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# Cloning and Overexpression of Gene Encoding the Pullulanase from *Bacillus naganoensis* in *Pichia pastoris*

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**Abstract** The expression of a pullulanase gene in *Pichia pastoris* was investigated. The gene encoding pullulanase was cloned by PCR using the chromosomal DNA of *Bacillus naganoensis* as the template. The expression vector pPIC9K-Pu was constructed by inserting the pullulanase gene into plasmid pPIC9K and then transformed into *Pichia pastoris* SMD1168 by electroporation. Activity determination, SDS-PAGE, and PCR amplification indicated that the gene of the pullulanase from *B. naganoensis* had successfully been expressed in SMD1168 and the molecular size of the expressed recombinant product was about 119.9 kDa. This is the first report on the successful expression of the pullulanase from *B. naganoensis* in *P. pastoris*. The transformant secreted recombinant pullulanase with the activity of 350.8 IU/ml in shake-flask culture. The properties of the recombinant pullulanase were characterized.

**Key words:** *Bacillus naganoensis*, pullulanase, *Pichia pastoris*, expression, enzymatic properties

Pullulanases (pullulan 6-glucanohydrolase; EC 3.2.1.41) are debranching enzymes that are able to hydrolyze the α-1,6 glycosidic linkages in pullulan, starch, amylopectin, and related oligosaccharides. Starch, the essential constituents of amylose and amylopectin, is a polymer of glucose, in which the glucose units are joined by either alpha-1,6-glucosidic linkages or alpha-1,4-glucosidic linkages. The enzymatic conversion of starch into glucose, maltose, and fructose for use as food sweeteners represents an important area in industrial enzyme usage. Enzymes known as the alpha-amylases can cleave the alpha-1,4-glucosidic linkages, but cannot cleave the alpha-1,6-glucosidic linkages. In order to cleave these linkages, and thereby breaking the large carbohydrate molecules into smaller molecules to obtain of high conversion of starch, it has already been proposed to use pullulanases that hydrolyze alpha-1,6-glucosidic bonds together with alpha-amylases. Therefore, the enzyme has been widely used in the starch-processing industry. The most important industrial application of pullulanase is in the production of glucose or maltose syrups, when it is used in combination with glucoamylase or β-amylase, respectively, in the saccharification process.

Because of a large demand for the pullulanase, much work has been done by many investigators to identify novel enzymes. Many microorganisms have been shown to produce pullulanases. However, owning to the restricted conditions such as pH and tempreture, most of these pullulanases have no commercial value: The conditions have to be optimized for function at the elevated temperatures and acidic conditions that are required in many commercial manufacturing protocols. In 1985, Nielsen *et al.* [7] reported a pullulanase produced by *Bacillus acidopullulyticus* that has improved thermoduric and aciduric properties. It is the only commercially available pullulanase, named PROMOZYME (Novo), up to now, thus costing exorbitant prices [15]. In 1991, Tomimura [13] described a pullulanase isolated from *B. naganoensis* (ATCC No. 53909). This pullulanase is more thermo- and acid-tolerant than that from *B. acidopullulyticus*. At least 50% of its pullulan-hydrolyzing activity retains after 232 h in aqueous solution at 60°C and pH 4.5. Teague *et al.* in 2001 [12] cloned the pullulanase gene from *B. naganoensis* into a *B. subtilis* host for the first time. However, the expression product had low pullulanase activity. There has so far been no report of the cloning and overexpression of the pullulanase gene in *P. pastoris*.

In this article, we report the cloning and overexpression of the pullulanase gene from *B. naganoensis* in *P. pastoris* and the properties of the recombinant enzyme.

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## MATERIALS AND METHODS

### Strains and Plasmids

The *E. coli* strain used was DH5α. *B. naganoensis* (ATCC53909) was the source of DNA from which the pullulanase was isolated. *P. pastoris* SMD1168 (Mut+, His−, pep4−) and pPIC9K (Invitrogen Corp.) were used for the expression of *B. naganoensis* pullulanase.

### Growth Media

*E. coli* DH5α were grown aerobically at 37°C in Luria-Bertani (LB) medium (consisting of 0.5% yeast extract, 1% Bacto tryptone, and 1% NaCl). *B. naganoensis* was grown aerobically at 30°C in LB medium as previously described [11]. In this culture condition, *B. naganoensis* can produce 1.3 IU/ml pullulanase in shake-flask culture. *P. pastoris* was grown in YPD medium (consisting of 1% yeast extract, 2% Bacto tryptone, and 2% glucose) and MD plate [consisting of 2% glucose, 1.34% yeast nitrogen base (YNB), and 2% agar]. When required, the following antibiotics were added: for *E. coli*, ampicillin; for *P. pastoris*, G418. *P. pastoris* carrying recombinant plasmid was cultured in BMGY (consisting of 1% yeast extract, 2% peptone, 1% glycerol, 1.34% YNB, 0.00004% biotin, pH 6.0, and 10% 1 mol/l potassium phosphate buffer) and BMMY (consisting of the same composition as BMGY medium, except that 1% methanol was added instead of glycerol).

### Extraction of the Genomic DNA from *B. naganoensis* and Cloning the Pullulanase Gene of Mature Peptide by PCR

The genomic DNA was extracted [9] from *B. naganoensis* grown overnight in LB broth at 37°C. The information about the pullulanase gene from *B. naganoensis* [12] was used to design oligonucleotides for PCR amplification of the pullulanase gene directly from *B. naganoensis* chromosomal DNA. The oligonucleotide primers were as follows (restriction enzymes are indicated in parentheses):

Pu1-forward 5'-CGGAATTCGATGGGAACACGACAA-CGATC-3' (EcoRI);
Pu2-reverse 5'-CGGAATTCTTACCGTGGTCTGGGCT-TACCT-3' (EcoRI);

PCR amplification was carried out at the following temperature profile: 30 cycles of 94°C for 1 min, 50°C for 90 s, and 72°C for 2 min 30 s. A final extention was carried out at 72°C for 20 min after the last amplification cycle.

For the analysis of DNA, agarose gel electrophoresis was carried out under standard conditions [9].

### Plasmid Construction

For recombinant expression, the 2.8-kb PCR product of the *B. naganoensis* pullulanase gene was digested with EcoRI and cloned in the vector pPIC9K. The resulting construct was pPIC9K-Pu, carrying the pullulanase gene. Plasmid DNA was isolated using Promega spin columns (Promega). The DNA was sequenced by TaKaRa Co. Ltd.

### Transformation of Plasmid DNA into *P. pastoris*

Linearized recombinant plasmid DNA (5 μl), which was digested with SacI, was electroporated into 80 μl of competent *P. pastoris* cells using a Bio-Rad Gene Pulser instrument following the manufacturer's instructions. After electroporation, the samples were plated on the dextrose-based medium, containing the antibiotic Geneticin (G-418 Sulfate), without histidine supplementation (MD plate). After 3 days of incubation at 30°C, colonies of transformed *P. pastoris* appeared on the plates. As a negative control, *P. pastoris* cells were also transformed with the vector plasmid pPIC9K.

### Cultivation of *P. pastoris*

*P. pastoris* transformants were grown at 30°C in 25 ml of BMGY medium in a 250-ml flask in a shaking incubator until $OD_{600}$ reached to 4. The culture was centrifuged and the supernatant was discarded. Subsequently, the cells were resuspended in 100 ml of BMMY medium to an $OD_{600}$ of 1, and induction was then started. The culture was incubated at 30°C for 4 days with the addition of 1 ml methanol once a day to maintain induction.

### Identification of High-Expression Transformants

Transformants were cultured in BMGY and BMMY media in turn at 30°C. Identification of pullulanase activity was carried out by plating the culture medium (10 μl) on plates that consisted of 1% red pullulan and 2.5% agar (pH 4.5): Pullulanase cleaves red pullulan into a colorless product. The size of the halo developed on the plate enabled easy identification of transformants that efficiently secreted recombinant pullulanase.

### Preparation of Crude Enzyme

After the culture broth was centrifuged for 10 min at 10,000 rpm, ammonium sulfate (60% saturation) was added to the suspension, and these were allowed to stand overnight at 4°C. The precipitate was collected by centrifugation (10,000 rpm, 10 min) and suspended in sodium acetate buffer (pH 4.5). Then, the crude enzyme suspension was obtained by dialysis.

### Enzyme Assay

Pullulanase activity was determined by measuring the amount of reducing sugars released during incubation with pullulan [11]. The assay mixtures (total volume, 2 ml) contained 1 ml of 0.5% (wt/vol) pullulan (Sigma P4516, Sigma Chemical Co., St. Louis, MO, U.S.A.) dissolved in 0.2 M sodium acetate buffer (pH 4.5) and 1 ml of appropriately diluted enzyme suspension. After incubation at 60°C for 30 min, the reaction was stopped by addition of 3 ml of

cold dinitrosalicylic acid reagent, followed by boiling for 7 min. It was then cooled to room temperature and mixed with distilled water (10 ml) for the reaction. The amount of reducing sugars released was determined by absorbance at 550 nm. Sample blanks were used to correct for nonenzymatic release of reducing sugars. One unit of pullulanase activity was defined as the amount of enzyme that released 1 μmol of reducing sugars per min under the assay conditions used.

**Polyacrylamide Gel Electrophoresis**

The crude enzyme preparation was analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to determine the apparent molecular size of the enzyme. Proteins were stained with Coomassie brilliant blue.

**Characterization of Hydrolysis Products**

Thin-layer chromatography (TLC) was performed on 0.2-mm silica gel plates with a solvent system consisting of chloroform, methanol, and water (13:7:1.5, vol/vol/vol). Sugars were detected by heating the TLC plates for a few minutes in a hot dry oven after dipping the plates in a alcohol-concentrated sulfuric acid mixture (85:15, vol/vol). Glucose and maltotriose were used as controls.

**Effect of Temperature and pH on Pullulanase**

The effect of temperature on pullulanase activity was determined at temperatures ranging from 40 to 80°C, for 30 min, using the pH described below. Thermostability at 60°C was determined by incubating the enzyme in sodium acetate buffer (pH 4.5) for the periods ranging from 0 to 64 h, followed by measurement of remaining activity.

To determine the effect of pH on the enzymatic activity, assay reaction mixtures were prepared by diluting pullulan in 0.2 M sodium acetate buffers with pH ranged from 3.0 to 7.0. The assays were performed at 60°C. For pH stability, the enzyme was preincubated for 2 h at 60°C in a suitable buffer (pH 3.0–7.0), and aliquots were then transferred into the standard reaction mixture to determine the amount of remaining enzyme activity.

**Effect of Metal Ions and Other Reagents on Pullulanase Activity**

Effects of EDTA, SDS, and ions on pullulanase activity were investigated after preincubation of the enzyme with various agents for 10 min at 60°C, followed by measurement of remaining activity.

## RESULTS

**Cloning the Pullulanase Gene of Mature Peptide from *B. naganoensis* by PCR**

Using the genomic DNA extracted from *B. naganoensis* as the template, PCR amplification yielded a 2.8-kb fragment that was identical to the sequence of the corresponding pullulanase gene fragment described before [12] (Fig. 1).

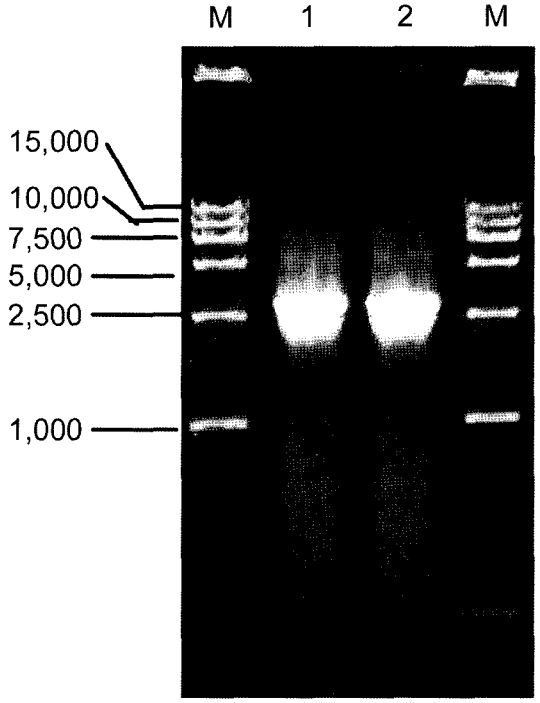


**Fig. 1.** Amplification the pullulanase gene fragment of mature peptide by PCR.
M, DL15000 marker; Lanes 1–2, amplified products of PCR.

**Construction of Pullulanase Expression Vectors**

The construction of pullulanase expression vector pPIC9K-Pu is shown in Fig. 2. The pullulanase gene fragments were fused in-frame with the 3'-end of an α-factor coding sequence under the control of the alcohol oxidase promoter (AOX1) and AOX1 terminator on the Pichia high-efficiency expression vector pPIC9K. The correct direction of the inserted pullulanase gene fragments was confirmed by PCR analysis.

**Transformation of *P. pastoris* with Recombinant Vector pPIC9K-Pu**

The plasmid pPIC9K-Pu was transformed into *P. pastoris* strain SMD1168 by electroporation. In most cases, high copy number integration has been shown to be important for heterologous high-level expression of enzyme. In order to identify suitable clones, transformants were selected by growth on MD plates containing increasing concentrations of G-418 (0.5 to 4.0 g/l), since there is a relationship between the copy number of integrated vector and the level of resistance to G-418 [1].

The individual colonies resistant to high concentration of G-418 were picked out. After induction by 1% methanol for 48 h, the culture medium of the recombinant yeast

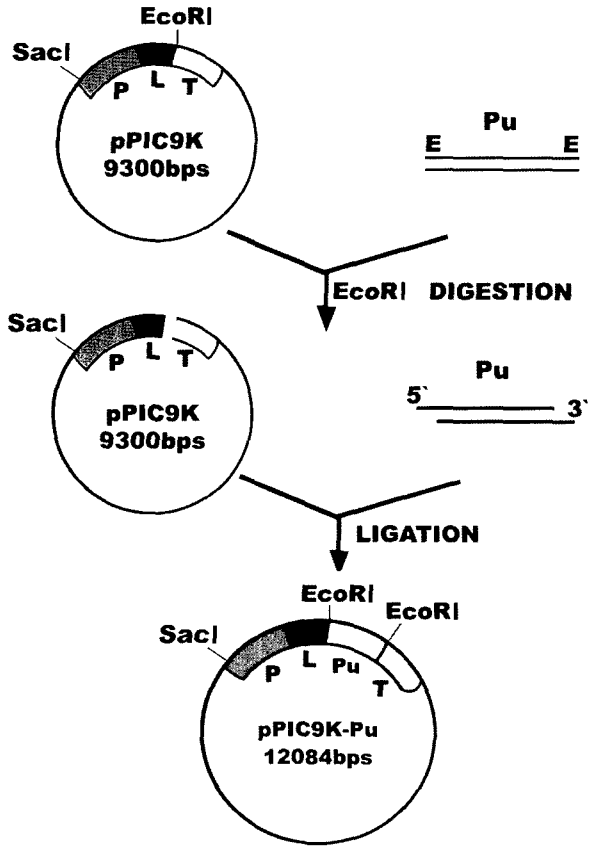


**Fig. 2.** Construction of pullulanase expression vector.
E, EcoRI; L, leader sequence; P, AOX1 promoter; Pu, pullulanase gene; T, AOX1 terminator.

strains were plated on plates, using red pullulan for substrate (Fig. 3).

Results showed that pullulanase from recombinant *P. pastoris*, named as P8, obviously hydrolyzed pullulan,

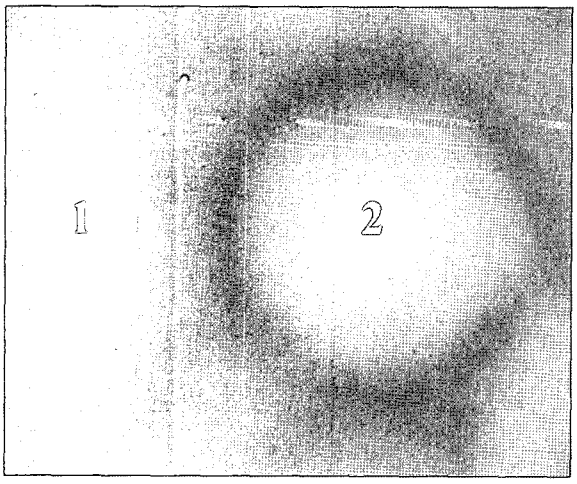


**Fig. 3.** Detection of the recombinant pullulanase by pullulan plate.
1, SMD1168-pPIC9K (negative control); 2, P8 (SMD1168-pPIC9K-P8).

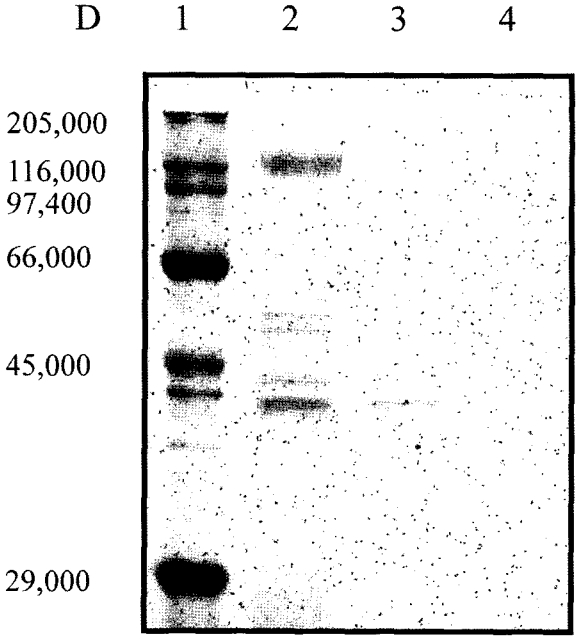


**Fig. 4.** Identification of the expression product from recombinant yeasts by SDS-PAGE.
1, Marker; 2, culture supernant of strain P8; 3, culture supernant of SMD1168-pPIC9K (negative control); 4, culture supernant of strain P8 after incubating for 6 h at 60°C.

whereas the negative control could not hydrolyze it. P8 secreted recombinant pullulanase, which had the highest enzyme activity of 350.8 IU/ml when the activity of pullulanase in the culture supernants was measured.

## Characterization of the Recombinant Pullulanase and its Hydrolysis Products

The results of SDS-PAGE are shown in Fig. 4. The culture supernant of strain P8 showed an obvious band, whereas

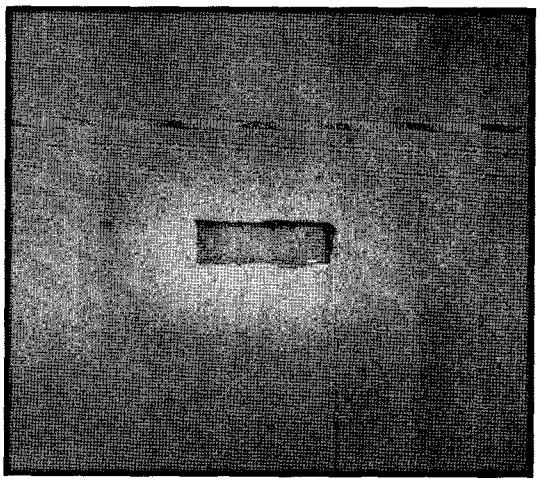

**Fig. 5.** Assay of the pullulanase activity of the renaturation SDS-PAGE band.

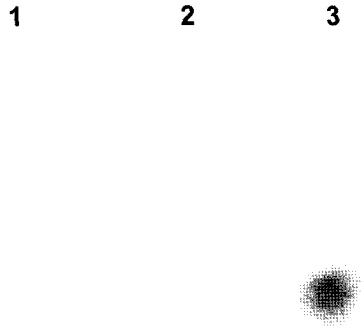


**Fig. 6.** TLC analysis of product formation with pullulan.
1, The samples of the pullulan degradation reaction, which were prepared after reaction with the recombinant enzyme for 2 h; 2, Control (Maltotriose); 3, Control (Glucose).

SMD1168-pPIC9K (negative control) did not. The molecular size of this protein band was found to be 119.9 kDa, which is close to that of the pullulanase from *B. naganoensis* [6], as determined by SDS-PAGE analysis. For further identification, the band was cut, renatured in 0.1 M Tris-HCl buffer, and the renatured band was then placed on a 1.5% red-pullulan plate (pH 4.5). The halo appeared after incubation for 6 h at 60°C (Fig. 5), showing that the band represents pullulanase. When the culture supernant of strain P8 was incubated for 6 h at 60°C, other protein bands disappeared. Only the band that corresponded to pullulanase persisted, indicating that the recombinant pullulanase was thermoduric.

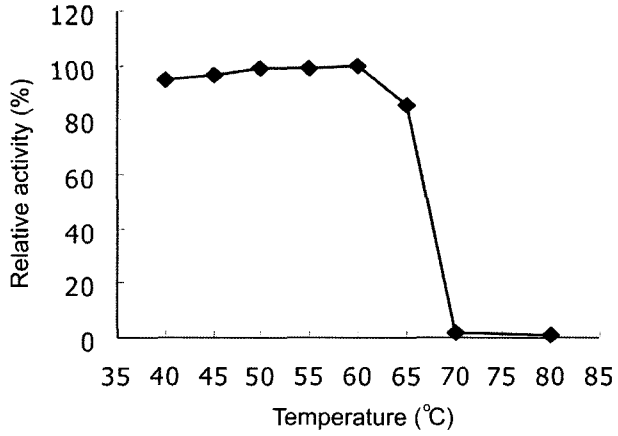


**Fig. 7.** Influence of temperature on pullulanase activity.

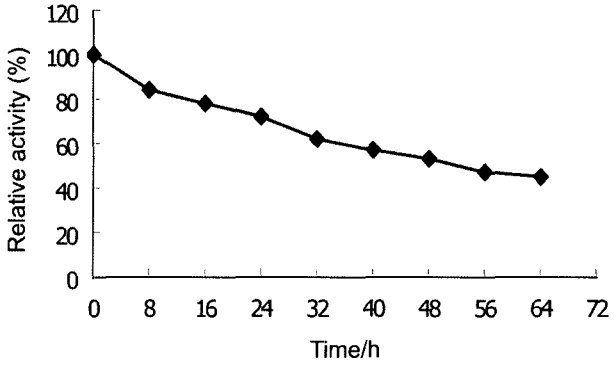


**Fig. 8.** Influence of temperature on pullulanase stability.

TLC analysis showed that hydrolysis of pullulan by the recombinant enzyme yielded maltotriose, but not glucose (Fig. 6). Thus, the product profiles indicated that the recombinant enzyme is pullulanase, which is different from other enzymes (*e.g.*, glucoamylase and cyclomaltodextrinase) that could not yield maltotriose, but yield glucose.

**Enzymatic Properties of the Recombinant Pullulanase**

The temperature and pH profiles of the *B. naganoensis* pullulanase produced by *P. pastoris* were determined with pullulan as substrate. The recombinant pullulanase was active at temperatures between 40°C and 65°C, where over 80% of the maximal activity remained. The temperature optimum of the enzyme was 60°C when measured at pH 4.5 (Fig. 7). The recombinant enzyme still retained most of the original activity (≥72%) after 24 h of incubation (Fig. 8).

The recombinant pullulanase showed activity over a broad pH range, ranging from 3.5 to 6.0, and the highest rate of pullulan hydrolysis was observed at pH 4.5 when measured at 60°C (Fig. 9). The activity was stable in a wide acidic pH range of 3.4–6.2, and in fact, it still retained the maximal activity (=100%) after incubation for 2 h at 60°C in pH 4.5 (Fig. 10).

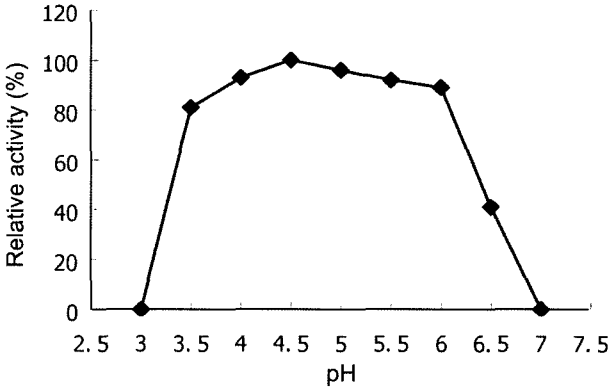


**Fig. 9.** Influence of pH on pullulanase activity.

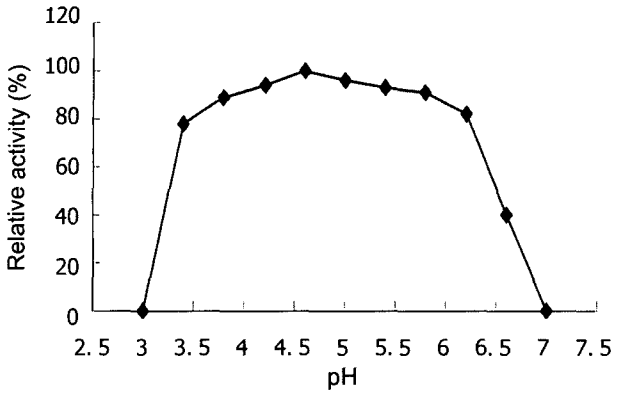


**Fig. 10.** Influence of pH on pullulanase stability.

The effects of various chemicals on pullulanase activity are shown in Table 1. The activity increased in the presence of $Mg^{2+}$ and $Ni^{2+}$, and was partially inhibited by $Ag^{2+}$, $Cu^{2+}$, $Zn^{2+}$, $Co^{2+}$, $Mn^{2+}$, $Fe^{2+}$, $Cd^{2+}$, and $Pb^{2+}$. The enzyme was totally inhibited by $Hg^{2+}$ and SDS, and was slightly affected by $K^{+}$, $Na^{+}$, $Li^{+}$, $Ca^{2+}$, $Ba^{2+}$, and EDTA.

## Discussion

Expression of the pullulanase gene is commonly carried out by using *E. coli* and *B. subtilis* strains as the host. However, the gene products are mainly intracellular enzyme, which is not released into the culture fluid and has low pullulanase activity. Presently, there is only one commercially available pullulanase, PROMOZYME (Novo), in the world, which is produced by *B. acidopullulyticus*. Owing to high cost of the enzyme, however, only a limited amount of commercial pullulanase has been used in the starch-processing industry.

The methylotrophic yeast *P. pastoris* has been developed as an efficient system for high-level production of foreign proteins. It has several advantages such as high productivity, stable inheritability, secretable product, and mature fermentation techniques. A number of proteins have thus far been successfully expressed in this system [2]. Proteins partially are secreted to exceptionally high levels [2–5, 8, 10, 16].

This is the first report on the heterologous high-level expression of pullulanase from *B. naganoensis* in the methylotrophic yeast *P. pastoris*: The level of the pullulanase activity from recombinant yeast P8 was about 310 times that of the wild-type strain. Furthermore, high-density fermentation in the fermentor would significantly improve the expression of the recombinant enzyme.

Characterization of the recombinant pullulanase showed that the pH optimum of the enzyme lies between pH 4.0 and 5.5, which is broader than that of the wild-type pullulanase, when measured at 60°C. The probable reason is that the structure, stability, and function of the pullulanase secreted were affected by the glycosylation [14].

The result described above indicates that the pullulanase produced by the recombinant yeast was more productive than that of the wild-type strain. Furthermore, the thermoduric and aciduric properties of the recombinant pullulanase are very propitious in applying to the starch-processing industry. Therefore, the recombinant *P. pastoris* that we have constructed has potential to be useful in large-scale industrial production.

**Table 1.** Effects of EDTA, SDS, and ions on recombinant pullulanase activity. Compounds were tested at 5 mM concentration.

| Chemical | Relative activity (%) | Chemical | Relative activity (%) |
|---|---|---|---|
| Control | 100 | | |
| KCl | 101.6 | $MnCl_2 \cdot 4H_2O$ | 34.5 |
| NaCl | 104.8 | $BaCl_2 \cdot 2H_2O$ | 99.9 |
| $LiCl \cdot H_2O$ | 105.0 | $NiCl_2 \cdot 6H_2O$ | 108.3 |
| $Ag_2SO_4$ | 39.3 | $HgSO_4$ | 0 |
| $CuCl_2 \cdot 2H_2O$ | 35.9 | $FeSO_4 \cdot 7H_2O$ | 58.5 |
| $ZnCl_2$ | 86.4 | $3CdSO_4 \cdot 8H_2O$ | 79.9 |
| $CaCl_2 \cdot 2H_2O$ | 92.4 | $Pb(CH_3COO)_2 \cdot 3H_2O$ | 15.5 |
| $CoCl_2 \cdot 6H_2O$ | 82.3 | EDTA | 94.8 |
| $MgCl_2 \cdot 6H_2O$ | 110.7 | SDS | 0 |

## Acknowledgments

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