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Optimization of solid fermentation of cellulase from *Trichoderma koningii*

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Abstract: To exploit peashrub resources in Ordos as fodders, it is very crucial to realize industrial production of cheap cellulase of high activity by optimizing culture technology, especially culture substrate. In this study, a new prescription experiment based on uniform design ideal was invented and successfully applied in the solid fermentation of *Trichoderma koningii* F244, which was performed with two different temperature degrees. The activities of FPA, cotton lyase, CMCase and β-glucosidase were assayed and then mathematical models of enzymatic activities, which were figured out by Unconstraint Mathematical Programming, were developed by Multivariate Regression Program of SPSS10.0. Enzymatic activities of optimized substrate prescriptions corresponding to mathematical models were forecasted to determine an ideal substrate prescription. It is revealed that in solid fermentation, Tween80 has negative effect on cellulase production. Furthermore, the ideal prescription for cellulase complex production by *Trichoderma koningii* F244 was straw powder 16.9%, wheat bran 26.5%, $(NH_4)_2SO_4$ 9.5% and water 47.1%, whose corresponding cellulase activity was expected to be at the same high level with that of *Trichoderma reesei* Q9414 on its own recommended substrate. Especially, goats mainly fed on peashrub tissues mixed with cellulase complex of this prescription and culture technology, got an incremental ratio of 0.3 kg/d, which brought a very promising feeding prospect for local peashrub resource. By populization of this cellulase complex, it can integrate living standard, economic construction of local residents into vegetational restoration tightly and thus this paper will be very meaningful to be use for reference for western China like Ordos to realize its sustainable development of economy, society and environment.

Keywords: *Trichoderma koningii*; substrate prescription; FPA; CMCase; cotton lyase; β-glucosidase

Introduction

Ordos Plateau lies between north latitude 37°—41° and east longitude 106°—110°, where average annual precipitation is about 300 mm while average annual evaporation can reach 2167 mm amazingly (Micael, 2000). Only shrub and weed can become dominant natural vegetation in such an ecologically vulnerable area. The severe damage on native environment, as a result of natural depasturage and over-grazing of long history, has not only made this area become an important seedbed of dust and sand, but also let thousands of native people have to make ecological emigration (Zhang, 1994). To achieve effective vegetational restoration and environmental protection, local governments had widely planted peashrub(*caragana*), which has an amazing vitality, adversity resistant characteristics. Peashrub can survive in the area where annual precipitation is only 150 mm and effective accumulated temperature is over 1500℃ and has become an important virescence specie in western China by its high survival forestation ratio and its effect on soil melioration, windbreak, sand stabilization and soil-water conservation. To rejuvenate, peashrub need to be periodically fell(i.e. stumped) and its above-ground biomass can be used as fodder, fuel, fertilizer or material of paper mill or fiberboard(Niu, 1999). Now, peashrub resources in Ordos have been up to 290953 hm^2, which plays a very important role in ecological protection for local environment.

With implementation of the strategy of western exploitation, the ecological environment of western China has received an unprecedented attention and thus rotation grazing and facility grazing have become common ideals in Ordos, where fodder deficiency is a serious limiting factor for local stockbreeding development. Traditionally, peashrub was lifesaving forage or accessorial grass for ruminants to go through winter or spring and had a very great feeding potential for its very high coarse protein content. But the coarse fiber ratio in biomass was far above the tolerance of ruminants and impeded its full exploitation as fodders. The enzymatic hydrolyzation is main channel to biodegrade coarse fiber. But the cellulase activity of cellulase complex on domestic market is far below that of the same products from foreign countries. For example, the highest FPA activity of native cellulase complex can not break through 40 FPU/g while that of foreign products can be several times of 40. In addition, the high price of cellulase complex is always far above the purchasing ability of local dweller (El-Hawary, 2001; Szengyel, 1997; Selinger, 1996). Therefore, to develop cellulase complex of high activity, low price and our own intellectual property

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right and to apply it efficaciously on facility breeding of ruminants by peashrub fodders have became one of the key points relating to the realization of rotation grazing and facility grazing in Ordos.

Cellulase is mainly produced from *Trichoderma* and *Aspergillus* and its industrial production has already been realized (Rodriguez, 2001; Bravo, 2001). *Trichoderma koningii* represents alluring prospect in cellulase production at low cost (Lonsane, 1992). Optimization of substrate and culture technics is one of the most important steps to increase activity and decrease cost in industrial production of cellulase complex, although strain is of great importance either(Byoo, 1999; Ingram, 1999). Moreover, cellulase is induced enzyme and mechanism of cellulase biosynthesis is regulated by inducer, N source and C source. Therefore, substrate for cellulase production often contains some kinds of agricultural byproducts abundantly containing N, C or cellulose, such as yeast extract, bean cake powder, dextrose, rice bran, rice and wheat straw powders, wheat bran, grass powder, hazel hull, willow rag, poplar leaves etc. (El-Hawary, 2001; Wang, 1996; Hu, 1998; Bakir, 2001). Any kind of natural cellulose castoff can be used in solid fermentation of cellulase whose production is 2—3 times higher than that of liquid fermentation and therefore solid fermentation cost can be decreased largely(Byoo, 1999).

In solid fermentation, wheat bran is specially favored by aerobiotic microorganisms because it has abundant N, C, inorganic salts while is loose, easy to ventilate and of great surface area. Rice straw powder is one of the mostly accepted C source or inducer for cellulase production in solid fermentation. Moreover, $(NH_4)_2SO_4$ and Tween80 are typical inorganic N source and surfactant respectively and they have been reported to be helpful for cellulase production (El-Hawary, 2001; Parminder, 1996). In the present study, *Trichoderma koningii* was selected as cellulase production strain and rice straw powder, wheat bran, $(NH_4)_2SO_4$, Tween80 and water were chosen as substrate ingredients, according to the principles of low-cost and extensive-source. By aiming at attaining simple, applied technics for cellulase complex production, this study has brought an insight into the optimization of solid substrate and corresponding culture conditions for cellulase production from *Trichoderma koningii*.

1 Materials and methods

1.1 Microorganisms

Trichoderma koningii F244, preserved in Institute of Applied Ecology, Chinese Academy of Sciences.

1.2 Precultivation and inoculum preparation

Trichoderma koningii was inoculated to PDA medium (potato 20%, dextrose 2%, agar 2%) in tubes. After static culture of 5 d at 25℃, *Trichoderma koningii* was transfered into flasks (500 ml) containing PDY substrate(potato 20%, dextrose 2%, yeast extract 1%) of 100 ml by 10 ml sterile distilled water. Then flasks were agitated on a rotary shaker at 28℃ for 3—4 d with aeration and a ration speed of 120—150 r/min.

1.3 Solid substrate optimization and cellulase production by solid fermentation

The ideal of uniform design was applied in this prescription experiment, each treatment weighed 50 g with natural pH(about 6.5), as shown in Table 1(Bravo, 2001). Then 2 ml *Trichoderma koningii* conidial suspension from flask was inoculated to 50 g pasteurized solid substrate. After incubation, the fermentation process was performed with two different temperature degrees which was 32℃ during the first 30 h and 27℃ for another 66 h continuously (Parminder, 1996), at the condition of natural oxygen supply and an environmental moisture capacity about 70% to keep substrate humid.

1.4 Coarse enzyme solution preparation

When fermentation was completed, about 2 g solid substrate and 20 ml distilled water was stirred in beaker, agitated at 130 r/min and 40℃ for 1 h and then leached by filter paper. Next, the filtrate was preserved at 4℃ after centrifugation (3000 r/min for 15 min) to remove mycelium.

1.5 Enzyme assay (Wu, 1998)

The widely accepted DNS method was applied in enzymatic activity assay and enzymatic activity unit is defined below.

Definition of FPA (filter paper lyase) activity: with Whatman No. 1 filter paper as degrading object, under the condition of pH 4.8, constant 50℃ for 1 h, the quality of cellase hydrolyzing cellulose to form 1mg glucose per 60 min was one U(U = mg dextrose/h).

Definition of CMCase(carboxymethyl cellulase) activity: with CMC-Na as degrading object, under the ondition of pH 4.8, constant 50℃ for 30 min, the quality of cellase dydrolyzing cellulose to form 1mg glucose per 60 min was one U(U = mg dextrose/h).

Definition of cotton lyase activity: with degreased cotton as degrading object, under the condition of pH 4.8, constant 50℃ for 24 h, the quality of cellase hydrolyzing cellulose to form 1 mg glucose per 24 h was one U(U = mg dextrose/24 h).

Definition of β-glucosidase activity: with salicin as degrading object, under the condition of pH 4.8, constant 50℃ for 30 min, the quality of cellase hydrolyzing cellulose to form 1 mg glucose per 60 min was one U(U = mg dextrose/h).

1.6 Experimental design and data analysis

A totally new prescription experiment was constructed in the light of uniform design ideal and successfully applied in solid fermentation in this paper. And second-degree polynomial regression models of enzymatic activity, which were established by Multivariate Regression Program, were figured out by Unconstraint Mathematical Programming to find the partially optimal substrate prescription corresponding to cellulase activities. At last, the ideal solid substrate prescription was determined by enzymatic activity forcast with that of *Trichoderma reesei* Q9414 as comparison.

2 Results and discussion

2.1 Enzymatic activity

Cellulase activity of prescription experiment is shown in Table 1.

Table 1 Prescription experiment of cellulase production by solid fermentation from *Trichoderma koningii*

No.	Substrate constituents, g					Cellulase activities, U/g*			
	Rice straw	Water	Wheat bran	$(NH_4)_2SO_4$	Tween80	FPA	Cotton lyase	CMCase	β-glucosidase
1	28.64	11.45	6.78	2.82	0.31	21.7	26.9	54.0	39.9
2	21.88	12.64	9.16	4.42	1.9	19.9	14.0	44.3	40.2
3	18.05	12.28	8.9	8.98	1.8	27.8	31.4	68.5	54.5
4	15.25	23.57	5.78	4.14	1.26	47.1	63.7	150.5	102.8
5	13.00	14.22	18.62	3.19	0.97	39.1	38.0	96.7	78.8
6	11.09	12.86	15.41	10.28	0.35	14.4	17.5	30.5	26.1
7	9.43	27.51	10.67	1.99	0.4	53.9	110.7	287.3	62.4
8	7.96	22.53	8.83	10.33	0.36	16.9	19.3	96.0	29.3
9	6.62	14.34	15.01	9.82	4.21	14.4	17.9	42.4	72.4
10	5.4	23.90	14.16	4.58	1.96	76.6	93.1	208.5	94.4
11	4.27	20.57	20.57	4.44	0.15	94.0	105.4	246.0	184.0
12	3.21	17.98	14.89	12.52	1.39	16.9	19.2	33.8	26.3
13	2.23	32.4	9.10	5.65	0.63	48.2	74.0	257.2	79.3
14	1.3	21.9	12.12	11.25	3.43	11.6	14.6	25.5	23.1
15	0.42	16.39	22.69	8.75	1.75	11.4	16.7	26.7	19.9
Aver.	9.92	18.97	12.85	6.88	1.39	34.3	44.2	111.2	62.2

Note: * Enzymatic activity of one g substrate

2.2 Data analysis and mathematical model construction

After enzyme assay, converted the dosage of each ingredient in Table 1 into corresponding centralized ratio and then Multivariate Regression Program of SPSS10.0 was applied to get ideal second-degree polynomial regression models of enzymatic activity.

$$\begin{aligned}\text{FPA} = {} & 130.639 + 96.265x_2 - 1647.173x_2^2 - \\ & 3209.029x_2x_5 + 123.3x_3 - 3027.562x_2^3 + \\ & 1710.415x_3x_4 - 10377.0x_4^2 + 24203.48x_4x_5 - \\ & 1298.614x_5 - 29375.5x_5^2 \qquad (0.852,\ 0.219)\end{aligned}$$

$$\begin{aligned}\text{Cotton lyase} = {} & 171.438 + 160.084x_2 - 2220.651x_2^2 - \\ & 6279.748x_2x_5 + 178.137x_3 - 4422.748x_3^2 + \\ & 3543.708x_3x_4 - 13385.2x_4^2 + 35756.81x_4x_5 - \\ & 1911.63x_5 - 42284.0x_5^2 \qquad (0.941,\ 0.045)\end{aligned}$$

$$\begin{aligned}\text{CMCase} = {} & 349.298 + 430.912x_2 - 3772.495x_2^2 - \\ & 16536.3x_2x_5 + 304.427x_3 - 8504.358x_3^2 + \\ & 5476.735x_3x_4 - 26430.9x_4^2 + 69360.37x_4x_5 - \\ & 4212.38x_5 - 79019.6x_5^2 \qquad (0.964,\ 0.018)\end{aligned}$$

$$\begin{aligned}\text{β-glucosidase} = {} & 186.842 + 95.781x_2 - 2458.692x_2^2 - \\ & 6599.625x_2x_5 + 117.44x_3 - 3333.778x_3^2 + \\ & 1312.94x_3x_4 - 15394.6x_4^2 + 34991.78x_4x_5 - \\ & 2007.999x_5 - 27092.8x_5^2 \qquad (0.703,\ 0.574)\end{aligned}$$

Note: x_2 = water/50 − 0.3794;

x_3 = wheat bran/50 − 0.2569;

x_4 = $(NH_4)_2SO_4$/50 − 0.1375;

x_5 = Tween80/50 − 0.0278.

CMCase, cotton lyase and β-glucosidase are all of great importance in cellulase complex and FPA is considered to embody the synergy of CMCase, cotton lyase and β-glucosidase. The four individual enzymes are fitted to degrade coarse fiber of different crystallinity or different degrees of polymerization. Therefore it is very necessary to take them all into consideration in cellulase complex production (Fu, 2000). In view of the great difference among the average activity of each enzyme and the simulation situation of each regression equations, it was inevitable to sum each enzyme equation with weight of the quotients of total correlation coefficient and the average activity value to get an ideal model of overall cellulase activity.

$$\begin{aligned}\text{Cellulase} = {} & \text{FPA} \times 0.852/34.3 + (\text{cotton lyase}) \times \\ & 0.941/44.2 + \text{CMCase} \times 0.964/111.2 + \\ & \text{β-glucosidase} \times 0.703/62.3 \\ = {} & 12.04106 + 10.6231x_2 - 148.765x_2^2 \\ & - 431.54x_2x_5 + 10.82823x_3 - 280.927x_3^2 \\ & + 180.362x_3x_4 - 946.349x_4^2 + \\ & 2360.487x_4x_5 - 132.234x_5 - 2622.7x_5^2.\end{aligned}$$

2.3 Model solution and prescription determination

By the Unconstraint Mathematical Programming, the solutions of enzyme models are shown in Table 2.

Then the corresponding optimized prescription of solid substrate for each model can be figured out by mathematical transmission, as shown in Table 3.

Table 2 Solutions of enzyme models corresponding to maximal dependents

Mathematical model	x_2	x_3	x_4	x_5	Dependent maximum
FPA	0.078455	0.003802	−0.05863	−0.05054	167.5
Cotton lyase	0.15011	−0.02432	−0.11097	−0.08067	258.4
CMCase	0.455357	−0.0609	−0.24473	−0.18171	820.8
β-glucosidase	0.561624	−0.0734	−0.46222	−0.40395	615.0
Cellulase	0.166907	−0.01748	−0.11448	−0.09046	18.8

Table 3 Optimized prescriptions corresponding to maximal dependents of enzyme models (%)

Optimized prescription	Rice straw	Water	Wheat bran	$(NH_4)_2SO_4$	Tween80
FPA	0.203	0.457	0.261	0.079	0(−0.023)
Cotton lyase	0.211	0.529	0.233	0.027	0(−0.053)
CMCase	0(−0.03)	0.804	0.196	0(−0.107)	0(−0.154)
β-glucosidase	0(−0.125)	0.816	0.184	0(−0.324)	0(−0.376)
Cellulase	0.192	0.546	0.239	0.023	0(−0.063)

Note: The value without parentheses is applied ratio of substrate constituent in prescription, the values in parentheses is theoretic ratio

Table 3 shows that Tween80 has negative effect on enzyme production and should be abandoned, although surfactant was usually reported to be helpful for enzyme biosynthesis(El-Hawary, 2001; Viesturs, 1996). By solid fermentation, Tween80 had been reported to have little positive, or negative effect on CMCase, FPA production from *trichiderma*(Hu, 1998; Cheng, 2000). And similar negative effect of 0.1% Tween80 on extracellular beta-glucosidase had been reported by Lucas R *et al*. too(Lucas, 2000).

In solid fermentation, low a_w(water activity) is induced by water compression in solid substrate as a result of water evaporation, water absorbance by new cells and monocase sugar accumulation (Nagel, 2001; Taragano, 1997). Therefore, it is reasonable to assume that the positive effect of Tween80 to help enzyme to be released from fungi cell by improving membrane permeability becomes repressed at the condition that the sugar transportation is restricted and the membrane permeability of fungi cell is transferred by low a_w (Taragano, 1997; Angelova, 1995; Chiyomi, 2002). Otherwise, surfactant will affect enzymatic activity by associating with enzyme protein or inhibiting mycelia growth if it is above the critical micelle concentration (Chiyomi, 2002). Hence, Tween80 is not a proper ingredient in solid substrate.

Straw powder and $(NH_4)_2SO_4$ have repressed the production of CMCase and β-glucosidase and this result was in line with the conclusion of Türker M. *et al*. (Türker, 1987). Further, our result implied it clearly that rice straw and $(NH_4)_2SO_4$ will only restrain part of the cellulase whose degrading object is not natural fiber. Otherwise, straw powder and $(NH_4)_2SO_4$ can activate FPA, cotton lyase as well as overall cellulase activity and so they are necessary in application.

2.4 Forecast of enzymatic activity and appraisal of ideal substrate prescription

Generally, *Trichoderma reesei* Q9414 has been famous for its high cellulase activity (Muhannad, 2001; Mandels, 1976). To select an ideal substrate prescription, it is a reasonable way to compare their forecasted enzymatic activities with that of Q9414 as comparison. Firstly, transformed the ratios of substrate constituents of each prescription into corresponding independents of their regression equations, as shown in Table 4.

Table 4 Transformation of constituent ratios of optimized prescriptions to the corresponding independents of enzyme models

Prescription	Item	Water	Wheat bran	$(NH_4)_2SO_4$	Tween80
FPA	Ratio, %	0.457	0.261	0.079	0
	Independent	0.0776	0.0041	-0.0585	-0.0278
Cotton lyase	Ratio, %	0.529	0.233	0.027	0
	Independent	0.1496	-0.0239	-0.1105	-0.0278
Cellulase	Ratio, %	0.546	0.239	0.023	0
	Independent	0.1666	-0.0179	-0.1145	-0.0278

Note: CMCase prescription and β-glucosidase prescription is not taken into account because substrate of their prescription is not in solid state

Secondly, calculated the dependants of the mathematical models on condition that x_2, x_3, x_4, x_5 equals the corresponding independent levels above. Finally, the dependants were transformed into enzymatic activity of IU/g, as shown in Table 5.

Table 5 Forecast of enzymatic activities of optimized prescriptions

Prescription	Unit	FPA	Cotton lyase	CMCase	β-glucosidase	C_1/C_X
FPA	U/g	152.4	216.7	473.9	232.9	0.457
	IU/g	14.1	20.1	43.9	21.6	
Cotton lyase	U/g	82.4	141.2	346.8	126.7	0.407
	IU/g	7.6	13.1	32.1	11.7	
Cellulase	U/g	70.5	127.0	326.3	109.1	0.389
	IU/g	6.5	11.8	30.2	10.1	
Q9414*	U/g	-	-	-	-	0.297
	IU/g	14.0	41.0	138	19.6	

Notes: IU/g = μmol dextrose/(min·g dry matter); * *Trichoderma reesei* Q9414 is cultured on its recommended solid substrate in the experiment of Wang *et al*. (Wang, 1996)

Cellulase activity assay with filter paper as degrading object is considered to be a widely accepted and typical method while the deficiency of β-glucosidase is a restrictive factor in the saccharification of cellulose, especially for *Trichoderma*(Brumbauer, 1999; Ryu, 1991). Moreover, a 1/1 mixture of CBH1/EG1, approximately cotton lyase/CMCase, was found to be most effective for degradation and digestion of coarse fiber(Henrissat, 1985). It is clear that the forecasted enzymatic activities from *Trichoderma koningii* F244 of FPA prescription is apparently higher than that of the rest prescription and no less than that of *Trichoderma reesei* Q9414 on its own recommended substrate on these key indexes. So the prescription of rice straw 20.3%, water 45.7%, wheat bran 26.1% and $(NH_4)_2SO_4$ 7.9% is ideal substrate prescription.

2.5 Feeding effect of cellulase complex of FPA prescription on goats

To verify the weight gaining effect of cellulase complex of FPA prescription on ruminants, a feeding experiment was carried out in Ordos Handicapped Breeding Center after coarse cellulase complex were prepared by solid fermentation and FPA prescription with two temperature degrees under the condition of natural oxygen supply and an environmental humidity of 70%. Beforehand, one week of adaptive feeding was applied on goats and the formal feeding experiment was carried out from July 7, 2002 to August 15, 2002, during which the goat weight was weighed periodically. Three goats was treated as contrast and their feedstuff is corn flour 10%, wheat bran 7%, and oddments of the above-ground tissues of *caragana intermedia* (a typical kind of peashrub) 83%. Another three goats, as experimental object, were fed the same feedstuff mixed with 20 g crude cellulase complex of FPA prescription each time. As shown in Table 6, the weight incremental ratio of experimental goats is two times of that of contrast goats.

Table 6 Effect of cellulase complex on weight increment of goats fed on peashrub biomass

Feeding effect	Experimental goats			Contrast goats		
Weight on July 21, kg	62	61	48	47	54	72
Weight on August 15, kg	70	68	56	52	57	75
Net increment, kg	8	7	8	5	3	3
Incremental ratio, kg/d	0.32	0.28	0.32	0.20	0.12	0.12
Average incremental ratio, kg/d	0.31			0.15		

3 Conclusions

While solid fermentation of *Trichoderma koningii* F244 is performed under the condition of two different temperature degrees of 96 h, natural aeration, natural substrate pH(about 6.5) and an environmental humidity of 70% for cellulase complex production, Tween80 is only to restrain enzyme production in solid substrate. Further, a prescription of straw powder 20.3%, wheat bran 26.1%, $(NH_4)_2SO_4$ 7.9% and water 45.7% is found to be ideal for cellulase complex production and enzymatic activity of this prescription is expected to be the same high level with that of *Trichoderma reesei* Q9414 on its own recommended substrate.

Moreover, feeding experiment implied that with peashrub tissues as main feedstuff, the weight incremental ratios of goats fed with a small quantity of cellulase complex of FPA prescription can reach 0.3 kg/d, which is two times of that of goats with the same peashrub feedstuff alone. To exploit peashrub resources as fodders, it is reasonable and inevitable to produce cellulase complex by this FPA prescription and culture conditions, which has simple technics, low cost, independent intellectual property right and the characteristic of easy popularization in underdeveloped area. In this way not only the situation of frequent snow disaster and fodder deficiency in spring and winter in Ordos can be transformed thoroughly, but also the living standards, economic construction of local residents are integrated tightly into vegetational restoration and the enthusiasm on ecological construction and elimination of sand and dust devil of local people is enhanced effectively. In view of representative characteristics of Ordos, the research results of this paper will be very meaningful to be use for reference for western China to realize its sustainable development of economy, society and environment.

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