A novel pullulanase from a fungus *Hypocrea jecorina* QM9414: Production and biochemical characterization

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Pullulanase production from a fungus *Hypocrea jecorina* QM9414 that produces native extracellular hydrolases having industrial applications was carried out in a shaking flask culture containing 0.5% amylopectin at a pH of 6.50 at 30°C. The enzyme was purified 11-fold by ammonium sulfate fractionation, anion-exchange and gel-filtration chromatographies with a yield of 10.12% and a specific activity of 1.36 ± 0.14 U/mg protein. The molecular mass of pullulanase was estimated to be 130.56 kDa by PAGE and SDS-PAGE, indicating that the native enzyme was a monomer. The optimum pH and temperature for purified enzyme was 6.5 and between 35°-65°C, respectively. The $K_m$ values for amylopectin, starch and pullulan as substrates were 10.7, 15.5 and 38.4 mg/mL, respectively. The $V_{max}$ values were found to be 3.32, 3.32 and 3.82 ΔA/min for amylopectin, starch and pullulan, respectively. The enzyme was stable at 40-70°C for 30 min, but lost about 33% of its activity at 80°C and about 43% of activity at 90°C and 100°C for the same incubation period. Pullulanase activity was stimulated by CoCl$_2$, NiCl$_2$, KI, NaCl, MgCl$_2$, and LiSO$_4$. The enzyme was slightly inhibited by urea, CaCl$_2$ and β-mercaptoethanol. The enzymatic characteristics, substrate specificity and the products of hydrolysis indicated that the enzyme was similar to those of type II pullulases.

**Keywords:** *Hypocrea jecorina* QM9414, Culture conditions, Induction, Type II pullulanase, Purification

Starch-degrading enzymes, such as pullulanases, amylases and glucoamylases play an important role in food, chemical and pharmaceutical industries$^1$. Pullulanases (pullulan-6-glucanohydrolase [EC 3.2.1.41]) are divided into type I that exclusively hydrolyze α-1,6 glycosidic linkages and produce branched dextrins and type II that hydrolyze both α-1,4 and α-1,6 linkages and produce mainly maltose, maltotriose and glucose. Type II pullulanases (also termed as amylopullulanases) have been used commercially, mainly in the saccharification process$^2$. All pullulanases known to date are unable to degrade cyclodextrins. In contrast, pullulan hydrolyase type I (neopullulanase) and pullulan hydrolyase type II (isopullulanase) are able to cleave α-1,4 glycosidic linkages in pullulan, releasing panose and isopanose, respectively and are highly active against cyclodextrins. Recently, pullulan hydrolyase type III has also been reported. This enzyme attacks α-1,4 as well as α-1,6 glycosidic linkages in pullulan, forming maltotriose, panose and maltose$^3$.

Industrial processes prefer microorganisms which secrete enzymes extracellularly and are constitutive in nature. However, many potentially important enzymes are regulated by induction/repression systems. Therefore, identification of the underlying mechanisms of these systems would help in designing an effective medium with rapid induction of the enzyme, as well as to construct mutants that are resistant to regulation by substrate and hydrolysis product. The genes encoding such regulatory enzymes are activated by certain inducers. Starch, amylopectin and pullulan are inducers for pullulanase$^4,5$.

Starch is the most abundant form of storage polysaccharide in plants. Thus, it constitutes an inexpensive source for production of syrups containing glucose, fructose or maltose which are widely used in food industries$^6$. Industrial glucose production from starch is a two-step process, involving a number of amylolytic enzymes, each having a different pattern of action on starch. The first step (i.e. liquefaction) converts a concentrated starch suspension (30-40%) into a solution of soluble dextrins having different degrees of polymerization. During second step (i.e. saccharification), these dextrins are hydrolyzed to glucose$^7$. Amylopectin, in general, is the major component of starch
(representing a 75% to 85% weight fraction). It is an α (1-4) linked glucopyranose polymer, but in addition also contains α (1-6) glycosidic linkages, representing branch points occurring at every 17 to 26 residues. It is a highly branched, high molecular weight polymer. Pullulan is the generic name given to the water-soluble homopolysaccharide that is a linear α-D-glucan which consists of maltotriose repeating units interconnected via linkages α (1-6).

In recent years, a considerable number of type II pullulanases (amylopullulanases) have been isolated from a wide variety of microorganisms, particularly thermophilic ones, due to scientific interest in industrial applications. The filamentous fungus Hypocrea jecorina (Anamorph: Trichoderma reesei) is a potent producer of cellulolytic and hemicellulolytic enzymes. However, no reports are available on the pullulanases from H. jecorina, so far. In this study, we report for the first time, the purification and characterization of a novel pullulanase from H. jecorina. We have optimized the environmental conditions to enhance pullulanase production using different culture conditions, such as different amount of carbon sources, temperature and pH. The kinetic properties, molecular mass determination, optimum temperature and pH, substrate specificity, thermal stability and inhibition studies are also reported for the novel extracellular pullulanase.

Materials and Methods

Fungus and growth conditions

Hypocrea jecorina QM9414 was obtained from Institute of Biochemical Technology and Microbiology, Technical University of Vienna, Austria. The fungus was grown on minimal medium (MM) at 30°C for 7 days. The slants were stored at 4°C and subcultured every 8-9 weeks. The pullulanase production medium consisted of 10.0 g casein peptone, 10.0 g meat extract, 5.0 g yeast extract, 2.0 g ammonium citrate, 1.0 g Tween 80, 5.0 g sodium acetate, 20.0 g glucose, 2.0 g K_{2}HPO_{4}, 0.2 g MgSO_{4}.7H_{2}O and 0.05 g MnSO_{4}.H_{2}O per liter. pH of culture medium was adjusted to 6.50. The cultures were incubated at 30°C on a rotary shaker at 160 rpm for 48 h.

Production of pullulanase in shaking flasks

In order to determine optimum composition and conditions of the medium for pullulanase production, the replacement experiments were developed. For this purpose, the mycelia were filtrated from enzyme production medium after 48 h incubation time and washed twice with sterile distilled water. Then, instead of using glucose, the mycelia were transferred into 500 mL Erlenmeyer flask containing 100 mL of pullulanase production medium, supplemented with 3 different concentrations (0.1, 0.5 or 1.0%, respectively) of pullulan, amylopectin or starch as the carbon sources. Pullulanase production was examined at 20, 25, 30, 35 and 40°C and at different pH values (between 4.5-8.5). The culture of H. jecorina was incubated on a rotary shaker at 160 rpm for 120 h. The samples were collected from each shaking flask with 24-h intervals.

Isolation of the enzyme

H. jecorina was grown in the enzyme production medium for 24 h. After 24 h of incubation, the culture broth was filtered to remove the cells and the filtrate was centrifuged at 10,000 x g for 30 min at 4°C by using Sigma 3K centrifuge. The supernatant was subjected to fractionated ammonium sulfate precipitation. Ammonium sulfate crystals were added to the supernatant to bring the saturation to 40-80% in an ice bath and the precipitate was collected by centrifugation at 10,000 x g for 30 min at 4°C. For ammonium sulfate precipitation, addition of solid ammonium sulfate was carried out slowly to completely dissolve it after each addition during 1 h. The pellet was redissolved in a small volume of 0.1 M acetate buffer at pH 5.0 and dialyzed at 4°C in the same buffer in cellulose dialysis tubing. The dialysis buffer was changed five-times at 7 h intervals. The dialyzed solution was kept in stoppered test tubes at -20°C and used as crude enzyme extract.

DEAE-cellulose ion-exchange chromatography

The crude enzyme extract was loaded on to a DEAE-cellulose column (1.8 x 20 cm), pre-equilibrated with 0.5 mM sodium phosphate buffer, pH 7.0 at 4°C. The column was thourghly washed with the equilibration buffer until the UV absorbance of the column eluate returned to the base line. The bound protein was eluted with a linear gradient of 0-100 mM NaCl in the same buffer at a flow rate of 0.5 mL/min. Active fractions were pooled and purified further by Sephadex G-100 chromatography.

Sephadex G-100 gel-exclusion column chromatography

The active fractions obtained from DEAE-cellulose ion-exchange chromatography were rechromatographed on a Sephadex G-100 gel-exclusion column (1.8 x 50 cm), previously equilibrated with sodium
phosphate buffer (0.5 mM pH 7.0) containing NaCl (1.0 mM). The pullulanase was eluted with the same buffer at a flow rate of 0.5 mL/min. The fractions were collected from the column in 3 mL volumes. Elution was continued until no absorbance was observed at 280 nm. The protein amounts and pullulanase activities of each fraction were determined. The fractions having pullulanase activity were collected and their purification degrees were determined by measuring specific activity.

Analytical assays
Pullulanase activity was determined by measuring the amount of enzyme required to release reducing sugar from pullulan. One mL of reaction mixture consisting of 100 µL of a 1% (w/v) pullulan in 0.1 M sodium acetate (pH 6.50) and 100 µL enzyme solutions was incubated for 30 min at 37°C. The amount of released reducing sugar was measured using dinitrosalicylic acid method. One unit of enzyme activity was defined as amount of enzyme required to produce reducing sugar equivalent to one µmol of glucose per min under the used assay conditions and expressed as U/mL. Protein concentration was determined by the Bradford method using different concentrations of bovine serum albumin as a standard.

Electrophoretic methods
Non-dissociating polyacrylamide gel electrophoresis (PAGE) was performed on 4% acrylamide gel. The subunit molecular mass of the enzyme was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 10% gel. Protein bands were visualized with Coomassie brilliant blue R-250 (Sigma). The molecular mass of the enzyme was estimated using a high range (36-200 kDa) molecular weight calibration kit as a standards (Sigma Marker S8320) consisting of rabbit phosphorylase B (97 kDa), chicken ovalbumin (45 kDa), bovine glutamic dehydrogenase (55 kDa), bovine serum albumin (66 kDa), rabbit phosphorylase B (97 kDa), E. coli β-galactosidase (116 kDa) and porcine myosin (200 kDa). Subunit molecular mass was determined from standard curve plotted between Rf values of the standards. Zymography was used to confirm the enzyme activity in the native-PAGE. It was done by incubating substrate with the native-PAGE having the band of the pullulanase. The activity zone was formed by staining with lugol reagent.

Biochemical properties of pullulanase

Substrate specificity
Pullulanase activity was determined using three potential substrates (pullulan, amyllopectin and starch). All substrates solutions were prepared at 0.1 M sodium acetate buffer at pH 6.5 as 1% (w/v) concentration. Activity assays were performed in triplicate measurements, employing the standard reaction mixture, but using different substrates.

Effect of pH and temperature
The optimum pH of pullulanase activity was determined using three different substrates (pullulan, amyllopectin and starch) and 0.1 M sodium acetate buffer (pH 4.0 to 6.0) or 0.1 M Tris/HCl buffer (pH 7.0 to 9.0). In order to determine optimum temperature, the pullulanase activity was measured in the temperatures ranging between 25-85°C by using different substrates.

Thermal stability
The temperature stability of pullulanase was determined by placing 2 ml of enzyme solution in a test tube in a water bath at the appropriate temperatures ranging between 10-100°C. Enzyme solution (100 µl) was withdrawn at different time points and rapidly cooled in ice bath. Then, the pullulanase activity was assayed at pH 5.0 and 37°C using 1% (w/v) starch solution as a substrate.

Determination of \( K_m \) and \( V_{max} \)
The effect of substrate concentration and enzyme kinetics of pullulanase were studied by using starch, amyllopectin and pullulan as substrates and the rate of pullulanase reaction was measured at various substrate concentrations (0.5-2.5%, w/v) under optimum conditions. The kinetic data were plotted between activities and substrate concentrations. The Michaelis-Menten constant (\( K_m \)) and maximum velocity (\( V_{max} \)) were determined from Lineweaver-Burk curve.

Effect of metal ions, denaturing reagents and enzyme inhibitors
The influence of various metal ions (20 mM) on the enzyme activity was investigated using CaCl₂, FeCl₃, MgCl₂, CoCl₂, NiCl₂, NaCl, CuSO₄, LiSO₄, AgNO₃ and KI. Activity in the absence of ions was taken as 100%. The effect of some surfactants, such as Triton X-100 and Tween 20 (1%, v/v) on the enzyme stability was studied by pre-incubating the enzyme. The activity of the enzyme without any additive was taken as 100%. The effect of enzyme
inhibitors on pullulanase activity was studied using β-mercaptoethanol, dithiothreitol, EDTA and urea at a final concentration of 20 mM. The purified enzyme was pre-incubated with inhibitors. The activity of the enzyme without any additive was taken as 100%.

**Statistical analysis**

All experiments and parameters were done in triplicate and the results presented as mean ± SEM (standard error of mean). Statistical comparisons were made using the Student’s t-test. Differences were considered significant at p < 0.01.

**Results and Discussion**

**Optimization of culture conditions and pullulanase production**

Production of extracellular pullulanase in shaking cultures from *H. jecorina* in different concentrations of amylopectin, starch and pullulan [% (w/v)] was examined and the results were given as specific activity (U/mg protein) (Fig. 1). The maximum enzymatic activity was observed after 24 h of incubation with 0.5% (w/v) of amylopectin. A high amylopectin concentration (1.0%, w/v) appeared to repress the production of pullulanase. Starch exhibited moderate activity than amylopectin, while pullulan showed the lowest pullulanase activity. These results suggested that pullulanase from *H. jecorina* was inducible with amylopectin and starch, similar to pullulanases from the bacterial sources.

The effect of pH on pullulanase production is shown in Fig. 2. Pullulanase activity was the highest at pH 6.5, which was the optimum pH for *H. jecorina* growth. The effect of temperature on pullulanase production of the fungus is presented in Fig. 3. The maximum pullulanase activity (0.598 ± 0.032 U/mg protein) was observed at 30°C. Under optimized conditions, pullulanase was produced from *H. jecorina* in shaking batch cultures. Protein and pullulanase activity was monitored for 120 h. The highest activity was obtained at first 24 h of incubation.
Purification of pullulanase from culture broth of *H. jecorina* QM9414

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Recovery (%)</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>59.66 ± 4.52</td>
<td>7.30 ± 0.93</td>
<td>0.12 ± 0.006</td>
<td>100</td>
<td>1.00</td>
</tr>
<tr>
<td>60% (NH₄)₂SO₄ Precipitation</td>
<td>9.08 ± 0.96</td>
<td>2.05 ± 0.18</td>
<td>0.23 ± 0.006</td>
<td>28</td>
<td>1.85</td>
</tr>
<tr>
<td>DEAE-cellulose ion-exchange chromatography</td>
<td>3.23 ± 0.37</td>
<td>1.83 ± 0.04</td>
<td>0.57 ± 0.03</td>
<td>25</td>
<td>4.63</td>
</tr>
<tr>
<td>Sephadex G-100 gel-exclusion chromatography</td>
<td>0.44 ± 0.07</td>
<td>0.60 ± 0.07</td>
<td>1.36 ± 0.14</td>
<td>10.12</td>
<td>11.00</td>
</tr>
</tbody>
</table>

*Means with different superscript are significantly different (*P* < 0.01: a > b > c > d).
the optimum enzymatic activity was observed at pH 6.5. This value was similar to that of pullulanases from bacterial sources\textsuperscript{15-17}. The optimum pHs for different bacterial pullulanases have been generally reported as between pH 4.5-6.5 and these values differ according to the enzyme source and the substrate used.

The optimum temperature for maximum pullulanase activity was determined by using various substrates at seven different temperatures between 25 and 85°C. Pullulanase was active in a broad range of temperatures. As shown in Fig. 7, the optimum temperature for \textit{H. jecorina} pullulanase activity was between 35 and 65°C. Earlier, the optimum temperature for the pullulanases of various thermophilic bacteria has been reported between 70 and 90°C\textsuperscript{15,16}.

**Thermal stability**

The knowledge about the thermal stability of an enzyme is useful for exploring the potential applications of the enzyme. The thermostability of pullulanase from \textit{H. jecorina} was studied at 10-100°C and the results are presented as relative percentage activity (Fig. 8). \textit{H. jecorina} pullulanase was stable between 40-70°C for 30 min. About 84% and 75% of the enzymatic activity was retained after 30 min incubation at 30°C and at 20°C, respectively. However, the enzyme lost about 33% of its activity at 10°C and 80°C and about 43% of its activity at 90°C and 100°C for the same incubation period. These results suggested that \textit{H. jecorina} pullulanase was a relatively thermostable enzyme, thus can be used in hydrolysis processes which require the high temperatures.

**Kinetic characterization**

Lineweaver-Burk plots were used to show the linear relationships and kinetic parameters of \textit{H. jecorina} pullulanase. The Michaelis constants (\(K_m\)) for amylopectin, starch and pullulan were 10.7, 15.5 and 38.4 mg/mL, respectively. The maximum reaction rate (\(V_{\text{max}}\)) values were found to be 3.32, 3.32 and 3.82 ΔA/min for amylopectin, starch and pullulan, respectively. In \textit{Bacillus subtilis}, the \(K_m\) for pullulan is reported to 1.284 mg/mL and the \(V_{\text{max}}\) value as 27.609 U/min\textsuperscript{18}. In \textit{Fervidobacterium pennavorans} Ven5, the pullulanase has shown \(K_m\) and \(V_{\text{max}}\) values of 0.4 mg/mL and 1.58 U/mg, respectively using pullulan as substrate\textsuperscript{19}. 

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Fig. 6—Optimum pH of \textit{H. jecorina} pullulanase for different substrates

Fig. 7—Optimum temperature of \textit{H. jecorina} pullulanase for different substrates 

Fig. 8—Effect of temperature on stability of \textit{H. jecorina} pullulanase. [Enzyme solutions were incubated at the different temperatures (10-100°C) for 5, 10, 15, 20, 25 and 30 min]
Effect of metal ions, denaturing reagents and enzyme inhibitors
The activator or inhibitor effects of various metal ions on the enzyme activity were studied. The results were expressed as percentage of the activity obtained without the ions. Fe$^{3+}$ (4.29%) and Cu$^{2+}$ (4.29%) were found to be weak activator of the enzyme. Fe$^{3+}$ (10.48%), Pb$^{2+}$ (14.84), Ag$^{+}$ (15.40%), Li$^{2+}$ (22.37%), Mg$^{2+}$ (23.28%), Na$^{+}$ (23.35%), K$^{+}$ (23.91%), Ni$^{2+}$ (23.95%) and Co$^{2+}$ (26.90%) had moderate activator effect on the enzyme activity. However, Ca$^{2+}$ (2.26%) slightly inhibited the enzyme activity.

The stability of pullulanase was also studied by incubating the enzyme in the presence of surfactants Tween-80 and Triton X-100. The enzyme was highly stable in the presence of the non-ionic surfactants. The activation percent was found as 1.40% and 11.50% in the presence of Tween 80 and Triton X-100, respectively.

The effect of various chemical reagents on pullulanase activity was also examined. β-Mercaptoethanol and urea inhibited the enzymatic activity by 1.52% and 8.31%, respectively. Addition of EDTA and dithiothreitol to the reaction mixture increased the enzymatic activity by 1.75% and 23.70%, respectively. Addition of EDTA and urea inhibited the enzymatic activity by 1.52% and 8.31%, respectively. Addition of EDTA and urea inhibited the enzymatic activity by 1.52% and 8.31%, respectively. Addition of EDTA and urea inhibited the enzymatic activity by 1.52% and 8.31%, respectively.

In conclusion, the optimum production conditions for the purified enzyme could be used for potential industrial purposes.

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References