *Xho*I, separated on 1% agarose gel and purified as mentioned above and named *Coh4159*.

2.3. Cloning and expression of Coh4159

pET-26b(+) plasmid vector supplied in the bacterial cell culture was isolated using a High Pure Plasmid Purification kit according to the manufacturer. The plasmid was restricted with *Nco*I and *Xho*I as stated earlier at 37 ˚C for 2 h, separated on 1% agarose gel and purified using a High Pure PCR Product Purification kit according to the manufacturer. *Coh4159* (25 ng/µl) was ligated with pET-26b(+) (37 ng/µl) using T4-DNA ligase (5 U/µl) in the presence of 1 µl 10× T4-DNA ligase buffer at 22 ˚C for 2 h and 1 h at 25 ˚C. The construct was used to transform competent BL21 cells via the heat-shock method (Green and Sambrook, 2012). The cloned plasmid, pET-Amylo, was isolated as mentioned above and sequenced using M13 primers for sequence confirmation.

BL21 cells containing pET-Amylo were cultured in 5 ml LB containing 25 µg/ml kanamycin for 16 h at 37 ˚C. The culture was used to seed a 50 ml LB until OD_{600} = 0.6. IPTG (0.5 mM) was used to induce the T7 promoter at 25 ˚C on a rotary shaker at 150 rpm for 6 h. The bacterial cells expressing pET-Amylo were precipitated at 6000 ×g for 10 min at 4 ˚C. The cells were resolvated in lysis buffer (50 mM NaH_2PO_4, 500 mM NaCl, 10 mM imidazole, 0.05% v/v Tween20) and incubated for 45 min at 4 ˚C. The cells were broke open using a sonifier at 70% strength and five 0.5 pulses on ice. The mixture was centrifuged at 6000 ×g for 10 min at 4 ˚C. The supernatant was used for recombinant protein purification via affinity

chromatography using Ni-NTA resin according to the manufacturer. The isolated recombinant protein was dialyzed for 24 h against 20 mM NaH_2PO_4 (pH = 7.5) at 4 ˚C with buffer change every 8 h.

2.4. Activity assays for recombinant amylopullulanases

Amylopullulanse activity was determined according to the 3,5-dintrosalisilic acid (DNS) method (Bernfeld, 1955).The substrate was 1% pullulan in mixed buffer (50 mM of each sodium phosphate, sodium acetate and glycine, pH = 5.5). To assay the protein activity, enzyme (50 µl) was added to the mixture of 50 µl 1% pullulan and incubated for 20 min at 60 ˚C. DNS reagent (100 µl of 1% w/v DNS, 30% w/v sodium potassium tartarate, 1.6% w/v sodium hydoxide) was added to stop the progression of reaction. The reaction was incubated at 100 ˚C for 10 min and after cooling down to 22 ˚C, centrifuged at 6000 ×g for 10 min, and the production of reduced sugars was read at 530 nm. By definition, 1 unit of amylopullulanse can release 1 µmole of reduced sugar in 1 min and in our case at 60 ˚C (pH = 6.0), when maltose is being used as the standard.

2.5. Definition of Biochemical and kinetic parameters

A pH profile was established with 1% pullulan in 50 mM mixed buffer at pHs 3-12 and an enzyme assay was performed as mentioned above. For pH stability, enzymes were incubated for 3 h at pH 3, 7 and 11 in 50 mM mixed buffer. The substrate prepared at optimum pH was added to the enzyme and the activity was measured as stated earlier. Furthermore, relative enzyme activities were determined in temperatures ranging from 20-90 ˚C. For this, 200 µl enzyme with 200 µl substrate were incubated separately at the temperatures for 60 min and enzyme activity was assayed. For temperature stability measurement, 600 µl of enzyme was incubated at 60, 70 and 80 ˚C for 3 h. Every 10 min, 20 µl of amylopullulanase incubated in different temperatures, was added to 20 µl substrate and the enzyme activity was assayed.

2.6. Effects of chemical compounds and metal ions on Coh4159 activity

Metal cations, 5 mM of each $MgCl_2$, $CaCl_2$, KCl, $FeCl_2$, $MnCl_2$, $ZnCl_2$, $CuCl_2$, and NaCl, were analyzed to check the effect on the enzyme activity. V_{max} and K_m were established for both recombinant enzymes with Lineweaver-Burk (Cook and Cleland, 2007) using pullulan as substrate. Every experiment had three replicates and the data were plotted in Prism5 (Motulsky, 2007).

2.7. Zymogram analysis

Native-PAGE (10%) including pullulan was used to separate the isolated recombinant enzyme. Triton X100 (2.5%) was used to wash the gel for 1 h with 50 mM sodium acetate (pH = 6.0) at 4 ˚C. The gel was incubated at 60 ˚C for 1 h then Lugol's reagent was added to the gel and enzyme activity was photographed.

3. Results and discussion

3.1. Expression and purification of the recombinant amylopullulanase

The *Coh4159* gene was PCR amplified, restricted with *Nco*I and *Xho*I and cloned in pET-26b(+) and transformed into BL21(DE3). Recombinant Coh4159 was heterologously expressed in the presence of 0.5 mM IPTG and purified via affinity chromatography (Figure 1, lane 3). The enzyme relative molecular weight was 82, kDa which can be considered as a relatively small to medium size

amylopullulanases (Table 1). Enzyme activity was demonstrated via zymogram analysis in the presence of pullulan (Figure 1, lane 5).

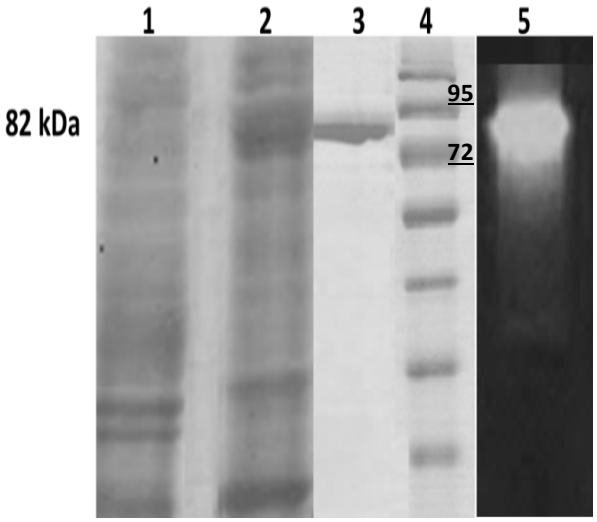

Figure 1. Heterologous expression of recombinant Coh4159 in *E. coli* and zymogram analysis. Lane 1: un-induced bacterial cell culture, Lane 2: induced bacterial cell culture with prominent band around 82 kDa. Lane 3: purified Coh4159 via affinity chromatography using Ni-NTA column, Lane 4: protein ladder, Lane 5: zymogram analysis is illustrative of enzyme activity in hydrolyzing pullulan.

3.2. Coh4159 kinetic and biochemical parameters

Enzymes kinetic parameters in the presence of pullulan were calculated with Prism5. Coh4159 had V_{max} = 2.85 µmol.min^{-1} and K_m = 0.5 mM (Figure 2a). Optimum pH for Coh4159 was 6.0 (Figure 2b), and the enzyme kept 50% of its activity for 2 h at pH = 7.0 and for 50 min at pH 3, and 11 (Figure 2c). Thus, the pH optima was similar to other previously reported amylopullulanases, except for Zebradast *et al*. (2017) where the greatest pH activity was obtained at basic pH = 8.0. The highest activity for Coh4159 was seen at 60 ˚C (Figure 2d). The recombinant enzyme kept 50% of its activity for 60 min, 100 min and 120 min incubated at 80, 70 and 60 ˚C, respectively (Figure 2e). Therefore, the enzyme had great temperature stability when compared to homologous enzymes from other bacterial sources. Although, some other amylopullulanases with greater tolerance to higher temperature have been previously reported (Table 1).

Table 1. Some properties of characterized amylopullulanases from thermophilic bacteria

Bacterial species	MW† (kDa)	T. O‡ (˚C)	pH♯	$Ca^{2+\Delta}$	Reference
Bacillus flavocaldarius KP1228	55	75-80	6.3	-	Suzuki *et al.*, 1991
Pyrococcus furiosus	89	105	5.5	d	Dang *et al.*, 1997
Thermoanaerobacterium thermosaccharolyticum	150	60	5-5.5	-	Ganghofner *et al.*, 1998
*Thermoanaerobacterium ethanolicus*39E	109	80	6.0	d	Lin and Leu, 2002
Staphylothermus marinus	73.5	105	5	d	Li *et al.*, 2013
Geobacillus thermoleovorans (gt-apu)	182	60	7.0	-	Nisha and Satyanarayana, 2013
Geobacillus thermoleovorans (apu105)	105	80	7.0	i	Nisha and Satyanarayana, 2014
Coh00831	70	60	6.0	d	Zebardast *et al.*, 2017
Coh01133	127	70	8.0	d	Zebardast *et al.*, 2017
Coh4159	82	60	6.0	d	This study

† Molecular weight

‡ Temperature optimum

♯ pH optimum

Δ Calcium dependency, d: dependent, i: independent

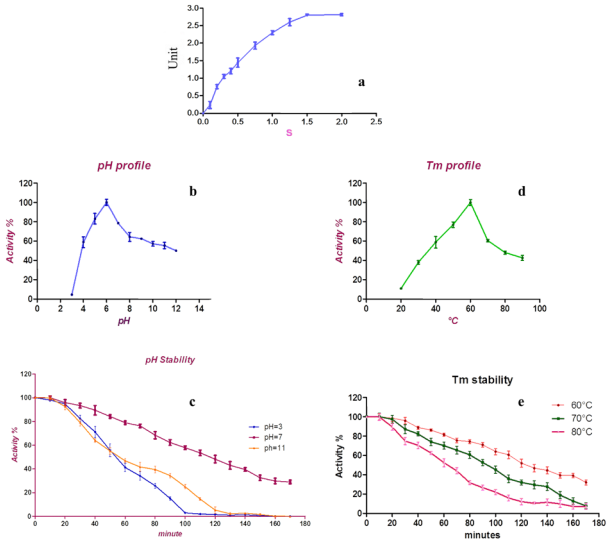

Figure 2. Kinetic parameters of Coh4159. a) Michaelis-Menton graph, b) pH profile, c) pH stability, d) temperature profile, and e) temperature stability.

3.3. Metal ions and chemical compounds effects

Amongst metal ions tested, Mn^{2+} and Ca^{2+} have improved the enzyme activity both at 5 and 10 mM. So far the only reported Ca^{2+} independent amylopullulanase was from *Geobacillus thermoleovorans* (Nisha and Satyanarayana, 2014), while similar to this study the others were dependent (Table 1). Mg^{2+} increased the enzyme activity at 5 mM, while 10 mM Mg^{2+} was no different than the control. Other cations significantly decreased the enzyme activity (Figure 3).

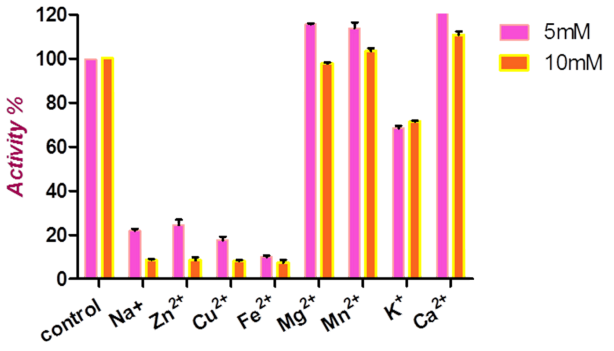

Figure 3. The effect of 5 and 10 mM cations on amylopullulanase activity. The thin bars are 10 mM of each metal cations.

4. Conclusion

Due to high amylopullulanase activity and thermostability, we found the recombinant enzyme suitable for use in industries such as baking, liquid sugar and bioethanol. In the future, we plan to transform the plasmid in an industrial strain and further optimize the culture condition for greater production of the recombinant enzyme.

Conflict of interest

The authors declare that there is no conflict of interest.

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Ethical approval

This manuscript does not contain any studies with human participants or animals performed by any of the authors.

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