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Novel Properties for Endoglucanase Acquired by Cell-Surface Display Technique

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In order to improve the stability of endoglucanase under thermal and acidic conditions, the endoglucanase gene was fused to the N-terminus of the *Saccharomyces cerevisiae pir* gene, encoding the cell wall protein PIR. The fusion gene was transformed into *Pichia pastoris* GS115 for expression. A resulting strain with high expression and high activity was identified by examining resistance to Geneticin 418, Congo red staining, and quantitative analysis of enzyme activity. SDS-PAGE analysis revealed that the endoglucanase was successfully displayed on the yeast cell surface. The displayed endoglucanase (DEG) showed maximum activity towards sodium carboxyl methyl cellulose at approximately 275 IU/g cell dry weight. DEG exhibited greater than 60% residual activity in the pH range 2.5–8.5, higher than free endoglucanase (FEG), which had 40% residual activity at the same pH range. The highest tolerated temperature for DEG was 70ºC, much higher than that of FEG, which was approximately 50ºC. Moreover, DEG showed 91.1% activity at 65ºC for 120 min, while FEG only kept 77.8% residual activity over the same period. The half-life of DEG was 270 min at 65ºC, compared with only 150 min for FEG. DEG could be used repeatedly at least three times. These results suggest that the DEG has broad applications as a yeast whole-cell biocatalyst, due to its novel properties of high catalytic efficiency, acid-thermal stabilities, and reusability.

Keywords: Endoglucanase, cell-surface display, *Pichia pastoris*, acid-thermal stabilities, reusability

Introduction

Endoglucanases (endo-1,4-β-D-glucanases or EGs, E.C. 3.2.1.4) play key roles in the hydrolysis of cellulose [11], and are widely used in the food industry for increasing fruit juice yields, filtering beer, and extracting oils. EGs have also been employed to improve the nutritive quality of animal feed and for the fermentation of biomass into biofuels [3, 13]. EGs with strong stability in acidic-thermal environments are required in these industrial processes. It is well known that EGs have been widely detected in bacteria and filamentous fungi and expressed in *Escherichia coli* or yeast [26, 27], but some drawbacks to the recombinant EG have restricted their application in various biotechnological processes, including the low expression levels, and the relatively poor temperature and pH stabilities [1, 17].

The cell-surface display system provides a feasible approach for improving the stability of natural enzymes [5, 23]. This approach displays enzymes on the surface of microorganisms, such as *Zymobacter palmae* [10], *Saccharomyces cerevisiae* [14, 19], and *Pichia pastoris* [9, 24], by fusing them to a cell membrane or cell wall protein. Moreover, surface-immobilized enzymes retain their native catalytic activity while having enhanced stability. For example, the short peptide Scr35 anchored by α-agglutinin was more tolerant to acid [15]. The thermostability of lipase Lip2 was improved by displaying it on *S. cerevisiae* cells using Cwp2 as an anchor [12]. Therefore, using surface-displayed enzymes as whole-cell biocatalysts is more cost-effective and

advantageous for industrial bioconversion processes [20]. Various anchor proteins have been reported in yeast, such as α-agglutinin, Flo1p, GPI (glycosylphosphatidylinositol), PIRs (proteins with internal repeats), and CWPs (other cell wall proteins) [22]. PIRs are extensively conserved yeast cell wall proteins that contain a varying number of internal repeat sequences (SQIGDGQIQAT) and are covalently link to cell wall β-1,3-glucan through alkali-sensitive ester linkages [21]. The repeating sequences are essential for transmembrane insertion and have recently been applied to cell-surface display systems [4]. In our previous work, β-galactosidase and green fluorescent protein were displayed successfully on the *P. pastoris* cell surface using PIR as an anchor [25], which demonstrated that PIR could be used for display of other heterologous enzymes while retaining their native activity.

The yeast *P. pastoris* is particularly suited to foreign protein expression because it can yield high levels of protein expression and possesses the ability to perform post-translational modifications, including glycosylation and disulfide bonds. Based on these advantages, some researchers are trying to use *P. pastoris* as a cell-surface display system to construct whole-cell biocatalysts, in order to obtain an excellent engineering strain with high protein yield and biocatalysts with stable characteristics [22].

We had previously done gene shuffling of EG from the thermophilic fungus *Thermoascus aurantiacus* (GenBank Accession No. KJ481835) and heterologous expression of mutant EG in *p. pastoris*. The mutant EG was active over a wide pH range and had high temperature optima, but it did not show high thermal and pH stabilities. This EG had only 41% residual activity after 30 min at 70ºC and 40% of residual activity after 24 h in the pH range of 2.5 to 8.5 at room temperature [2]. In this study, the mature peptide of mutant EG was fused to the N-terminus of the anchor protein PIR, generating a novel cell-surface EG with a high level of CMCase activity, thermal stability, pH stability, and reusability.

Materials and Methods

Strains, Plasmids, and Growth Media

Strains and plasmids used in this study are listed in Table 1. *E. coli* DH5α was aerobically grown at 37ºC in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, and 0.5% sodium chloride) or on LB agar plates. *S. cerevisiae* EBY100 and *P. pastoris* GS115 were cultivated at 28ºC in YPD medium containing 1% yeast extract, 2% peptone, and 2% dextrose. The transformants were grown at 28ºC in MD medium (containing 1.34% yeast nitrogen base, 4×10^{-5}% biotin, 0.5% dextrose, and 1.5% agar), whereas MM medium was supplemented with 0.5% methanol instead of dextrose. The recombinant strain PEG-6 was cultivated at 28ºC in BMGY and BMMY medium for growth and induction studies, respectively (BMGY medium: 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5}% biotin, 2% peptone, 1% yeast extract, and 1% glycerol; BMMY medium was supplemented with 0.5% methanol instead of glycerol).

DNA Manipulations and Transformation

Isolation of plasmid DNA from *E. coli* was performed using the Qiagen Mini Spin isolation kit according to the manufacturer's instructions (Qiagen Inc., Valencia, CA, USA). Chromosomal DNA of *S. cerevisiae* EBY100 was prepared as described by Harju *et al*. [6]. Plasmids were introduced into *E. coli* DH5α by standard heat-shock transformation [18]. Electroporation was used for plasmid transfer into yeast cells as previously described [1].

Table 1. Strains and plasmids used in this study.

Strains / plasmids	Description	Source
Strains		
E. coli DH5α	lacZΔM15, Δ(lacZYA-argF)U169, Ap^r	Takara
S. cerevisiae EBY100	Wild type containing *pir* gene	This lab
P. pastoris GS115	Host for cell-surface expression (arg4 his4 aox1:: arg4)	Invitrogen
P. pastoris PEG-6	Displaying EG on cell surface (His^+mut^+, pPIC9K-pir-eg)	This study
Plasmids		
pMD18-T	Gene cloning and sequencing	Takara
pPIC9K	Protein expression	Invitrogen
pMD-pir	Containing *pir* gene	This study
pPIC9K-eg	Free expression of EG	This lab
pPIC9K-pir-N	No expression (control plasmid)	This study
pPIC9K-pir-eg	Surface displaying of EG	This study

Restriction endonuclease digestions and DNA ligation were performed according to the supplier's instructions (Takara, Beijing, China). DNA sequencing was performed with the Bigdye Terminator cycle sequencing kit (Sangon, Beijing, China).

Isolation of the Anchor Sequence *pir*

Anchor sequence *pir* was amplified by PCR from the chromosomal DNA of *S. cerevisiae* EBY100 using the forward primer 5′-TATATGAATTCACTAGTGCCGCTGCTATCTCTCAAATTGG-3′ and reverse primer 5′-TATATGCGGCCGCTTAAGAAGTTAAAG TTGTGGCTTGG-3′. Primers were designed according to the DNA sequence (GenBank Accession No. NM001179730). Restriction sites used for subsequent cloning are underlined: *Eco*RI and *Spe*I for the forward primer and *Not*I for the reverse primer. PCR amplifications with Taq polymerase were performed by pre-denaturation at 95°C for 5 min, followed by 30 cycles of 94°C for 45 sec, 61°C for 45 sec, and 72°C for 1 min, and post-elongation at 72°C for 10 min. The PCR product was digested by *Eco*RI and *Not*I, and then inserted into the vector pMD18-T by ligation. The ligation mixture was transformed into *E. coli* DH5α and transformants were screened on LB medium with ampicillin (50 μg/ml). The recombinant plasmid, designated pMD-pir, was sequenced and further analyzed with the DNAMAN software package was developed by Lynnon Corporation (USA).

Construction of Fusion Expression Vector

The plasmids pMD-pir and pPIC9K were digested with *Eco*RI and *Not*I and ligated by T_4 DNA ligase to generate a recombinant vector pPIC9K-pir-N. Then the gene *eg* in the recombinant plasmid pPIC9K-eg was cloned into the vector pPIC9K-pir-N as a *SnaB*I and *Spe*I fragment. The recombinant plasmid, designated as pPIC9K-pir-eg, was sequenced and further analyzed with the DNAMAN software package was developed by Lynnon Corporation (USA). The construction strategy is shown in Fig. 1.

Yeast Transformation and Screening of Multicopy Transformants with High Activity

The recombination plasmid pPIC9K-pir-eg was linearized with *Sal*I and transformed into *P. pastoris* GS115 by electroporation. The transformation mixture was spread onto MD plates. According to the multicopy expression kit, the His^+ transformants were grown in microtiter plates until all clones were at the same density. Then the His^+ transformants were spotted on YPD plates containing Geneticin 418 (G418) at a final concentration of 0, 1.0, and 3.0 mg/ml. The plates were incubated at 28°C and were checked after 2–3 days for G418-resistant clones. The colonies on the YPD agar plates containing 3.0 mg/ml G418 were picked onto fresh MM plates containing 0.5% methanol. The MM plates were incubated at 28°C for about 48 h and then heated at 70°C for 30 min. The plates were subsequently overlaid with soft agar containing 0.5% sodium carboxylmethyl cellulose (CMCNa) and incubated at 65°C overnight. After Congo red staining and washing, the size of the yellow halo zone around a colony reflected endoglucanase activity on CMCNa and thermal stability. The colonies with larger halo zones were grown in 5 ml of BMMY culture medium for 2 days at 28°C and 250 rpm in order to screen for the transformants with maximal activity. Then the activities of surface-displayed EG were measured by the DNS method [16]. The optimum reaction temperature and pH were 70°C and 4.0, respectively. One unit (IU) of EG activity was defined as the amount of enzyme catalyzing the release of 1 μmol glucose equivalent per minute. The clone with highest enzyme activity was selected for further study.

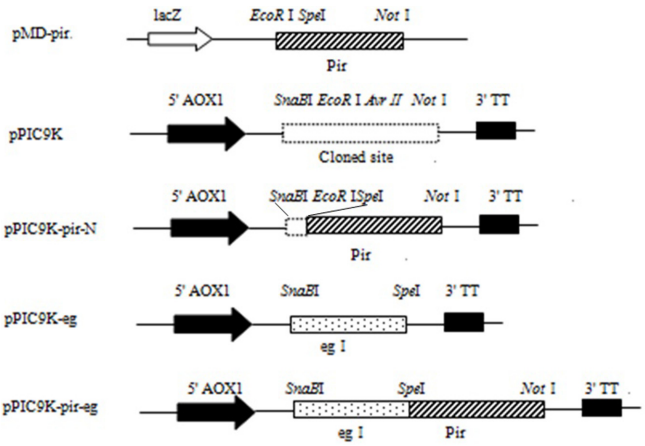

Fig. 1. Construction of fusion expression vector pPIC9K-pir-eg.

Analysis of EG of Recombinant Strain

The clone with the highest enzyme activity was inoculated in 25 ml of BMGY medium at 28°C until the optical density was 4.5–6.0 at 600 nm (OD_{600}). Then the cells were collected by centrifugation (8,000 rpm, 5 min). The cell pellet was resuspended in 50 ml of BMMY medium and transferred into a 500 ml culture flask. The culture was shaken for 240 h at 28°C. Methanol was added to a final concentration of 1.0% at every 24 h. The cell pellets were collected at every 24 h. After washing twice with sterile water, the cells were resuspended in 100 mM potassium phosphate (pH 6.0), followed by lyophilization. Then 10 mg of lyophilized samples was dissolved in 1 ml of sterile water as diluted EG. Part of the diluted sample was treated with alkaline (final concentration of 30 mM NaOH) at 4°C for 12 h. A 10 μl sample of the treating fluid was analyzed by SDS-PAGE. The other part of the diluted sample was used to determine the EG activity by the DNS method.

Acid-Thermal Stabilities of Enzyme Displayed on Cell Surface

The effect of temperature on enzyme activity was tested by incubating the diluted EG at 50–80°C for 30 min and at 65°C for different times, respectively. Then the residual CMCase activity was assayed by the DNS method.

The effect of pH on enzyme activity was tested by adjusting the diluted EG from pH 2.5 to 8.5 (at increments of 0.5 pH units) with sterile 1 M HCl and 1 M NaOH. After incubating for 24 h at room

temperature, the residual CMCase activity of the treated samples was assayed by the DNS method.

Reusability of Displayed EG

The displayed EG was assayed over six cycles of catalysis. For each cycle, 0.01 g of cell pellets was incubated with 800 μl of 1% CMCNa for 120 min at 65°C. The contents were then centrifuged at 5,000 rpm for 10 min and the supernatant was used for assaying enzyme activity by the DNS method. The pellet was used for the next cycle in a similar manner. The enzyme activity for the first reaction cycle was defined as 100% activity.

Results

Sequence Analysis of *pir* Anchor

The internal repeating anchor sequence gene *pir* was obtained by PCR with specific primers from the chromosomal DNA of *S. cerevisiae* EBY100. The expected 500 bp PCR product was purified and cloned into vector pMD18-T to generate a recombinant plasmid, designated pMD-pir (Fig. 1). Sequencing revealed that the DNA of *pir* was 540 bp long and encoded 180 amino acids. The amino acid sequence of the amplified PCR product showed 86% homology with Pir1p from *S. cerevisiae* S288c (GenBank Accession No. NM001179730). The *pir* gene contained the internal repeating sequence (SQIGDGQIQATT), so it would be used for construction of the fusion expression vector.

Screening of Multicopy Transformants with High Activity

The multicopy His^+ transformants were screened for resistance to G418. The G418 screening results indicated that the numbers of strains resisting G418 decreased with the increasing concentration of G418. Only 12 colonies were able to grow at the highest concentration of G418 (3.0 mg/ml), indicating the possibility of multiple copies in these strains. Furthermore, these transformants were grown on MM agar plates overlaid with a CMCNa soft agar layer. The No. 1, No. 6, and No. 10 colonies with larger halo zones, indicating high EG activity, were selected for further characterization. The EG activities of No. 1, No. 6, and No. 10 were 10.8, 18.2, and 16.3 IU/g, respectively. When considering both the halo zones and EG activity assays, the No. 6 clone designated PEG-6 was selected to investigate the expression of *eg*.

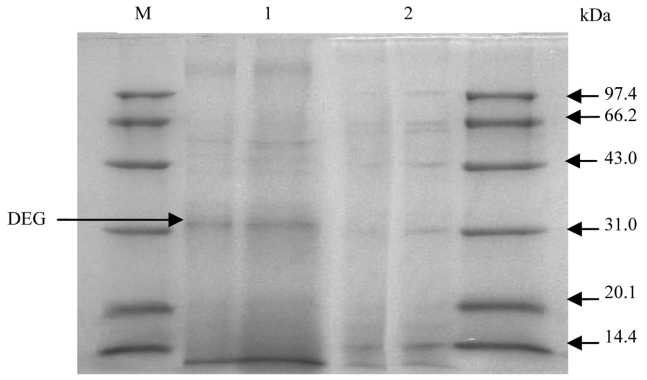

Fig. 2. Recombined protein detected by SDS-PAGE.
M: Protein Marker; Lane 1, 2: sample from PEG-6 for 4 days, 8 days; Lane 3, 4: sample from control for 4 days, 8 days, respectively.

Analysis of EG in Recombinant Strain PEG-6

SDS-PAGE demonstrated a target protein band at the expected size of EG (34.1 kDa) in the sample from recombinant strain PEG-6 (Fig. 2, lanes 1, 2). Other bands in the SDS-PAGE profile may represent other proteins released from the cell surface after alkaline treatment. No target protein band appeared in a control sample of *P. pastoris* GS115 transformed with pPIC9K-pir-N (Fig. 2, lanes 3, 4). These results demonstrated that EG was successfully displayed on the surface of *P. pastoris*. Furthermore, the EG displayed on cell surface (DEG) showed a maximal activity of 275 IU/g cell dry weight after induction by methanol for 8 days. No EG activity was detected in the control of *P. pastoris* GS115 transformed with vector pPIC9K-pir-N.

Acid-Thermal Stabilities of Displayed EG

The stability of DEG under the heat and acidic conditions was analyzed in this study. Free endoglucanase (FEG, 339 IU/ml) from the growth medium of recombinant *P. pastoris* GS115 harboring plasmid pPIC9K-eg was used as a control. The residual activities of DEG were retained in the range of 164.31–208.35 IU/g, above 60% of the original activity between pH 2.5 and 8.5, which was higher than that of FEG with 40% native activity (Fig. 3).

The highest tolerant temperature of DEG was 70°C, much higher than that of FEG, which was approximately 50°C.

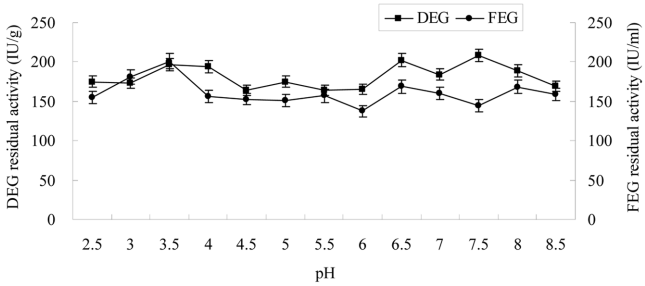

Fig. 3. Effects of pH on the stability of FEG and DEG.

The residual activities for DEG were 278.75 IU/g and 90.75 IU/g, about 100% and 33% at 70°C and 80°C, respectively. However, FEG activity declined dramatically at higher temperatures, and only retained 41% and 16% residual activity at 70°C and 80°C, respectively (Fig. 4A).

Since most processes for industrial applications of cellulases are carried out above 60°C for 90–120 min, and even longer, we analyzed the thermostability at 65°C. With incubating at 65°C for 120 min, DEG showed 250.5 IU/g residual activity, about 91.1% of the original activity, while FEG only kept 77.8% of its original activity. From Fig. 4B, it can be concluded that the half-life of DEG was 270 min at 65°C, but that of FEG was only 150 min at the same temperature (Fig. 4B). These results demonstrated that the thermal stability and pH tolerance of the surface-displayed enzyme were significantly higher than those of the free enzyme.

Reusability of Displayed EG

DEG could be used repeatedly at least three times. DEG activity decreased by less than 20% of the original activity by the third cycle and still exhibited 71.2% relative activity at the fourth round of recycling *via* cooling treatment of the recycled pellets (Fig. 5A). However, 64.9% of residual activity at the third time was detected if the recycled pellets were directly reacted with substrate (Fig. 5B). These results suggested that the enzyme underwent reversible denaturation under continuous reaction at 65°C and it was gradually restored to its original state through a low temperature treatment.

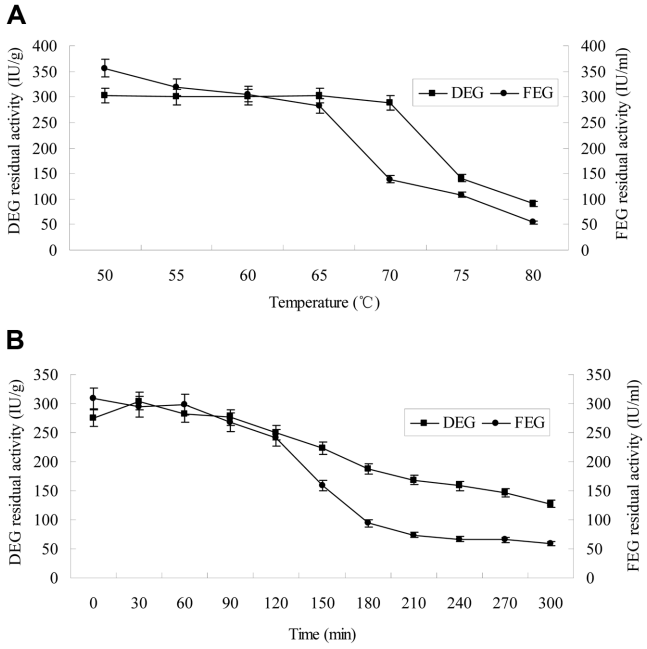

Fig. 4. Effects of temperature on the stability of FEG and DEG. (**A**) Incubating for 30 min at different temperatures; (**B**) incubating at 65°C for different times.

Discussion

In recent years, the yeast cell-surface display system has been the subject of extensive study. Many heterologous proteins have been immobilized on the cell surface and applied in various industrial processes [7, 8]. Choosing an appropriate anchor protein is an important consideration in cell-surface display development. Moreover, the site of fusion between a target and an anchor protein may significantly impact whether the target protein retains its native function when displayed [22]. In this study, PIR was used as an anchor for EG by fusing the C-terminus of EG to the N-terminus of PIR. This linkage best preserves the native conformation and activity of the N-terminal catalytic domain of EG. We found that the level of EG expression increased gradually and reached a maximal activity from the displayed EG of 275 IU/g cell dry weight at 8 days

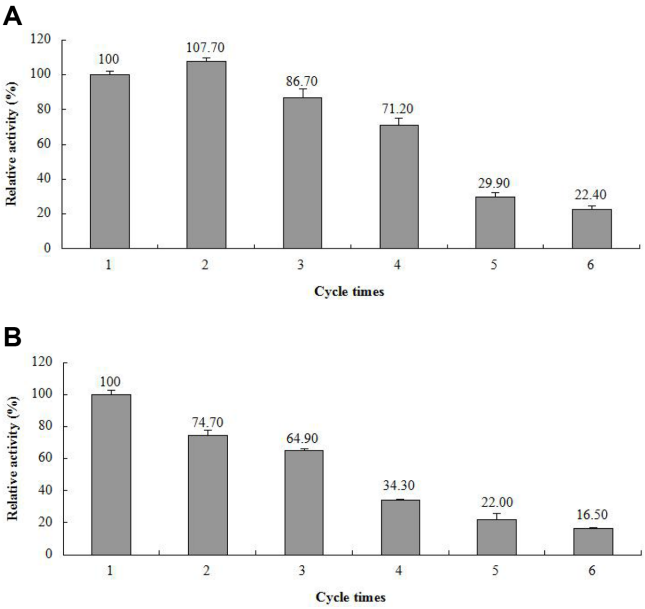

Fig. 5. Effects of repeated utilization on stability of DEG. (**A**) With cooling treatment; (**B**) Without cooling treatment. Whole-cell pellets were incubated with substrate CMCNa for 120 min at 65°C. The supernatant was used for assaying enzyme activity by the DNS method. The pellet was used for the next reaction cycle after cooling treatment for 2 h at 4°C (**A**). The pellet was then used directly for the next reaction cycle (**B**).

post-induction. However, those enzymes in which the active site is located near the C-terminus must be fused to the anchor sequence at the C-terminus [8].

One of the prerequisites for enzymes to be employed for industrial applications is that they must be robust and highly stable under hostile conditions of industrial processes, including extremes of temperature and pH [3]. Previous directed evolution studies successfully enhanced endoglucanase catalytic efficiency based on assays using soluble cellulose derivatives, but the stability in acid-thermal conditions needs to be improved [2]. In this study, the stability of DEG was greatly improved compared with the stability of FEG, which overcomes the current limitation of instability of FEG under the same acidic-thermal conditions. After the target enzyme is immobilized on the cell surface, the deformation energy and the molecular conformation stability of the enzyme would be increased, explaining the observed increased stability. In fact, the DEG is not only superior to the free enzyme in stability and maneuverability, but may also be used without any purification owing to immobilization on the yeast cell surface. Furthermore, DEG could be reused at least three times while retaining enzyme activity above 64.9%. Reusability is good for any application based on displayed EG, and the feasibility of regenerating endoglucanase activity after thermal treatment provides significant economic benefits for industrial use, reducing the costs associated with very expensive enzymes.

Taken together, these data suggest that whole-cell, high-expression surface display cell lines may exhibit a considerable production of a CMCNa hydrolytic enzyme with high acid-thermal stabilities. Because of its high catalytic efficiency, acid-thermal stabilities, and reusability, the DEG of strain PEG-6 may have broad applications for industrial processes under extreme environments.

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