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硕 士 学 位 论 文

纤维素酶高产菌株选育与发酵条件优化

Screening and Mutation of Wild Fungi for Enhanced

Cellulase's Production and Optimization of Culture

Condition by RSM

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摘要

纤维素是植物细胞壁的主要成分，广泛存在于自然界中，是世界上最丰富、最廉价的可再生资源，纤维素被彻底分解而无污染的一条有效途径便是利用纤维素酶将它水解为可发酵糖类。纤维素酶的生产主要是利用微生物发酵法，但一直以来，由于菌种选育和生产技术进展不大、酶活力低、成本高等原因，没有形成大规模生产。

本文采用甲基磺酸乙酯 (Ethyl Methyl Sulfonate, EMS) 与 UV-irradiation 结合的方法，对野生菌进行诱变，两轮诱变后，筛选到菌株 EU2-77，其产生的胞外蛋白浓度、滤纸酶活 (FPase)、 β -葡萄糖苷酶活 (BG) 及内切葡聚糖酶活 (CMCase) 比野生菌产生的分别提高了 67.3 %、149 %、161 % 和 116 %；该突变株 EU2-77 产生的滤纸酶活高于商业菌 JU-A10 和 EMS-UV-8，但产酶效率低于 JU-A10，基于 JU-A10 发酵只用四天，因此缩短 EU2-77 的发酵周期，可以较大程度地提高其产酶效率。

在辅助碳源和氮源的单因素优化后，采用 Plackett-Burman 实验设计和 Box-Behnken 实验设计进行优化，发现菌株 EU2-77 产纤维素酶的最佳培养基配方及发酵条件为：微晶纤维素 24.04 g/L、麦麸 30 g/L、 $(\text{NH}_4)_2\text{SO}_4$ 4.0 g/L、 KH_2PO_4 5.38 g/L、 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.9 g/L、酵母膏 0.6 g/L、蛋白胨 2.0 g/L、 CaCl_2 0.9 g/L、接种量为 10 %、培养时间为 118 小时、培养温度为 30 °C 及摇床转速为 150 rpm。在此优化条件下经三批摇瓶培养实验验证，预测值与验证实验平均值接近。与原始培养条件相比，纤维素酶产量提高了 97.07 %，FPase 活力达到 4.71 IU/mL。

以溶剂萃取法对园林废弃物进行预处理，利用绿色木霉 EU2-77 产生的纤维素酶对预处理前后的园林废弃物进行酶解，结果表明，经预处理过的园林废弃物的酶解产物总还原糖收率为未处理的 24.69 倍。EU2-77 纤维素酶分别与商业菌株 RUT C30 产生酶和商业酶进行酶解性能比较，发现该酶酶解总还原糖收率 (316.4 mg/g) 高于商业菌株 RUT C30 产生酶 (232.4 mg/g) 和商业酶 Celluclast 1.5L (239.6 mg/g)；而与商业酶 Celluclast 1.5L +Novozym 188 混合液酶解能力 (331.6 mg/g) 以及 Celic CTec 酶解能力 (303.2 mg/g) 相差不大。绿色木霉 EU2-77 产生的纤维素酶可望在木质纤维素能源转化利用方面得到推广应用。

关键词：纤维素酶 绿色木霉 诱变育种 条件优化 木质纤维素转换

厦门大学博硕士学位论文摘要库

Abstract

Cellulose, the main composition of the vegetal cell wall, exists widely in the nature and is the most abundant reproducible bio-polymer in the earth. The degradation catalysed with cellulase is the most effective way to hydrolyze cellulose to sugars thoroughly and causes no pollution. In the production of cellulase by micro-organism, owing to low productivity of strains, low hydrolysis efficiency of obtained cellulase and high price of production, large scale of production was not applicable yet. This research was mainly aimed at the improvement of the cellulase production with liquid state fermentation by *Trichoderma viride* using mutagenesis method and culture condition optimization, and analysis of the enzymatic hydrolysis of obtained cellulase against natural biomass.

Firstly, *Trichoderma viride* mutants were developed by using Ethyl Methyl Sulfonate (EMS) treatment and UV-irradiation followed by a semi-quantitative plate clearing assay on phosphoric-acid-swollen cellulose plates. Mutant EU2-77 proved to be the most promising extracellular cellulase producer among 20 mutants in a screening program performed in shake flask fermentation after plate screening. Soluble protein content, filter paper cellulase (FPase) activity, β -glucosidase activity and endoglucanase (CMCase) activity of the fermentation broths of the mutant strain were increased by 67.3%, 149%, 161%, and 116%, respectively, compared with the parent strain. This enzyme complex produced by mutant EU2-77 contained FPase (2.19 IU/ml), CMCase (16.46 IU/ml), β -glucosidase (4.04 IU/ml), xylanase (42.37 IU/ml), and β -xylosidase (0.12 IU/ml). The soluble protein concentration in the enzyme complex was 1.69 mg/ml.

Secondly, the medium composition was optimized using response surface methodology (RSM) after one-factor-one-factor optimization of co-carbon source and nitrogen source. A Plackett-Burman design was applied to elucidate the medium components that significantly affect cellulase production. The concentration of Cellulose and KH_2PO_4 in the medium and Culture time were significant factors. The

steepest ascent method was used to locate the optimal domain and a Box-Behnken design was used to estimate the quadratic response surface from which the factor levels for maximum production of cellulase were determined. The composition of fermentation condition optimized with response surface methodology was (in g/L): Cellulose 24.04; Wheat bran 30; (NH₄)₂SO₄ 4.0; KH₂PO₄ 5.38; MgSO₄·7H₂O; Yeast extract 0.6; Peptone 2.0; CaCl₂ 0.9; Inoculate size 10 %; Culture time 118 hour, Culture temperature 30 °C and Shake speed of incubator 150 rpm. Compared to the original medium, the filter paper activity increased by 97.07 % and reached to 4.71 IU/mL.

Finally, in order to enhance the biomass hydrolysis efficiency, horticultural waste collected from Singapore was pre-treated by organosolv method. The pre-treated and the non-pre-treated horticultural waste were hydrolyzed using the cellulolytic enzyme complex from *T. viride* EU2-77. After pretreatment, the reducing sugar yield was increased by 23.69 times due to the high β -glucosidase and xylanase contents in the enzyme samples. Biomass hydrolysis performances of the cellulolytic enzyme complex from the commercial strain RUT C30, commercial cellulase Celic CTec, celluclast 1.5L, and the mixture of celluclast 1.5L and Novozym 188 were compared. It was demonstrated that cellulolytic enzyme complex derived from strain EU2-77 yielded more reducing sugar (316.4 mg/g horticultural waste) than RUT C30 cellulases (232.4 mg/g horticultural waste) and Celluclast 1.5L (239.6 mg/g horticultural waste). However, its performance is close to that of Celic CTec (303.2 mg/g horticultural waste) and the mixture of celluclast 1.5L and Novozym 188 (331.6 mg/g horticultural waste), rendering the need for β -glucosidase supplementation in biomass hydrolysis unnecessary.

Key words: Cellulase; *Trichoderma viride*; Mutation; Optimization; Bio-conversion of lignocelluloses

目 录

第一章 文献综述	1
1.1 纤维素酶简介	1
1.1.1 纤维素酶的组成	1
1.1.2 纤维素酶的分子结构与功能	2
1.1.3 纤维素酶的理化性质	3
1.1.3.1 分子量	3
1.1.3.2 等电点	3
1.1.3.3 最适作用 pH 和温度	3
1.1.3.4 酶学活性	3
1.2 纤维素酶的来源	4
1.2.1 微生物来源	4
1.2.2 动物性来源	4
1.3 纤维素酶生产菌株的菌种选育	4
1.3.1 纤维素酶产生菌种的诱变选育及其研究进展	5
1.3.1.1 诱变的作用机理	5
1.3.1.2 诱变育种中的几个考虑因素	6
1.3.1.3 高产突变菌株的筛选	7
1.3.1.4 纤维素酶菌种诱变育种概况	7
1.3.2 原生质体融合	8
1.3.3 构建基因工程菌	8
1.4 纤维素酶的生产	9
1.4.1 纤维素酶的固态发酵生产	9
1.4.2 液态深层发酵生产纤维素酶	10
1.5 纤维素酶的应用	11
1.5.1 纤维素酶在燃料酒精生产中的应用	11
1.5.2 纤维素酶在饲料行业的应用	11
1.5.3 纤维素酶在纺织行业中的应用	12
1.5.4 纤维素酶在食品加工行业的应用	12

1.5.5 纤维素酶在天然产物提取中的应用.....	12
1.5.6 纤维素酶在基因工程等研究中的应用.....	13
1.6 本文立题依据和主要内容	13
1.6.1 立题依据.....	13
1.6.2 研究内容.....	14
第二章 材料与方法	15
2.1 实验试剂与仪器	15
2.1.1 实验试剂.....	15
2.1.2 缓冲溶液与分析试剂的配制.....	16
2.1.3 实验仪器.....	17
2.2 分析方法	18
2.2.1 酶活测定.....	18
2.2.2 总还原糖浓度的测定.....	19
2.2.3 蛋白质浓度的测定.....	21
2.2.4 葡萄糖、木糖及纤维二糖的测定.....	23
2.2.5 菌体干重的测定.....	23
2.3 实验方法	24
2.3.1 菌种.....	24
2.3.2 培养基.....	24
2.3.3 摇瓶培养.....	24
2.3.4 发酵罐培养.....	24
第三章 诱变育种	25
3.1 实验内容	25
3.1.1 诱变出发菌株的筛选.....	25
3.1.2 纤维素的磷酸化处理.....	25
3.1.3 筛选培养基的配制.....	25
3.1.4 致死曲线的制作.....	26
3.1.5 诱变菌的筛选.....	26
3.1.6 酶活测定.....	26
3.2 实验结果与讨论	26
3.2.1 诱变出发菌株的选择.....	26

3.2.2 NP13a 的细胞生长和产酶情况.....	27
3.2.3 致死曲线.....	27
3.2.4 诱变菌的筛选.....	28
3.2.5 诱变菌的摇瓶培养.....	29
3.2.6 诱变菌 EU2-77 的产酶能力与商业菌株的比较.....	31
3.2.7 诱变菌 EU2-77 的遗传稳定性考察.....	32
3.3 小结	33
第四章 发酵条件优化	34
4.1 实验内容	34
4.1.1 菌种培养和酶活分析方法.....	34
4.1.2 辅助碳源及其浓度的优化.....	34
4.1.3 氮源的选择及其浓度的优化.....	34
4.1.4 Plackett-Burman 设计法筛选纤维素酶发酵条件重要影响因素	34
4.1.5 响应面分析实验设计优化纤维素酶发酵条件.....	34
4.1.6 模型验证及小型放大实验.....	35
4.2 结果与讨论	35
4.2.1 最佳辅助碳源及其浓度的选择.....	35
4.2.2 最佳氮源及其浓度的选择.....	36
4.2.3 Plackett-Burman 设计法筛选纤维素酶发酵条件重要影响因素	37
4.2.4 响应面分析实验设计优化纤维素酶发酵条件.....	39
4.2.4.1 Box-Behnken 试验设计	39
4.2.4.2 二次回归拟合及方差分析	40
4.2.5 纤维素酶最佳发酵条件的确定及验证.....	43
4.3 小结	44
第五章 酶解特性分析	46
5.1 实验部分	46
5.1.1 酶源.....	46
5.1.2 酶活测定.....	46
5.1.3 园林废弃物的预处理.....	47
5.1.4 园林废弃物的成分测定.....	47
5.1.5 酶解实验及酶解产物分析.....	47

5.2 结果与讨论	47
5.2.1 酶活测定	47
5.2.2 处理与未处理过的木质纤维素材料的酶解效果分析	48
5.2.3 EU2-77 纤维素酶酶解能力与商业菌纤维素酶和商业酶的比较	49
5.3 小结	53
第六章 结论与展望	54
6.1 结论	54
6.2 展望	54
参 考 文 献	56
在学期间所发表的论文	63
致 谢	64

Table of contents

Chapter 1 Review of literatures	1
1.1 Introduction of cellulase	1
1.1.1 Components of cellulase.....	1
1.1.2 Molecular structure and function of cellulase.....	2
1.1.3 Physical and Chemical characterstize of cellulase.....	3
1.1.3.1 Molecular weight.....	3
1.1.3.2 Isoelectric points.....	3
1.1.3.3 Optimal pH and temperature.....	3
1.1.3.4 Enzymatic activities.....	3
1.2 Source of cellulase	4
1.2.1 Microorganisms.....	4
1.2.2 Animals.....	4
1.3 Strain improvement for enhanced cellulase production	4
1.3.1 Strain improvement by mutation and its research progress.....	5
1.3.1.1 Mechanism of mutagenesis.....	5
1.3.1.2 Principles of mutagenesis.....	6
1.3.1.3 Screening of hyper-producing mutants.....	7
1.3.1.4 Research progress of mutagenesis for enhanced cellulase production....	7
1.3.2 Protaplast fusion technology for enhaced cellulase production.....	8
1.3.3 Construction of genetic engineering strains.....	8
1.4 Production of cellulase	9
1.4.1 Production of cellulase under solid-state fermentation.....	9
1.4.2 Production of cellulase under liquid-state fermentation.....	10
1.5 The applications of cellulase	11
1.5.1 Application of cellulase in the bio-ethanol industry.....	11
1.5.2 Application of cellulase in the feed industry.....	11
1.5.3 Application of cellulase in the textile industry.....	12
1.5.4 Application of cellulase in the food processing industry.....	12
1.5.5 Application of cellulase in the natural products extraction.....	12

1.5.6 Application of cellulase in the reearch of genetic engineering	13
1.6 Aim, significance and contents of this thesis	13
1.6.1 Aim and significance of this research	13
1.6.2 Contents of this thesis	14
Chapter 2 Materials and methods	15
2.1 Reagents and instruments.....	15
2.1.1 Reagents.....	15
2.1.2 Preparation of buffer and reaction solution	16
2.1.3 Instruments.....	17
2.2 Analytic methods	18
2.2.1 Enzymatic activities assay	18
2.2.2 Reducing sugars	19
2.2.3 Protein.....	21
2.2.4 Glucose, xylose and cellubiose.....	23
2.2.5 Dry cell weight.....	23
2.3 Experimental methods	24
2.3.1 Strains	24
2.3.2 Medium.....	24
2.3.3 Shake-flask fermentation	24
2.3.4 Bio-reactor fermentation.....	24
Chapter 3 Strain improvement by mutation	25
3.1 Experiments	25
3.1.1 Screening of potential wild fungi for mutation.....	25
3.1.2 Preparation of the phosphoric-acid-swellon cellulose	25
3.1.3 Preparation of the screening medium	25
3.1.4 Curve of lethality rate	26
3.1.5 Screening of hyper-producing mutants	26
3.1.6 Enzymatic activities assay	26
3.2 Results and discussion.....	26
3.2.1 Screening of potential wild fungi for mutation.....	26
3.2.2 Mycelium growth and cellulase production by NP13a.....	27

3.2.3 Curve of lethality rate	27
3.2.4 Mutants after screening by plate-clearing method.....	28
3.2.5 Shake-flask fermentation of mutants	29
3.2.6 Comparing the mutant EU2-77 to the commercial strains.....	31
3.2.7 Hereditary stability of the mutant EU2-77.....	32
3.3 Summary	33
Chapter 4 Optimization of the fermentation condition.....	34
4.1 Experiments	34
4.1.1 Strain's cultivation and enzymatic activities assay	34
4.1.2 Optimization of co-carbon source.....	34
4.1.3 Optimization of nitrogen source	34
4.1.4 Plackett-Burman design	34
4.1.5 Response surface methology.....	34
4.1.6 Model validation	35
4.2 Results and discussion.....	35
4.2.1 Optimization of co-carbon source.....	35
4.2.2 Optimization of nitrogen source	36
4.2.3 Screening of parameter affecting cellulase production by Plackett-Burman design.....	37
4.2.4 Optimization of fermentation condition by the RSM design.....	39
4.2.4.1 Box-Behnken experiment design	39
4.2.4.2 Analysis of variance for the selected quadratic model	40
4.2.5 Validation of the optimal culture condition.....	43
4.3 Summary	44
Chapter 5 Hydrolysis performance of obtained enzymes	46
5.1 Experiments	46
5.1.1 Source of cullase	46
5.1.2 Enzymatic activities assay	46
5.1.3 Pretreatment of horticultural waste.....	47
5.1.4 Analyzing of the composition of horticultural waste	47
5.1.5 Enzymatic hydrolyzing and hydrolsate assays	47

5.2 Results and discussion	47
5.2.1 Enzymatic activities of the enzymes	47
5.2.2 Effect of pretreatment with the horticultural waste on hydrolyzing	48
5.2.3 Comparing the hydrolyzing performance by the cellulase derived from EU2-77 and commercial strain RUT C30 and commercial cellulases	49
5.3 Summary	53
Chapter 6 Conclusions and perspectives	54
6.1 Conclusions	54
6.2 Perspectives	54
References	56
Publications during graduate study	63
Acknowledgement	64

第一章 文献综述

纤维素是一种高分子量的碳水化合物，是由葡萄糖通过 β -1,4-糖苷键连结而成的直链聚合物。纤维素是植物细胞壁的主要成分，广泛存在于自然界中，是世界上最丰富、最廉价的可再生资源，通过植物的光合作用，地球上每年合成的植物总量约为 10^{10} t，其中纤维素占 40%^[1]，超过其他碳水化合物的总和。由于纤维素不溶于水，在环境中比较稳定，要把它水解成可利用的葡萄糖是相当困难的。所以到目前为止仍没有得到很好地利用，绝大多数纤维素不仅被白白浪费，还造成了环境污染。随着世界人口增长，为解决日益加剧的食品和能源危机，纤维素资源的利用已引起了世界各国的极大关注和高度重视。当前，新能源、新食品资源的开发利用是世界各国都在研究的重大课题。利用纤维素酶对纤维素生物质进行转化对解决世界能源危机、粮食短缺、环境污染等问题具有重大意义^{[2], [3]}。

1.1 纤维素酶简介

1.1.1 纤维素酶的组成

纤维素酶是指所有参与降解纤维素并将其转化为葡萄糖的各种酶的总称。它是一类复杂的复合物，故而又被称为纤维素酶系 (Cellulase System)，一般将其分为三类，如图 1-1^[4]：①内切葡聚糖酶 (CMCase, Endo-1,4- β -D-glucanase; EC 3.2.1.4)，来自于真菌简称为 EG，来自于细菌简称为 Len)，这类酶一般作用于纤维素内部的非结晶区，随机水解 β -1,4 糖苷键，将长链纤维素分子截短，产生大量带非还原性末端的小分子纤维素；②外切葡聚糖酶 (Cellobiohydrolase; EC 1.2.1.91)，来自于真菌简称为 CBH；来自于细菌简称为 Cex)，这类酶作用于纤维素线状分子非还原性末端，水解 β -1,4 糖苷键，每次切下一个纤维二糖分子；③ β -葡萄糖苷酶 (β -glucosidase; EC 3.2.1.21)，简称 BG，这类酶将纤维二糖水解开成葡萄糖分子。

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