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厦门大学

硕　士　学　位　论　文

β-1,3-1,4-葡聚糖酶重组毕赤酵母的构建及其产酶发酵工艺优化

Construction of recombinant *Pichia pastoris* for β-1,3-1,4-glucanase expression and optimization of fermentation process for glucanase production

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摘 要

β-1,3-1,4-葡聚糖酶(EC3.2.1.73)作为一种重要的工业用酶，被广泛应用于酿酒和饲料工业中。目前国内对葡聚糖酶的研究还处于初级阶段，天然菌株产酶量及酶性能满足不了实际应用。随着基因工程方法的应用，为实现目的蛋白的工程菌表达提供的了可能。现国内外对 β-1,3-1,4-葡聚糖酶的研究集中在构建工程菌株来提高酶的产量和性能方面。本研究的主要目的即通过基因工程及分子生物学方法来提高 β-1,3-1,4-葡聚糖酶表达量，研究其酶学特性，为生产中的问题提供有效的解决途径。主要研究内容及相应结果如下：

采用 PCR 法从载体 pLF3 中扩增出杂合 β-1,3-1,4-葡聚糖酶基因，并在基因两端引入酶切位点 *Eco*R Ⅰ / *Not* Ⅰ，插入带有 AOX 启动子和 α-信号肽的表达载体 pPIC9K 的多克隆位点，构建出重组载体 pPIC9K-*bgl*；经 *Sac* Ⅰ线性化重组载体后，通过电转化将其导入 *Pichia pastoris* GS115，使基因整合到酵母染色体上；最终筛选出甲醇利用型 His^+Mut^+，其最高抗遗传霉素浓度为 2 mg mL^{-1} 约有 7-8 个拷贝数的重组 GS115/pPIC9K-*bgl*。甲醇诱导重组菌表达 β-1,3-1,4-葡聚糖酶，对诱导的条件进行优化，确定诱导表达最佳条件为 pH 6.0，最适温度 30 ℃，表达时间 84 h。

利用 3 L 发酵罐进行重组毕赤酵母发酵优化。先以甘油为碳源，在甘油补加阶段，细胞干重达 47.5 g L^{-1}；甲醇诱导阶段，流加甲醇控制溶氧维持在 35%，诱导 84 h 后酶活达到最高值 115.3 U mL^{-1}，蛋白表达量 131.8 mg L^{-1}，细胞干重 88.5 g L^{-1}。并通过 SDS-PAGE 对重组蛋白进行分析，得出分子量约为 34 kDa，由于糖基化作用，比理论分子量大了约 8 kDa，目的蛋白占胞外总蛋白约 75%。

其次还确定了 β-1,3-1,4-葡聚糖酶的酶学性质。该酶在 pH 4.5-7.0 之间相对稳定，在 pH6.0 时酶活力达到最高，而在 pH 3.5-8.0 间保温 60 min，酶活仍然有 80%以上；最适反应温度 50 ℃，分别于 pH 6.0，30-60 ℃下，保温 150 min，酶活仍保留 80%以上；$CaCl_2$，$MnSO_4$，$CoCl_2$，$FeSO_4$ 对 β-1,3-1,4-葡聚糖酶有激活作用，而 EDTA，$CuSO_4$ 使酶活力降低。

关键词：杂合 β-1,3-1,4-D-葡聚糖酶；毕赤酵母；表达；流加发酵

Abstract

β-1,3-1,4-glucanase(EC 3.2.1.73), as an important industrial enzyme，has been widely used in the brewing and animal feed industry. Nowadays the domestic research on β-1,3-1,4-glucanase is in the beginning, and the expression level and properties of natural enzyme are not sufficient for industrial purpose. The development of molecular biology and genetic engineering provides a feasible way to resolve the problems. At present the researches on β-1,3-1,4-glucanase focus mainly on construction of genetically engineered strains and improvement of the expression level and enzyme properties. The purpose of this research is to improve the expression level and properties of β-1,3-1,4-glucanase with the methods of molecular biology and genetic engineering. It was proved to be an effective approach for accelerating the application of β-1,3-1,4-glucanase in industry. The studies and results about β-1,3-1,4-glucanase in this research are as follows:

In this paper, a pairs of primers with two enzyme sites *Eco*R I / *Not* I was designed and synthesized. The nucleotide sequence of hybrid β-1,3-1,4-glucanase was PCR-amplified from pLF3, and the gene fragment was inserted into the *Pichia pastoris* expression vector pPIC9K containing AOX1 promotor and α-secreting signal peptides, yielding the recombinant expression plasmid pPIC9K-*bgl*. After linearlized with *Sac* I, the recombinant expression plasmid was transformed into the host cell *Pichia pastoris* GS115 by electroporation, in which the plasmid would integrate into the genome by the crossover event of homological DNA fragments. A His^+Mut^+ phenotype of recombinant *Pichia pastoris* GS115/pPIC9K-*bgl* with the highest copies (7-8 copies) was screened under the highest G418 resistance concentration of 2 mg mL^{-1}. The optimal expression conditions of this recombinant GS115 were determined as pH 6.0, cultivation temperature 30 ℃, and expression time 84 h.

The optimization of fermentation with recombinant GS115/pPIC9K-*bgl* was

performed in a 3 L fermentor. Glycerol was selected as carbon souce. The dry cell weight reached 47.5 g L^{-1} at the end of the glycerol fed-batch phase. When all of the glycerol was consumed, the induction phase was started, and methanol was fed. The DO was maintained at 35% during the whole induction period. The highest β-1,3-1,4-glucanase activity was 115.3 U mL^{-1} with lichenan as substrate after 84 h of methanol induction, corresponding to a secreted protein concentration of 131.8 mg L^{-1}, and the dry cell weight reached 88.5 g L^{-1}. Taking into account the posttranslational modifications of *P. pastoris* expression system in amino acid (AA) sequence, the size of the recombinantβ-1,3-1,4-glucanase was approximately 34 kDa by SDS-PAGE analysis, which was about 8 kDa larger than the calculated molecular weight.

The properties of β-1,3-1,4-glucanase were also investigated. The pH optimum was 6.0, and the enzyme was relatively stable in a pH range of 4.5-7.5. After incubation at 50 °C and pH range of 3.5-8.0 for 60 min, the enzyme activity retained more than 80% of original enzyme activity. The temperature optimum was found to be 50 °C, and after incubation between 30 °C and 60 °C at pH 6.0 for 150 min，it retained approximately 80% of original enzyme activity. The recombinant enzyme activity was activated by $CaCl_2$, $MnSO_4$, $CoCl_2$ and $FeSO_4$, but inhibited by EDTA and $CuSO_4$.

Keywords: Hybrid β-1,3-1,4-D-glucanase; *Pichia pastoris*; Expression；Fed-batch fermentation.