HETEROLOGOUS EXPRESSION OF PULLULANASE FROM *FERVIDOBACTERIUM PENNIVORANS* IN *E. COLI*

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The thermophilic and mesophilic bacteria are a rich source of pullulan degrading enzymes. The gene encoding pullulanase enzyme from the thermophilic Fervidobacterium pennivorans was cloned, and its heterologous expression in E. coli *was achieved. The pullulanase gene encoding 544 amino acids has overexpressed under the control of a strong promoter. The recombinant enzyme was an optimal activity at 60 ºC and pH 4.5. The enzyme hydrolyzed 1.6 glycosidic linkages of pullulan, starch, amylopectin, glycogen and limited dextrin. The extraordinary high substrate specificity and its thermal stability makes this enzyme a good candidate for biotechnological applications in the starch processing industry.*

Keywords: thermophilic, pullulanase, recombinant

1. Introduction

Thermostable hydrolytic enzymes used in starch processing give 30% of the total industrial enzyme consumption worldwide [1] as starch requires a combination of enzymes for its complete hydrolysis. Exo- or endo- acting α-amylases, glucoamylases or β-amylases and isoamylases or pullulanases [3] are the main groups of enzymes used. The enzymes require different conditions for optimal activity, thus the processes in which they are involved require extensive control of pH and temperature variation, and, in several cases, added ions of adecvate cofactors [2, 3]. In the first step – liquefaction - alfa-amylase hydrolyses soluble starch (pH 4.5) at 90°C. The use of alfa-amylase is limited, however, to pH 6 and above, requiring a large amount of Ca2+ ions for its activity [4, 5]. The pH adjustment requires addition of salts. The pH has to be re-adjusted in the subsequent step, and the salts must be removed. All these processes increase the waste stream and the cost. In conclusion, for efficient applications of amylolytic enzymes, biocatalysts that work under similar conditions are required (4.5 pH, 60-

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90°C, without additional substances eg. Ca2+). Such enzymes can be produced from thermophilic microbial strains through cloning of fast-growing mesophiles by recombinant DNA technology. In the era of genomics, several hundreds of microbial genomes become available, consequently many DNA sequences coding for starch-converting enzymes are known. Isolation and cloning of several similar genes has been reported [7, 8, 9, 10, 11, 12]. Recently, an extensive study has been conducted for amylolytic enzyme isolation of several bifidobacterial strains [13]. However, specific properties of these enzymes can be concluded only from the growth-conditions of the host-organisms [6, 7] and several experiment have been conducted for expressing the recombinant amylolytic enzymes in several producer microorganisms.

Fervidobacterium pennivorans was isolated from a hot spring of the Azore Island in the Atlantic Ocean, belonging to the anaerobic *Thermotogales* and optimally growing at 70°C and pH 6,5. This extreme thermophile attracted our attention by they ability to degrade native feathers at high temperature.

2. Experimental

Construction of pullulanase expression plasmid

The coding sequence of the pullulanase was isolated from *Fervidobacterium pennivorans* by the polymerase chain reaction (PCR) using Forward 5' gCATATGGTTTAGAGATTCAAGTATGGGTCgg 3' and reverse 5' GGATCCGCTATGGTAATGTACAAAGG 3' primers. The PCR product and the pET20b plasmid were digested by NdeI and BamHI restriction enzymes and the digestion mixtures were separated and isolated from the 1% agarose gel by the Gel Extraction Kit (Fermentas), according to the instructions the manufacturer. The purity of large (pET20b digested by *NdeI* and *BamHI*) and small (PCR product digested by *NdeI* and *BamHI*) fragment isolates was verified by gel electrophoresis.

The ligation reaction was set up in 20 µL total volume, containing 14 µl of *NcoI/BamHI*-digested PCR product small fragment, 2 µL of pET20b large fragment and 1 U T4 ligase in 1x Ligation Buffer Fermentas, at 22^{o}C for 2 hours.

The ligation mixture was transformed into 100 µl of chemically competent *Escherichia coli* Top10F' cells (Invitrogen) and plated on Lysogeny Broth (LB) medium composed of 10 g/L Tryptone, 5 g/L Yeast Extract, 10 g/L NaCl and 100 mg/L ampicillin. The integration of the pullulanase in vector was verified by double digestion with *NdeI* and *BamHI* restriction enzymes, at 37^{o}C for 1 h.

Expression

Escherichia coli BL21 (DE3) RIL was used as host for enzyme expression. Chemically competent cells were transformed by 1 µL recombinant

vector (pET20b-pullulanase). Starter culture from a single colony was grown overnight at 37°C in LB medium containing 100 μg/mL ampicilline at shaking. To produce pullulanase, a volume of 200 mL LB medium supplemented with 100 μg/ml ampicilline was inoculated in the starter culture, and was grown at 37°C until OD_{600}=0.7. Expression was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) in a final concentration of 0.5 mM and culture was maintained at 30 and 37°C in a shaking incubator at 250 rpm in the same conditions for 6h and harvested by centrifugation (5000 g for 10 min).

Cells were resuspended in 10 mL 1x Phosphate Buffer Saline (1XPBS), and then lyzed by sonication, and centrifugated at 10000 g for 10 min. The supernatant was stored at 4°C. Purification of the pullulanase was carried out by affinity chromatography.

Influence of temperature and pH

Enzyme activity was determined in the pH range of 4.0 to 8.0. The assays were performed under the standard assay condition at 60°C. Thermostability of the enzymes was evaluated by incubation of pullulanase at temperatures between 20 and 90°C for 48 h at pH 5.0. Standard assays were conducted to determine the residual enzyme activity.

Substrate specificity

Substrate specificity was investigated using amylopectin (potato), amylopectin (corn), soluble starch, glycogen, amylase, pullulan and cyclodextrin as substrates.

3. Results and discussions

The pullulanase gene was isolated from *Fervidobacterium pennivorans* and inserted in pET20b expression plasmid as described in Experimental section. The scheme for the map of expression vector pET20b-pullulanase is outlined in Fig. 1.

Expression vector was introduced into chemically competent *Escherichia coli* BL21 (DE3) RIL. The expression was realized at 30°C and 37°C. Induction of the gene of pullulanase was realized by IPTG in a final concentration of 0.5 mM. The results of the expression were analyzed by SDS-PAGE, presented in Fig. 2.

The bacterial pellet from 250 mL production culture was resuspended in 30 ml 1XPBS and lysed by sonication.

In order to facilitate the protein purification, the pET20b contained a $(His)_6$-tag. Purification of the $(His)_6$-tagged pullulanase was carried out by affinity chromatography, then it was dialyzed in acetate (pH 4–6) and Na-phosphate (pH 6.5-8) buffers.

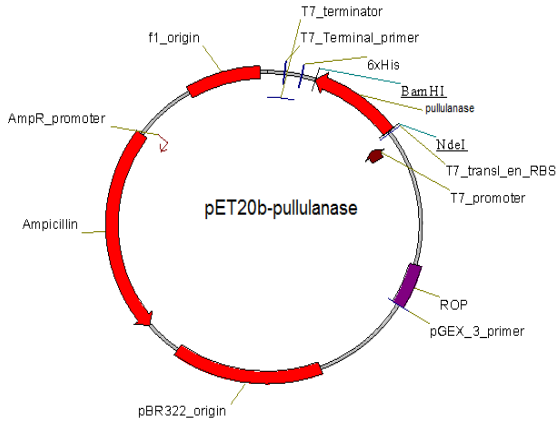

Fig. 1. Vector map of the pET20b-pullulanase recombinant plasmid

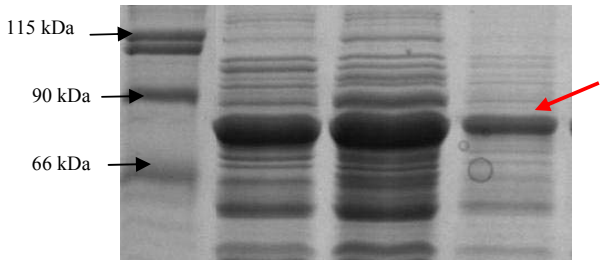

Fig. 2. SDS PAGE of the recombinant pullulanase. Lane 1 contains the molecular weight markers, Lane 2 contains the crude extract expressed at 30°C, 6 h, Lane 3 contains the crude extract expressed at 37°C, 6 h, and Lane 4 contains the soluble proteins from expression at 37°C, 6 h.

The results showed the yield of pullulanase was higher at 37^{o}C than at 30^{o}C after 6 h of expression. As identified by SDS-PAGE, approximately 40% of the expressed enzyme was in a soluble form.

The recombinant pullulanase activity was studied at 60^{o}C using buffer solutions with different pH values (sodium acetate between pH 4.0-6.0, sodium phosphate 6.5-8.0). The results are shown in figure 3.

The effect of temperature on amylolytic activity was evaluated in a 50 mM sodium - acetate buffer at different temperatures from 20^{o}C to 90^{o}C as shown on figure 4. The pH value was 5.0 under assays.

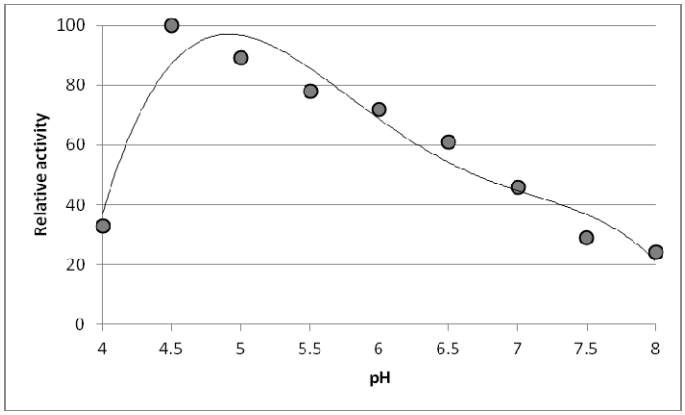

Fig. 3. Effect of pH on the activity of pullulanase at 60°C

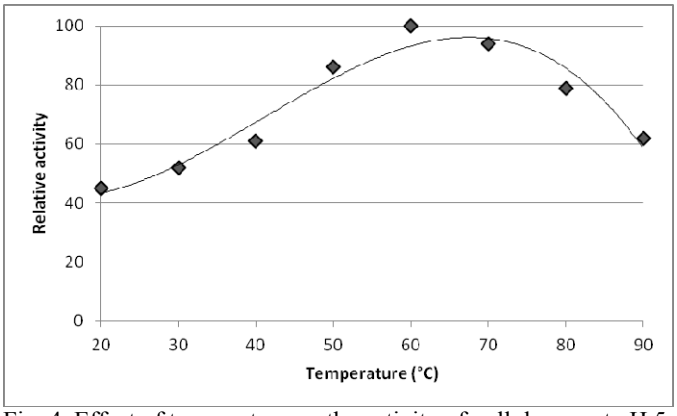

Fig. 4. Effect of temperature on the activity of pullulanase at pH 5.

Pullulanase was found to be active over a broad range, namely in the pH interval of 4 to 8. Maximal activity was found at temperature 60°C. The initial activity was increased with a few percents after incubation at high temperatures.

The recombinant pullulanase was found to liquefy various polysaccharides. Highest specific activity was measured with soluble starch and amylopectin from potatoes.

4. Conclusions

Production of the pullulanase from *Fervidobacterium pennivorans* in *Escherichia coli* was realized successfully. Using E. coli as expression host, a high efficiency in protein production was provided. Experimental results demonstrated that the recombinant pullulanase is active and stabile at 60°C.

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