End Product Analysis of Dried Grass Hydrolysis by Extracellular Cellulase of *Rhizopus oryzae*

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ABSTRACT

Extracellular cellulase was produced by *Rhizopus oryzae* PR7 MTCC 9642 and was used for bioconversion of dried grass. Extra cellular cellulase produced by the fungal isolate was found to contain all the component enzymes namely endoglucanase, avicelase and β- glucosidase. Product analysis revealed that all the three above mentioned components of cellulase enzymes acted synergistically to produce glucose as end product.

Keywords: Cellulase, *Rhizopus oryzae*, saccharification.

Introduction

Cellulose being an abundant and renewable resource is a potential raw material for the microbial production of food, fuel, and chemicals (Coughlan, 1985). Saccharification of cellulose to glucose by microbial cellulases has attracted the attention of the researchers, as this is the first step of bioconversion of cellulose material into valuable products such as sugar, fine chemicals and biofuels. Such process would not only solve modern waste disposal problem but also would alleviate shortages of food and animal feeds, and diminish man's dependence on fossil fuels. Moreover it would provide a convenient way to utilize the renewable source of energy in the form of glucose. Since bioconversion of the polymers by enzymes are proved superior to chemical techniques, research to acquire in depth knowledge on the biochemical and biotechnological aspects of the enzyme action and application becomes the need of the hour (Nguyen and Saddler, 1991; Jeffries, 1990).

Microorganisms are regarded to be the most important source of cellolytic enzymes and the majority of cellulose degradation has been done with fungi. The fungi generally secrete the cellulose degrading enzyme complex, acting synergistically to produce simpler sugars.

In the present paper production of all three enzymes of cellulase multienzyme complex from *Rhizopus oryzae* were tested and the end product of cellulose action on a cellulosic substrate collected from agro waste was analyzed to determine the nature of the sugar produced after saccharificaton.

Materials and methods

Microorganism: *Rhizopus oryzae* PR7 MTCC 9642, an endoglucanase producing strain was isolated from the soil of India and was identified by Institute of Microbial Technology (IMTECH), Chandigarh, India as *Rhizopus oryzae* MTCC 9642 (Karmakar *et al*, 2012).

Chemicals: All chemicals used were of analytical grade.

Cultivation of the strain: The strain *R. oryzae* PR7 MTCC 9642 was cultivated in 100 ml Erlenmeyer flasks each containing 20 ml of basal medium (BM) composed of (g/l): peptone 0.9; $(NH_4)_2HPO_4$ 0.1; KCl 0.1; $MgSO_4.H_2O$ 0.1and carboxymethyl cellulose (CMC) 0.5 (pH 6.0). 1.0% (w/v) salicin was added as inducer.

Extraction and assay of enzyme: The mycelial part was separated from the culture broth by filtering through filter paper and the culture broth was subjected to centrifugation at 15,000 rpm for 10min. The crude enzyme solution sieved through a 0.25 μm membrane filter.

To measure the activity of β-glucosidase and endoglucanase, tubes containing the assay mixture (1 mL) each containing an 0.5 ml of enzyme diluted with 0.1(M) phosphate buffer (pH 6) were incubated with 1%(w/v) avicel for cellobiose for 15 minutes and 1% (w/v) CM-cellulose for 10 minutes (Karmakar and Ray, 2010), respectively, at 33°C.

To measure the activity of avicelase, the assay mixture (1 mL) containing an equal volume of enzyme and 1% (w/v) avicel (Sigma) dissolved in 10 mM was incubated at 37°C for 10 min

The reducing sugar released in either case was measured by the dinitrosalicylic acid method (Bernfeld, 1955), taking glucose as standard. Blanks were prepared with inactivated enzymes. One unit of avicelase, β-glucosidase and endoglucanase was defined as that amount of enzyme that liberated 1μ mole of glucose per ml per minute of reaction from the respective substrate.

Saccharification of cellulosic substrates: A suspension of dried grass (5mg/ml) in 0.1M phosphate buffer (pH 7) was incubated with properly diluted cellulase enzyme in a screw capped tube for 30 minutes at 33°C. The hydrolysate was centrifuged at 2000 g for 2 minutes and the resultant supernatant was analyzed by DNSA method (Bernfeld, 1955) using glucose as standard.

Determination of end product of saccharification: The end products of saccharification of agrowaste dried grass by cellulase was analysed by injecting the supernatant in a Spherisorb-NH_2 column (0.46 X 25 cm) attached to HPLC (Shimadzu Prominence Series [CBM-20 A] with RI detector. The solvent system used was acetonitrile: water at a ratio of 3:1 (v/v). The sample was allowed to run and fractions were automatically collected at a flow rate of 1ml/min by GE FRAC-920 fraction collector. The standards used was glucose , cellobiose and salicin.

Results and discussion

The strain secreted multi-enzyme cellulase complex which consists of endoglucanase, avicelase and β-glucosidase (Fig. 1.).

The peaks of standard glucose, cellobiose and salicin were depicted in Fig 2, Fig 3 and Fig.4 respectively. The end product of saccharification of grass as cellulosic substrate could be identified from the Fig 5. Comparing with the peaks of the standard used, it could be concluded that the peak with retention time 6.23min clearly indicated the presence of glucose as the major end product. Intermediate products cellobiose indicated by the peak with retention time 7.67 min. Salicin used as the inducer the media could be detected by the peak of retention time 4.51 min, which further revealed that this was actually playing the role of a gratuitous inducer of the enzyme.

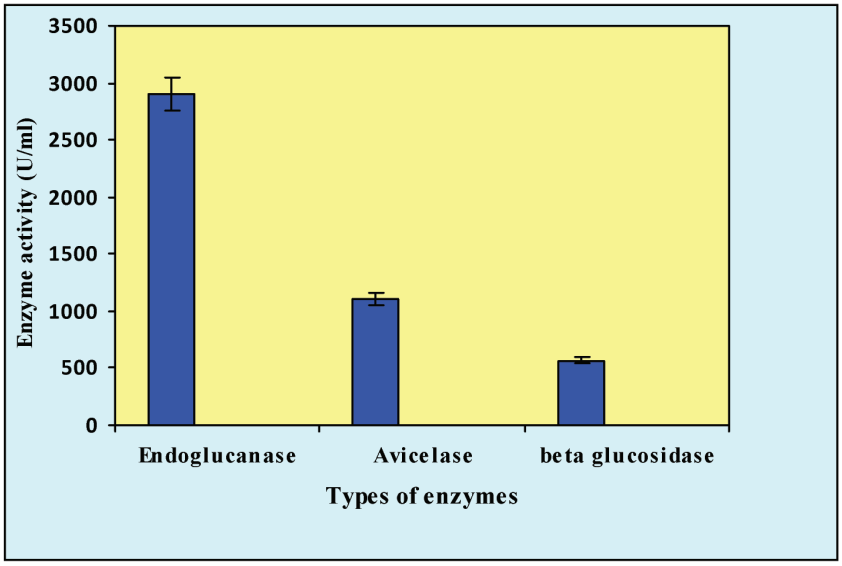
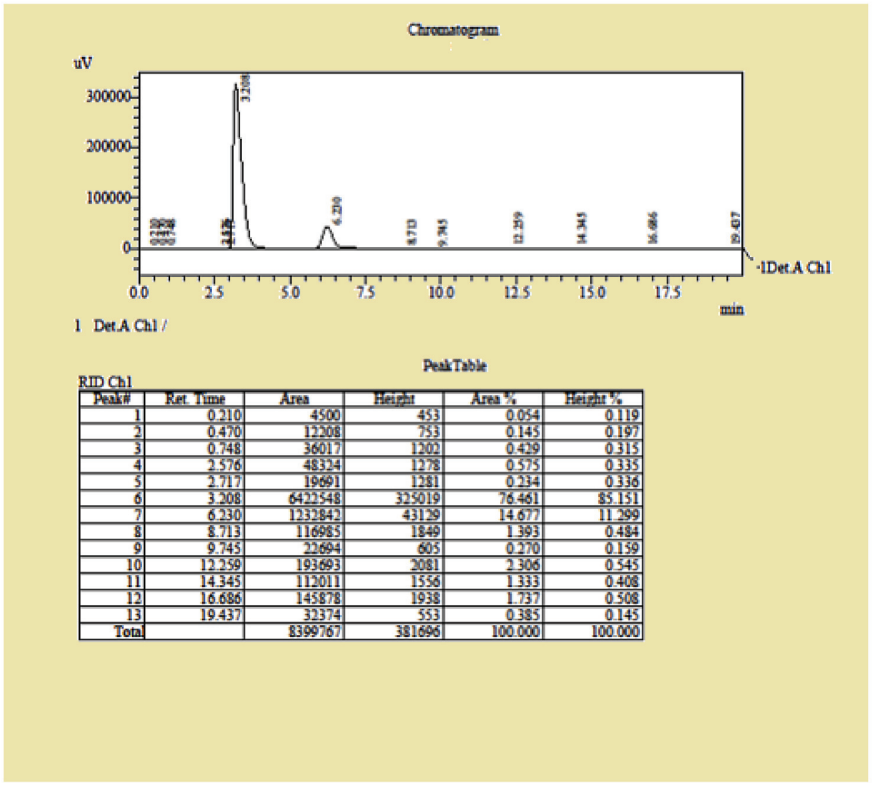


Fig 1. Different enzymes secreted by *R. oryzae* in presence of inducer salicin and CMC as substrate

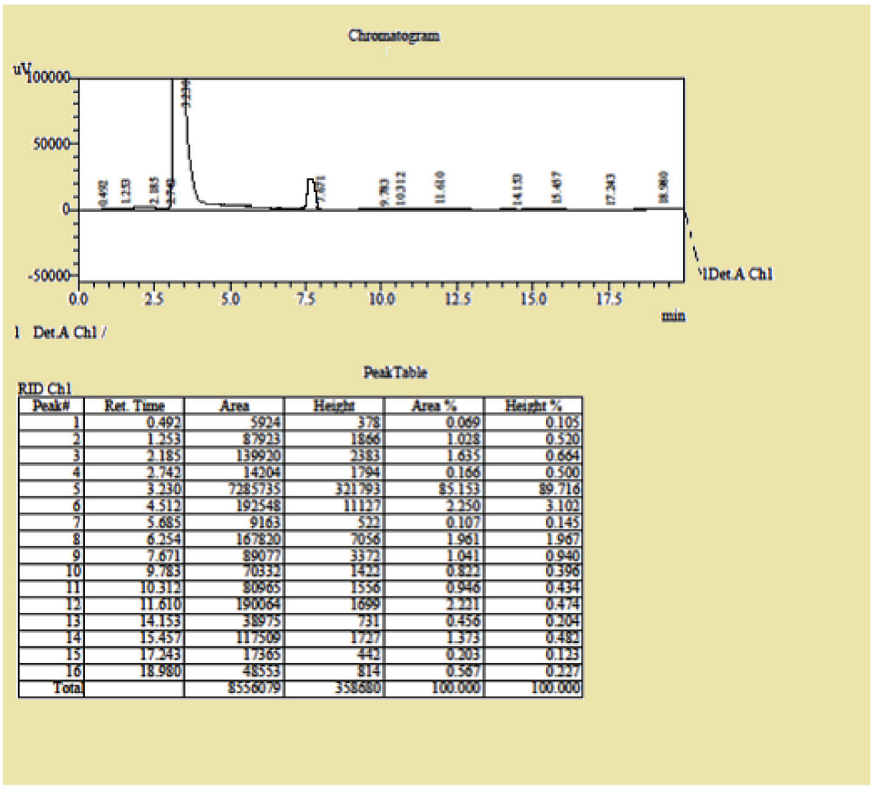


PeakTable

RID Ch1

Peak#	Ret. Time	Area	Height	Area %	Height %
1	0.210	4500	453	0.054	0.119
2	0.470	12208	753	0.145	0.197
3	0.748	36017	1202	0.429	0.315
4	2.576	48324	1278	0.575	0.335
5	2.717	19691	1281	0.234	0.336
6	3.208	6422548	325019	76.461	85.151
7	6.230	1232842	43129	14.677	11.299
8	8.713	116985	1849	1.393	0.484
9	9.745	22694	605	0.270	0.159
10	12.259	193693	2081	2.306	0.545
11	14.345	112011	1556	1.333	0.408
12	16.686	145878	1938	1.737	0.508
13	19.437	32374	553	0.385	0.145
Total		8399767	381696	100.000	100.000

Fig. 2. Glucose as standard in HPLC

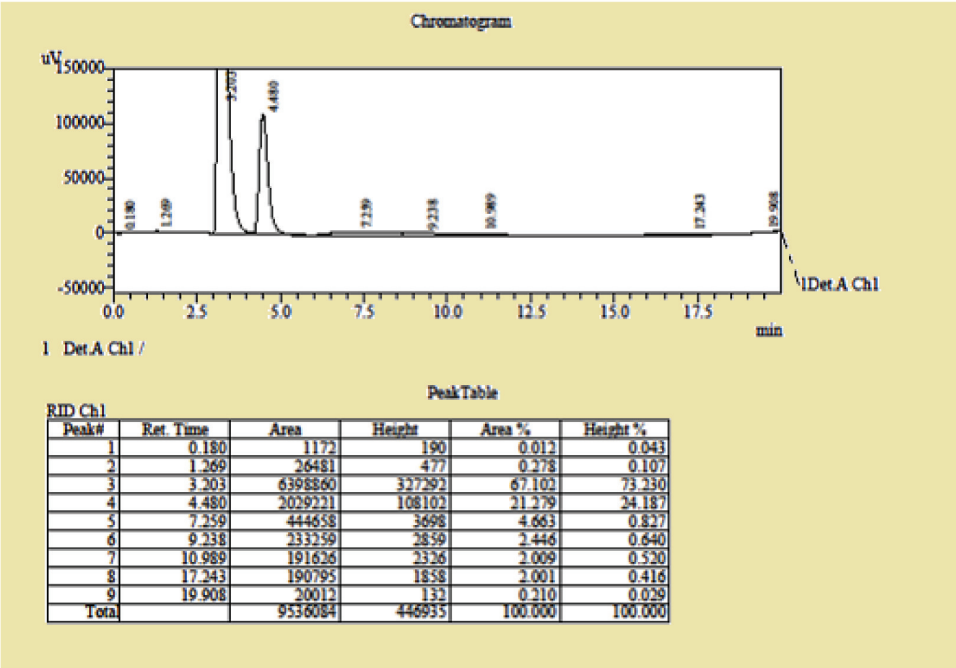


PeakTable

RID Ch1

Peak#	Ret. Time	Area	Height	Area %	Height %
1	0.492	5924	378	0.069	0.105
2	1.253	87923	1866	1.028	0.520
3	2.185	139920	2383	1.635	0.664
4	2.742	14204	1794	0.166	0.500
5	3.230	7285735	321793	85.153	89.716
6	4.512	192548	11127	2.250	3.102
7	5.685	9163	522	0.107	0.145
8	6.254	167820	7056	1.961	1.967
9	7.671	89077	3372	1.041	0.940
10	9.783	70332	1422	0.822	0.396
11	10.312	80965	1556	0.946	0.434
12	11.610	190064	1699	2.221	0.474
13	14.153	38975	731	0.456	0.204
14	15.457	117509	1727	1.373	0.482
15	17.243	17365	442	0.203	0.123
16	18.980	48553	814	0.567	0.227
Total		8556079	358680	100.000	100.000

Fig. 3. Cellobiose as standard in HPLC

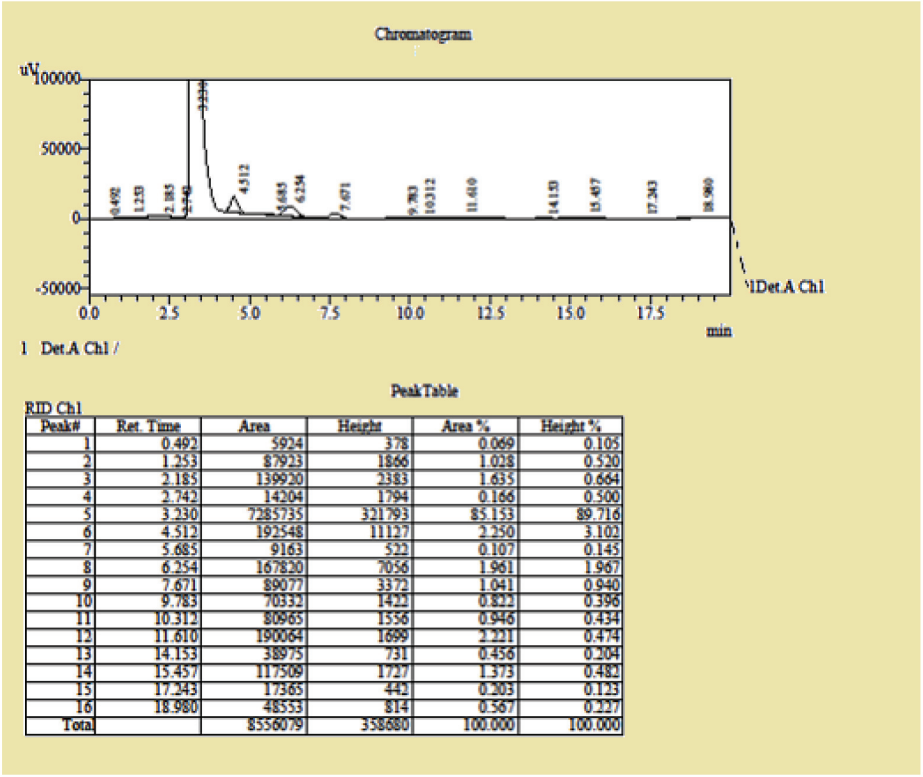


PeakTable

RID Ch1

Peak#	Ret. Time	Area	Height	Area %	Height %
1	0.180	1172	190	0.012	0.043
2	1.269	26481	477	0.278	0.107
3	3.203	6398860	327292	67.102	73.230
4	4.480	2029221	108102	21.279	24.187
5	7.259	444658	3698	4.663	0.827
6	9.238	233259	2859	2.446	0.640
7	10.989	191626	2326	2.009	0.520
8	17.243	190795	1858	2.001	0.416
9	19.908	20012	132	0.210	0.029
Total		9536084	446935	100.000	100.000

Fig. 4. Salicin as standard in HPLC



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Total		8556079	358680	100.000	100.000

Fig 5. Test sample of saccharification subjected to HPLC

CONCLUSION

The production of glucose as the major end product by the multienzyme cellulose complex of *R. oryzae* was confirmed by analyzing the end product of hydrolysis of cellulosic substrate (dried grass). Glucose thus produced may be used for production of other value added materials including alcohol (Bayer *et al,* 2007, Virendra and Ghose, 1981).Hence it might be considered to be a convenient and cost efficient way to produce glucose and other materials from agrowastes like dried grass using cellulose of *Rhizopus oryzae.*

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