Chapter 4

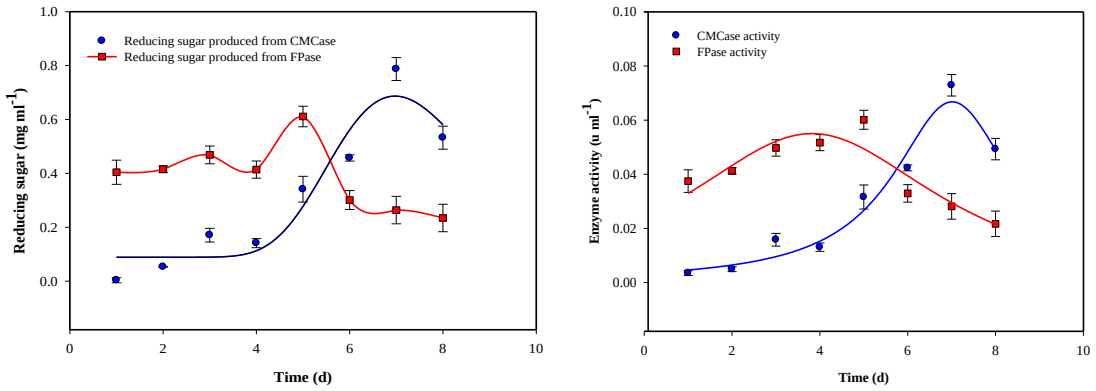
Results and discussion

4.1. Fungal cellulase production in modified media

In this part of the work, the production of cellulase from *P. sanguineus* in formulated liquid media was investigated. The cost-effectiveness and productivity of the medium composition were also compared. The goal of study is to explore a cost effective medium formulation enzyme production using *P. Sanguineus*.

Eight different media compositions were modified to study the production of cellulase enzyme by the fungus in shake flasks (Jaradat *et al*., 2008). The reducing sugars production by *P. sanguineus* from two different cellulolytic activities as a function of time (7 days) is shown in Figures 4.1 to 4.4 when the major carbon source is cellulose and in Figure 4.6 to 4.9 when CMC was a major carbon source. All the values presented in graphs were an average of triplicate measurements.

The cellulase enzyme produced is a mixture of crude enzyme from *P. sanguineus.* Hence, for determination of CMCase and FPase activities, the supernatant of each medium was added to different substrates i.e. Carboxymethyl cellulose for CMCase activity and filter paper for FPase activity measurements (3.4.2 and 3.4.3). The reducing sugars liberated by the respective enzyme reactions will be used as an indirect measure of a particular enzyme activity.



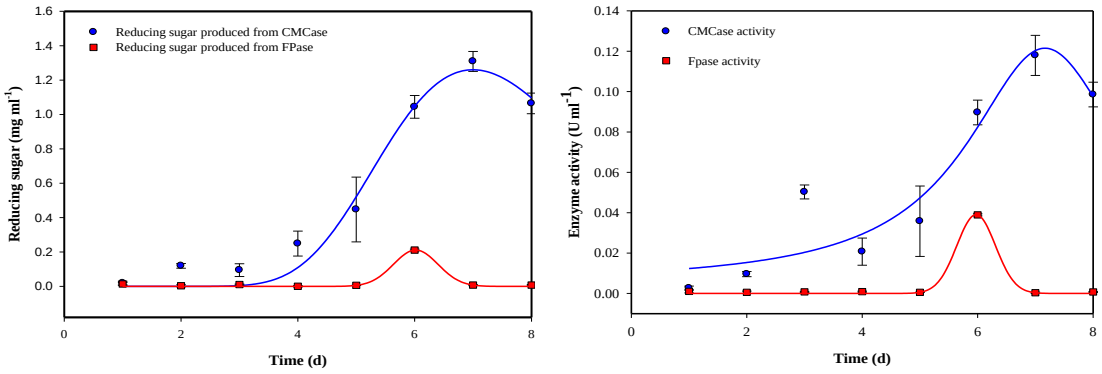
(a) (b)

Figure 4.1. (a) Reducing sugars production and (b) enzyme activities from CMCase and FPase when 20 g L^{-1} **cellulose** powder was used as the major source of carbon.

Cellulose powder has a strong crystalline structure. The digestibility of this form of polymer is lower compared to its digestibility in the amorphous form (Atlas 2004). At the initial stage the rate of digestibility of cellulose powder by cellulase enzyme complex was quite slow as reflected by low presence of reducing sugars in the medium. The presence of reducing sugars in the liquid medium and its evolution was used as an indirect representative of fungus's growth. The slow growth of the fungus was attributed to the relatively insoluble cellulose polymer as growth substrate.

In Figure 4.1.b, lower activity was obtained for FPase activity at high concentration of cellulose in the medium (20 g L^{-1}). As explained in section 2.1.3, it is likely that this is a result of sequential, cooperative action between the three enzyme components in a complex in which the product of one enzyme reaction develops into the substrate for another when the substrate has crystalline region like filter paper, suggesting some synergetic effects as the enzyme activity was lower than that obtained with the assay using cellulose. The same result has been reported according to Ahamed *et al*. (2008) with the fungus had been *Trichoderma reesei* RUT-C30. This may be due

to the produced enzymes being adsorbed on the solid cellulose surface at a similar rate than that of cellulase synthesis (Oashima *et al*., 1990).

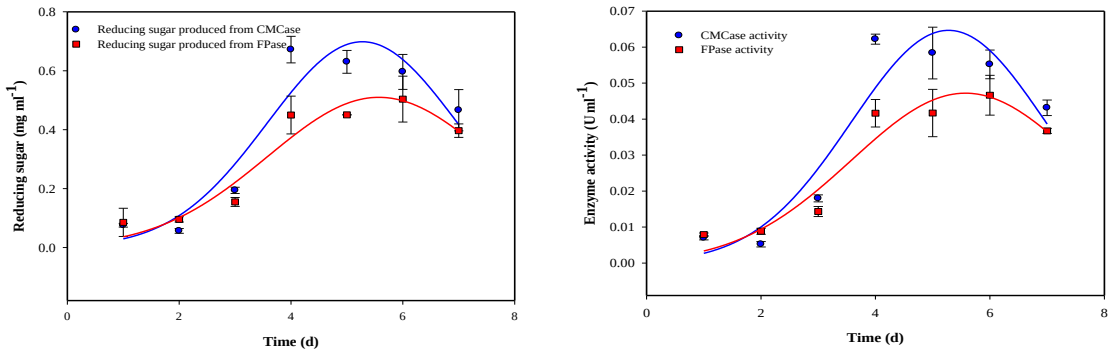


(a) (b)

Figure 4.2. (a) Reducing sugars production and (b) enzyme activities from CMCase and FPase when 20 g L^{-1} cellulose was as the major source of carbon and Tween 80 was included in the basal media.

Reducing sugars production was 1.2 g L^{-1} when Tween 80 was added to the media, which is twice the amount obtained in the media without Tween 80. The increased in activity after 6^{th} days, as shown by the increase in enzyme activities, indicated that as the cumulative growth increased with time, so was the production of cellulolytic enzyme which then hydrolysed the cellulose polymer generating shorter cellulose chains which are more soluble, thus more accessible to attacks by cellulase enzyme complex.

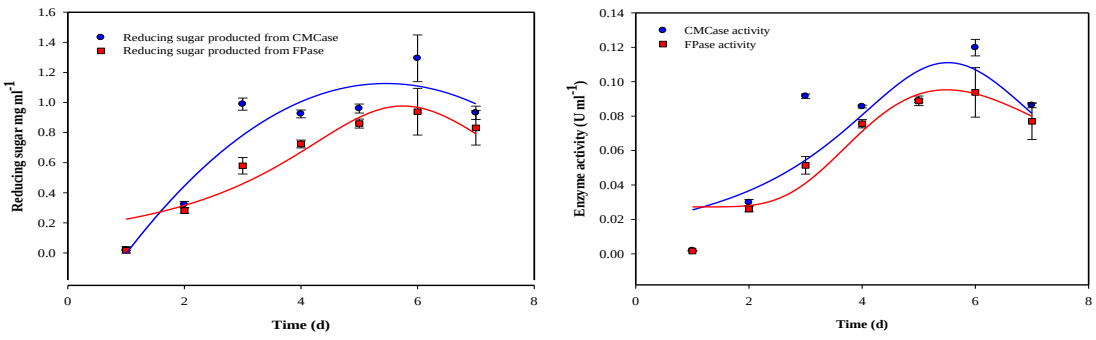
In Figure 4.3, 10 g L^{-1} cellulose is used as a main carbon source in the basal media and also yeast extract was supplemented in the media as a nutrient for the fungus.



(a) (b)

Figure 4.3. (a) Reducing sugars production and (b) enzyme activities from CMCase and FPase when 10 g L^{-1} **cellulose** was as the major source of carbon and 10 g L^{-1} **yeast extract** supplementation.

The amount of cellulose powder was used half as compared to those media shown in Figure 4.1 and 4.2 with 10 g L^{-1} of yeast extract added to the basal media. As shown in the graph 4.3.b, enzyme activities level was comparable to Figure 4.1, which was approximately 0.6 g L^{-1} in both media, with yeast extract and without it. Suggesting that the fungus was using cellulose powder for growth and enzyme production during cultivation period and yeast extract supplementation had little significant effect on enzyme activities level.



(a) (b)

Figure 4.4. (a) Reducing sugar production and (b) enzyme activities from CMCase and FPase when 10 g L^{-1} **cellulose**, 10 g L^{-1} **yeast extract** and **Tween 80** was included in the medium.

Similar effect was observed in the medium which incorporated cellulose powder, yeast extract and Tween 80 (Fig. 4.4). It is clear that the additional of yeast extract had little influence on the enzyme activities level as similar level of reducing sugars production (~ 1.2 g L^{-1}) was obtained in medium containing cellulose and Tween 80 only (Fig. 4.2). This suggests that the fungus is using cellulose powder for growth and enzyme production during cultivation period and yeast extract supplementation had little significant effect on enhancing enzyme activities level. On the other hand, the data reported by Ahamed *et al*., (2008) shows the additional yeast increase cellolase production in *T. Reesei*. Overall comparison for reducing sugars production among the cellulose media tested is shown in Fig. 4.5.

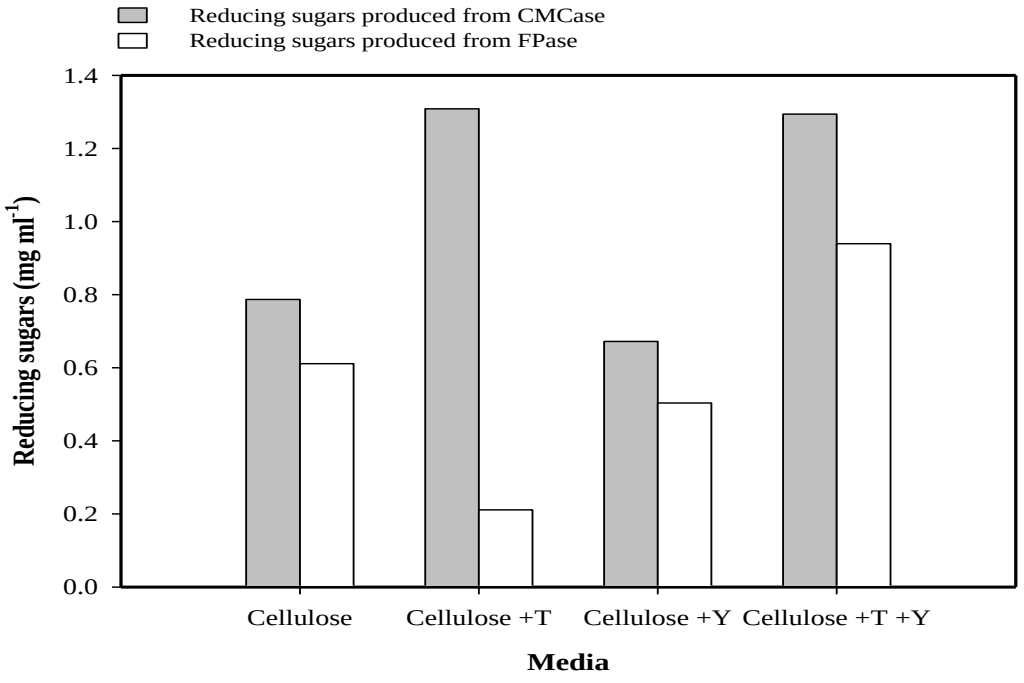
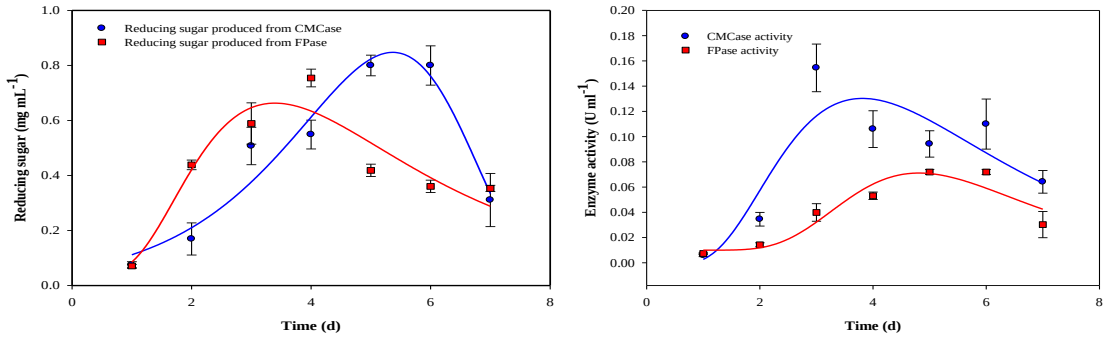


Figure 4.5. The reducing sugars production in different media composition with cellulose as a major carbon source. T: Tween 80, Y: yeast extract.

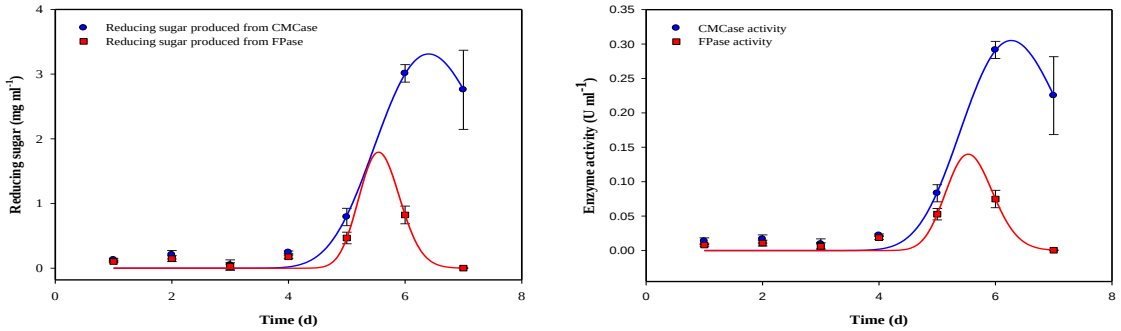
Comparison study was made between CMC and cellulose powder as a major carbon source where enzyme activities were investigated along with the effect of Tween 80 and yeast extract in the CMC media.



(a) (b)

Figure 4.6.(a) Reducing sugars production and (b) enzyme activities from CMCase and FPase when 20 g L^{-1} **CMC** was the major source of carbon in the media.

As shown in Figure 4.6, the CMCase activity level is higher than FPase activity and this is due to crystalline structure of filter paper that its utilization requires cooperative action between the three enzyme components in a cellulase complex in which the product of one enzyme reaction becomes the substrate for another. In this experiment, the amount of CMC is 20 g L^{-1}, at the same level of cellulose mass when it was used as a source of carbon.

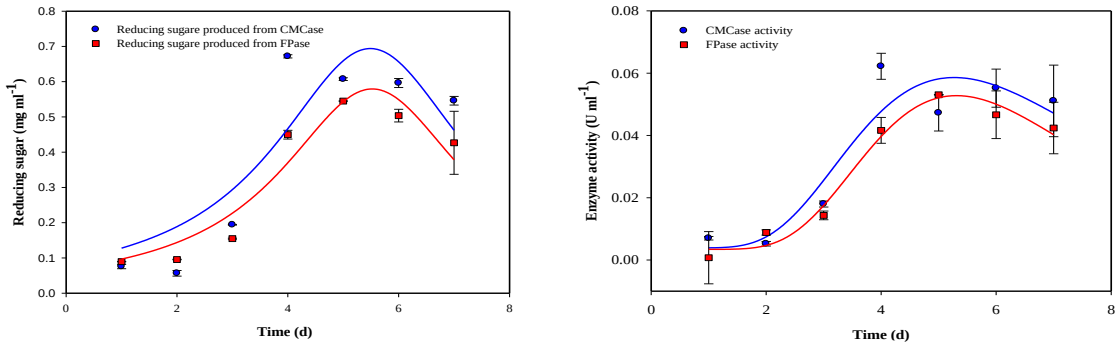


(a) (b)

Figure 4.7 .(a) Reducing sugars production and (b) enzyme activities from CMCase and Fpase when 20 g L^{-1} **CMC** was the major source of carbon and **Tween 80** was included in the basal media.

The highest enzyme activities in this study were obtained using CMC and Tween 80. CMC is an amorphous derivative of cellulose and Tween 80 is a strong detergent which helps amorphous group solubilisation. The enzyme activities were significantly increased in both cellulose-Tween 80 and CMC-Tween 80 media (Fig. 4.2, 4.7). This may arisen from the strong effect of Tween 80 as a powerful detergent, which allows higher solubilisation of polymeric carbon source for fungal consumption. The produced enzymes then quickly convert the amorphous structure of available cellulose into monomers hence the enzyme activity level was significantly higher than that of the cellulose or CMC without Tween 80 medium.

It has been reported that the addition of Tween 80 in the medium improved the cellulase yield in *Trichoderma* strains. The mechanism behind this increased yield, may be related to the improved permeability of the cell membrane, allowing more quick secretion of enzymes, which in turn leads to higher enzyme synthesis (Reese and Maguire 1977).

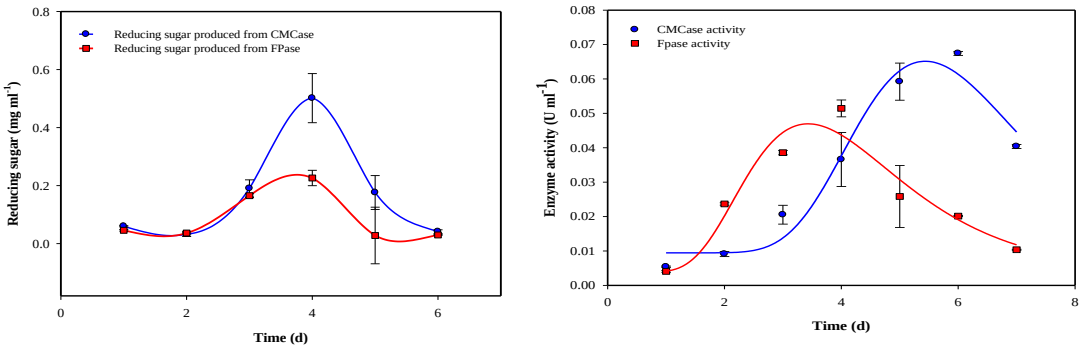


(a) (b)

Figure 4.8.(a) Reducing sugar production and (b) enzyme activities from CMCase and FPase when 10 g L^{-1} **CMC**, 10 g L^{-1} **yeast extract** was included in the medium.

In cellulose–yeast extract and CMC-yeast extract medium, the concentration of both cellulose and CMC was 10 g L^{-1}, as compared to 20 g L^{-1} of Cellulose and CMC, respectively, in cellulose and CMC medium. Similar enzyme activities in culture supernatant of the cellulose–yeast extract medium and CMC-yeast extract (Fig. 4.3 and Fig. 4.8) compared to those found in the cellulose and CMC media (Fig 4.1 and 4.6) indicated that yeast extract did not play a major role in enhancing growth and enzyme (s) production.

According to Ahamed *et al.* 2008 the addition of yeast in medium can induce enzyme production. It has been previously reported that yeast extracts stimulate both cell growth and cellulase production in *T. Reesei.*



(a) (b)

Figure 4.9. (a) Reducing sugars production and (b) enzyme activities from CMCase and Fpase when 10 g L^{-1} **CMC**, 10 g L^{-1} **yeast extract** and **Tween 80** were included in the medium.

The effect of Tween 80 and yeast extract were also studied and it showed that the enzyme activities level were similar to when CMC and yeast extract were included in the media.

Overall comparison for reducing sugars production among the CMC media tested is shown in Fig. 4.10. It can be concluded that Tween 80 supplementation to the medium had a positive effect on the enzyme activity levels.

The fungal cellulase enzyme production was compared in different media, and the cost of each medium was estimated. The highest amount of reducing sugar production was in medium containing CMC and Tween 80. This formulation was found to be the cheapest media among other formulations tested.

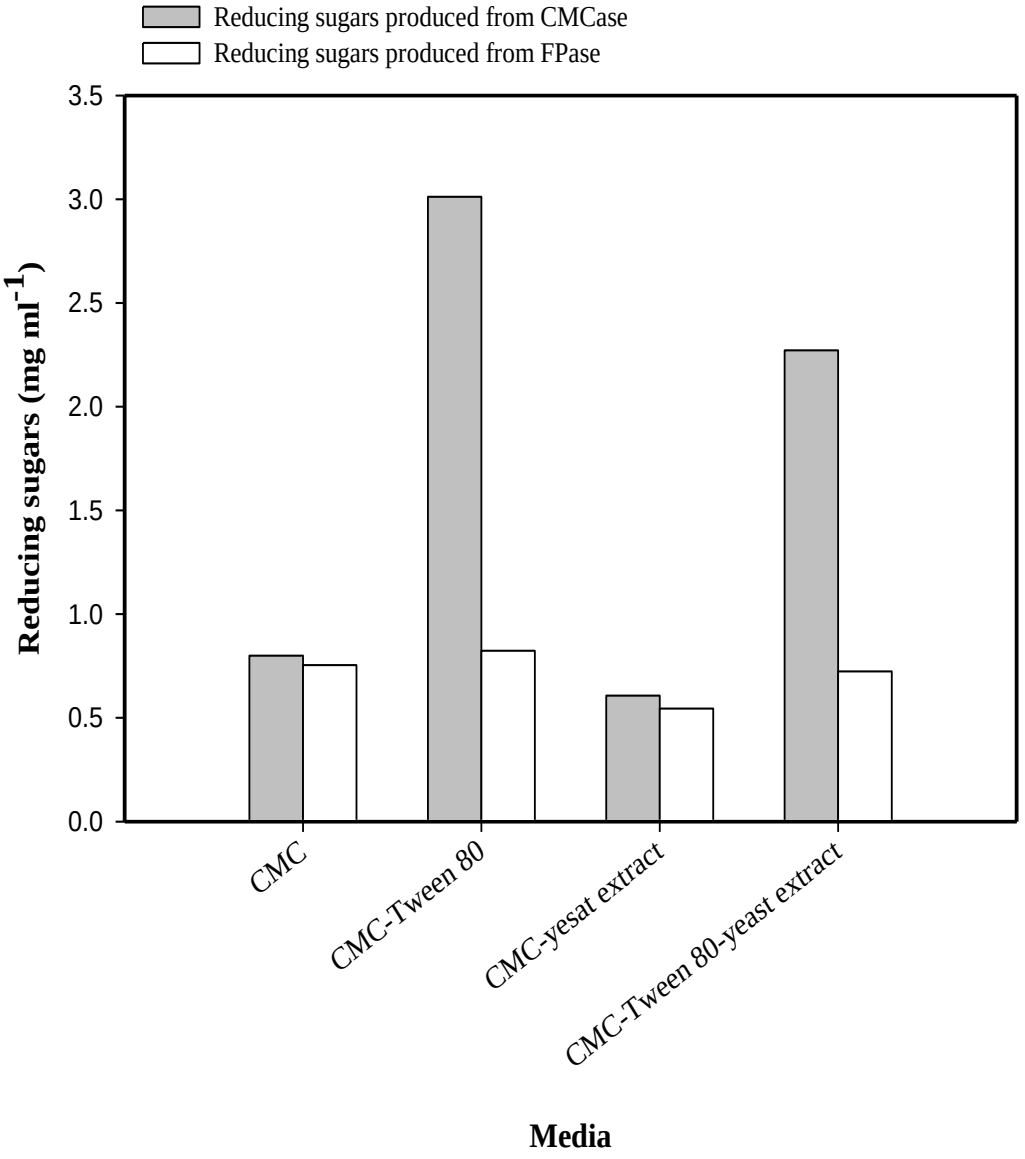


Figure 4.10. The reducing sugars production in different media composition in the presence of CMC as a major carbon source.

In all graphs, the increasing amount of reducing sugars production from CMCase was more than its production by FPase enzyme. This may be due to the ability of this fungus to produce endo-gluconase, which is expressed as CMCase activity, which is higher compared to its total complex enzyme production which is measured by FPase activity.

Maximum enzyme production was observed in 10 g L^{-1} CMC and 2 ml L^{-1} Tween 80 which is around 0.35 U mL^{-1} (Fig. 4.7). The amorphous structure of CMC and its interaction with Tween 80 as a detergent may help to allow better access of the cellulolytic enzyme (s) to the CMC.

Duff and Murray (1996) have reported that most of the *T. reesei* mutants produce cellulases with a specific filter paper activity between 0.5 U mL^{-1} per mg and 1 U mL^{-1} per mg of proteins and the highest reported activity was 3.6 U mL^{-1} per mg of proteins using VTT-D-79125 strai. There are no reports on the cellulase production by *Pycnoporus sanguineus* (Duff and Murray 1996).

4.2. CMCase and FPase activities in commercial cellulase preparation

In this study, commercial cellulase preparation was used in assessing the enzyme assays used in this study. The assay methods were for determining the activities of CMCase (substrate CMC) and Fpase (substrate filter paper). The use of commercial cellulase preparation also showed similar results as those cellulase preparations from fermentation broth i.e. CMCase assay resulted in higher activity than the filter paper assay. This curtailed activity shown by FPase was suggested to be due to the presence of crystalline region in the filter paper structure and insolubility of this substrate in the water (Fig. 4.11). Higher CMCase activity by commercial enzyme corroborated that the results obtained in section 4.1 which CMCase activity level was higher than FPase (Nieves *et al*., 1998).

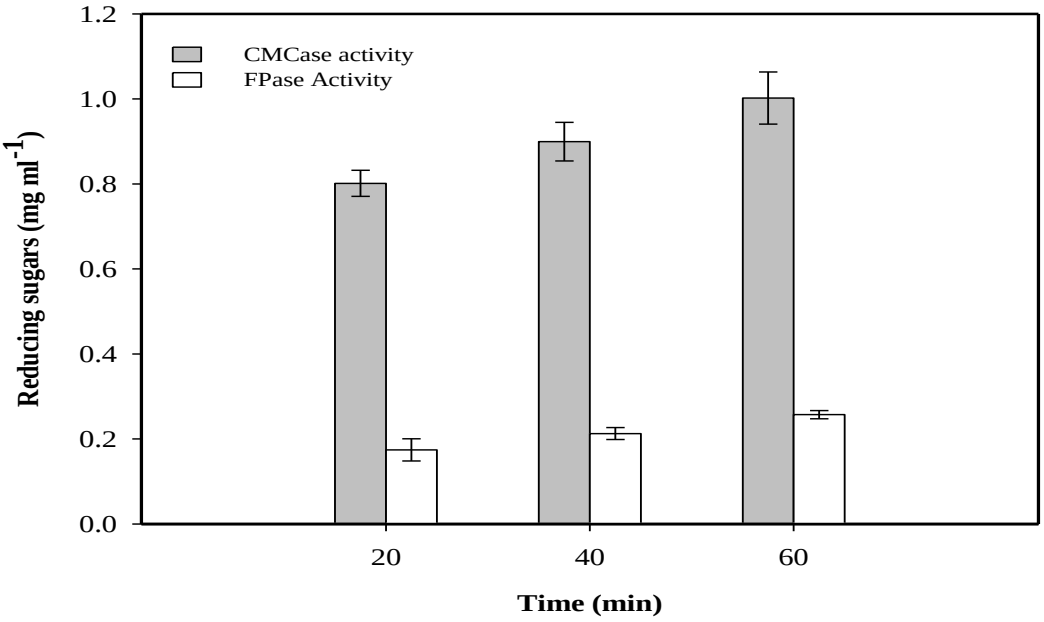


Figure 4.11. Reducing sugars production using commercial cellulase.

4.3. Comparison of costs of selected media

The costs for making 1 L of each culture medium were approximated for all tested media (Fig. 4.12). The cost estimates were based only on chemicals used to prepare the production media at laboratory-scale experiments. It is preferable that the most economical medium also exhibits highest enzyme activity level.

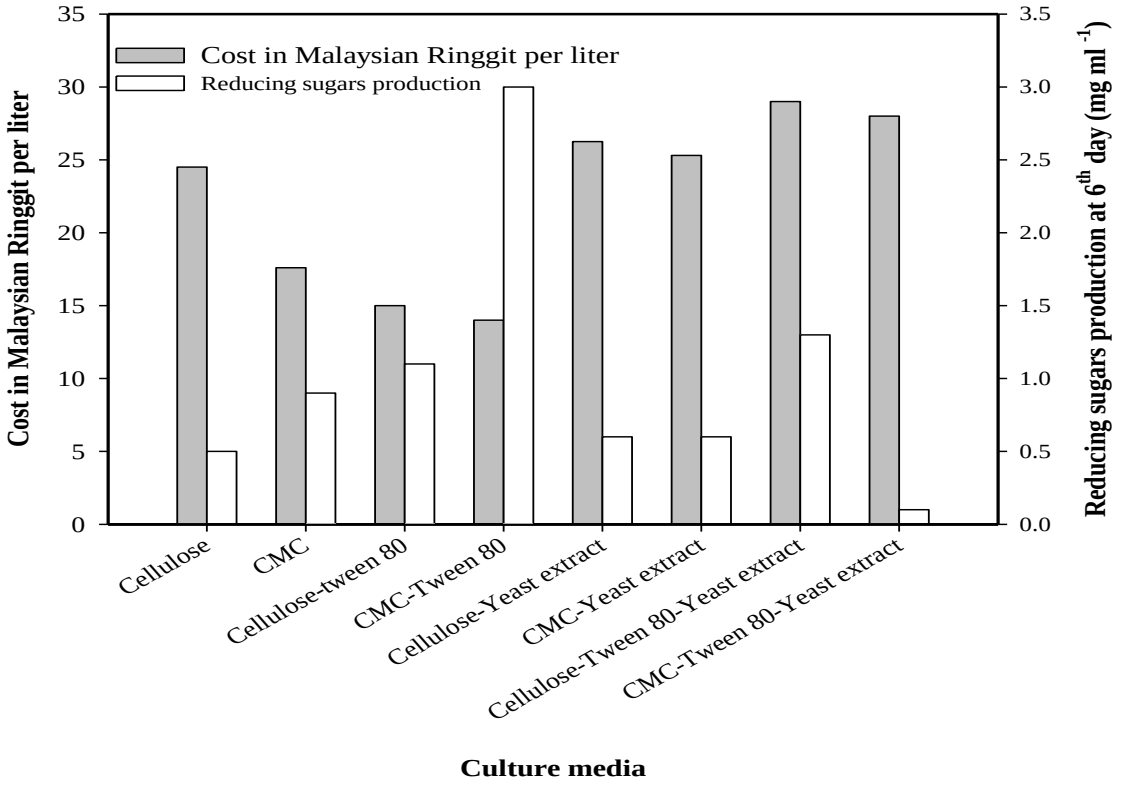


Figure 4.12. Costs in Malaysian Ringgit to produce 1 L of production media and reducing sugars production after 144 hours.

From Figure 4.12, the cost of CMC-Tween 80 medium was the lowest with highest reducing sugars production (i.e. indicative of high cellulase activities) when compared with the other tested media.

4.4. Mathematical modelling part

The second part of the study was to investigate the cellulase enzyme activities in different CMC concentrations and to find a mathematical model that relates viscosity measurements as direct indication of the prevailing cellulase activities. This is based on the observation that as the cellulase activities increase, the viscosity of the medium incorporating the CMC will concomitantly decreases.

Table 4.1. The viscosity and enzyme activities when inocula were prepared in GYMP (pH= 5.5 at 25°C).

Time	C_{CMC}= 5 (g L^{-1})		C_{CMC}= 10 (g L^{-1})		C_{CMC}= 15 (g L^{-1})		C_{CMC}= 20 (g L^{-1})		C_{CMC}= 25 (g L^{-1})	
	Vis	E_a	**Vis**	E_a	**Vis**	E_a	**Vis**	E_a	**Vis**	E_a
1	5.98	-	12.26	-	28.59	-	56.01	-	69.26	-
2	5.21	0.0044	9.62	0.0006	19.17	0.0065	47.34	0.0039	48.59	0.0028
3	3.19	0.01	7.4	0.0048	13.01	0.0088	31.69	0.0048	42.16	0.0035
4	3.65	0.013	4.8	0.005	9.28	0.0071	26.21	0.0113	24.27	0.0082
5	3.46	0.015	4.34	0.021	6.22	0.017	15.11	0.061	14.73	0.014
6	3.05	0.023	3.66	0.043	5.37	0.048	5.68	0.089	8.2	0.03
7	3.01	0.023	3.77	0.057	4.29	0.067	5.26	0.065	4.71	0.062
8	3.21	0.025	3.29	0.052	2.5	0.065	4.58	0.066	3.84	0.083
9	3.16	0.04	2.79	0.068	1.8	0.125	4.37	0.092	3.71	0.108
10	2.52	0.058	2.35	0.063	1.29	0.132	3.53	0.11	3.38	0.115
11	2.1	0.061	2.8	0.07	1.81	0.135	4.06	0.133	3.07	0.159
12	1.65	0.06	2.67	0.082	1.29	0.138	2.77	0.15	3.35	0.155
13	1.68	0.067	2.75	0.12	3.01	0.14	3.43	0.138	2.04	0.161
14	1.78	0.061	1.05	0.078	1.26	0.136	2.88	0.132	1.78	0.154
15	1.26	0.057	1.51	0.091	2.82	0.133	3.36	0.147	1.23	0.151
16	1.74	0.058	1.3	0.096	2.06	0.124	2.24	0.177	1.64	0.142
17	1.08	0.13	1.35	0.11	1.95	0.129	1.98	0.2	1.32	0.115

CMC: Concentration of CMC (g L^{-1}), Ea: enzyme activity (µmol ml-1min-1), Vis: viscosity (cP).

Table 4.2. The viscosity and enzyme activities when inocula were prepared in PDA media (pH= 5.5 at 25°C).

Time	C_{CMC}= 5 (g L^{-1})		C_{CMC}= 10 (g L^{-1})		C_{CMC}= 15 (g L^{-1})		C_{CMC}= 20 (g L^{-1})		C_{CMC}= 25 (g L^{-1})	
	Vis	E_a	**Vis**	E_a	**Vis**	E_a	**Vis**	E_a	**Vis**	E_a
1	6.43	-	10.23	-	48.17	-	49.01	-	79.42	-
2	5.34	0.0083	9.81	0.015	16.92	0.0026	32.16	0.015	54.48	0.0043
3	4.2	0.0093	8.39	0.0013	14.41	0.0031	22.13	0.0098	36.41	0.004
4	3.61	0.0076	6.31	0.0082	11.62	0.0047	18.06	0.0123	30.34	0.0125
5	3.1	0.0219	5.81	0.0168	14.09	0.018	15.36	0.034	20.24	0.023
6	2.77	0.023	3.94	0.04	3.09	0.048	6.5	0.058	7.49	0.036
7	2.2	0.048	3.36	0.073	3.02	0.068	3.3	0.09	5.13	0.066
8	1.75	0.066	3.35	0.079	2.75	0.088	2.65	0.125	3.13	0.12
9	1.78	0.057	2.03	0.107	3.55	0.1	2.72	0.1	2.86	0.148
10	1.09	0.0928	2.54	0.114	2.03	0.14	3.12	0.137	2.62	0.188
11	1.16	0.0647	2.3	0.11	2.18	0.117	1.25	0.165	2.43	0.168
12	1.25	0.051	1.95	0.098	3.01	0.092	1.84	0.136	2.29	0.116
13	0.82	0.068	2.3	0.11	2.39	0.088	2.31	0.13	1.46	0.104
14	1.74	0.116	1.35	0.067	2.42	0.081	2.82	0.139	1.68	0.151
15	1.14	0.082	1.35	0.059	2.75	0.09	1.07	0.134	1.6	0.13
16	0.85	0.093	2.38	0.088	1.84	0.05	1.32	0.124	1.3	0.154
17	1.11	0.1	1.64	0.058	1.36	0.045	1.63	0.09	1.24	0.118

C_{CMC}: Concentration of CMC (g L^{-1}), Ea: enzyme activity (µmol ml-1min-1), Vis: viscosity (cP).

Table 4.1 and 4.2 show that the viscosities and enzyme production data during 8 days cultivation when two initial cultures from two different inoculation media were used. It is shown that viscosity of the liquid medium decreased with the increase in enzyme activities. From the experimental results, a nonlinear modelling was carried out to evaluate a correlation between viscosity and enzyme activity in the two media. In this

study, experimental data was evaluated by DataFit scientific software (version 8.2 by Oakdale Engineering, USA). The nonlinear regression was conducted based on the Levenberg-Marquardt method with double precision. In training of the proposed models, various forms of input variables were tested to obtain the highest correlation between the experimental data and predicted values. Therefore, *t*-ratios and the corresponding *p* values were determined to evaluate the significance of the regression coefficient. Furthermore, the descriptive statistics of the residual errors were also provided to evaluate the model performance.

In the nonlinear modelling study, 240 different mathematical models were solved and automatically sorted according to the goodness-of-fit criteria (section 2.2.3) for each media. According to these criteria, the best fit was discriminated in the mathematical function for each media concentration, and the fit information is summarized for each CMC concentration in Table 4.3. The proposed model for media with GYMP inocula is defined as a function of two operating variables [Enzyme activities = *f* (viscosity, time)] is given in following equation:

$$y = a + bx_1 + cx_1^2 + dx_1^3 + ex_1^4 + fx_1^5 + \frac{g}{x_2} + \frac{h}{x_2^2} + \frac{i}{x_2^3} + \frac{j}{x_2^4} + \frac{k}{x_2^5} \qquad \text{Eq. (4.1)}$$

where y is enzyme activity rate (U ml^{-1}), x_1 is the fermentation time (12 hours interval) and x_2 is viscosity (cP).

The same model form was proposed for all concentrations of media tested with different coefficients. Summary of regression results for the equation for different CMC concentrations is given below (Table 4.3):

Table 4.3. Summary of regression results for the best fit modelling study for media with GYMP inocula.

Concentration ($g\ L^{-1}$)	SEE	SR	AR	RSS	R^2	R_a^2
5	5.3×10^{-3}	6.43×10^{-12}	3.781×10^{-13}	1.68×10^{-4}	0.999	0.973
10	5.82×10^{-3}	-2.94×10^{-12}	-1.73×10^{-13}	2.035×10^{-4}	0.999	0.976
15	9.66×10^{-3}	-6.47×10^{-12}	-3.8×10^{-13}	5.601×10^{-4}	0.989	0.979
20	9.83×10^{-3}	-8.08×10^{-11}	-4.75×10^{-12}	9.839×10^{-3}	0.99	0.975
25	7.26×10^{-3}	1.28×10^{-11}	7.57×10^{-13}	3.163×10^{-4}	0.995	0.987

SEE, standard error of the estimate; SR, sum of residual; AR, average residual; RSS, residual sum of squares; R^2, coefficient of multiple determinations; R_a^2, adjust coefficient of multiple determination.

According to the results obtained from the nonlinear study, a proposed function giving the highest correlation coefficient, R^2 was selected as the best-fit equation for the prediction of enzyme activities (Eq. 4.1).

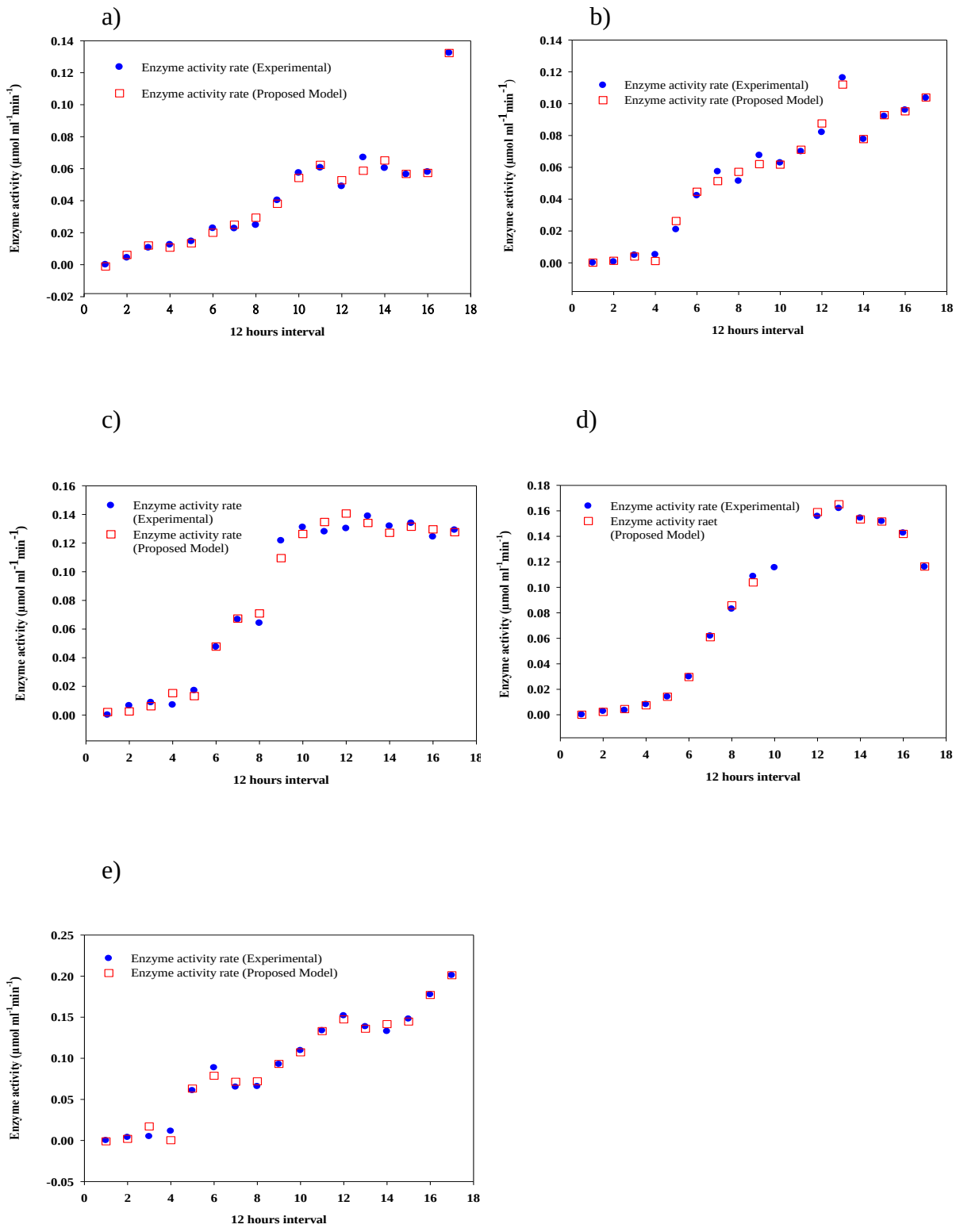


Figure 4.133. Agreement between the proposed model outputs and experimental data (**GYMP** inocula, a) **5** g L^{-1}, b) **10** g L^{-1}, c) **15** g L^{-1}, d) **20** g L^{-1}, d) **25** g L^{-1} CMC concentration).

Figure 4.13 (a) to (e) depict the agreement between the mathematical model outputs and the experimental data. To evaluate the model performance for each medium concentration, the coefficients were tested at 99% (Table 4.4 to 4.8). Other percentages of fit (68% to 95%) were also tested and the coefficients are given in Appendix A.

Table 4.4. 99% Confidence Intervals, GYMP inocula, 5 g L^{-1} CMC concentration.

Variable	Value	99% (+/-)	Lower Limit	Upper Limit
A	0.21488355816	0.845052875390	-0.63016931723	1.059936433550
B	$3.92971279805\times10^{-2}$	0.117665846527	$-7.836871854\times10^{-2}$	0.156962974508
C	$-1.1207328580\times10^{-2}$	$3.0812179721\times10^{-2}$	$-4.201950830\times10^{-2}$	$1.9604851141\times10^{-2}$
D	$1.48170182636\times10^{-3}$	$3.8814014745\times10^{-3}$	$-2.399699648\times10^{-3}$	$5.3631033009\times10^{-3}$
E	$-8.3480112102\times10^{-5}$	$2.3227964216\times10^{-4}$	$-3.157597542\times10^{-4}$	$1.4879953006\times10^{-4}$
F	$1.66170347065\times10^{-6}$	$5.2656623320\times10^{-6}$	$-3.603958861\times10^{-6}$	$6.9273658026\times10^{-6}$
G	-3.042507373591	11.06032506933	-14.1028324429	8.017817695746
H	12.932777950975	46.50852144324	-33.5757434922	59.44129939421
I	-24.18688615476	91.67190842813	-115.858794582	67.48502227336
J	19.815901585351	85.48156791882	-65.6656663334	105.2974695041
K	-5.603961391322	30.41661543837	-36.0205768297	24.81265404705

Table 4.5. 99% Confidence Intervals, GYMP inocula, 10 g L^{-1} CMC concentration.

Variable	Value	90% (+/-)	Lower Limit	Upper Limit
A	0.25845147045842	0.15139859534392	0.107052875114505	0.409850065802351
B	$2.273205321686\times10^{-2}$	$6.1740002226\times10^{-2}$	$-3.90079490098\times10^{-6}$	$8.44720554435\times10^{-2}$
C	$1.343478832927\times10^{-2}$	$1.35391398234\times10^{-2}$	$-1.04351494167\times10^{-4}$	$2.69739281527\times10^{-2}$
D	$-3.11565405382\times10^{-3}$	$1.7199258532\times10^{-3}$	$-4.8355799070\times10^{-3}$	$-1.39572820056\times10^{-3}$
E	$2.322481384460\times10^{-4}$	$1.1040398173\times10^{-4}$	$1.21844156709\times10^{-4}$	$3.42652120182\times10^{-4}$
F	$-5.63393037042\times10^{-6}$	$2.5839752176\times10^{-6}$	$-8.2179055880\times10^{-4}$	$-3.0499551527\times10^{-6}$

G	-5.2007018001441	3.5556131501782	-8.7563149503420	-1.645088649946
H	23.538631386817	17.3022637262957	6.23636766052172	40.840895113113
I	-47.68975975350	37.2165392942463	-84.906299047747	-10.47322045925
J	3.0753037590436	36.4002696911109	6.6750340679327	79.475573450154
Kp	-14.1845056465	13.2711965243889	-27.4557021708944	-0.913309122116

Table 4.6. 99% Confidence Intervals, GYMP inocula, 15 g L^{-1} CMC concentration.

Variable	Value	99% (+/-)	Lower Limit	Upper Limit
A	0.1025691328677	0.20310620706607	-0.1005370741983	0.305675339933772
B	$7.8190194420\times10^{-3}$	0.15509531352876	-0.1472762940867	0.162914332970806
C	1.974840871×10^{-2}	$6.0512027011\times10^{-2}$	$-4.076361837\times10^{-2}$	$8.02604357212\times10^{-2}$
D	$-2.831759184\times10^{-3}$	$8.7486418844\times10^{-3}$	$-1.1580401069\times10^{-2}$	$5.916882699877\times10^{-3}$
E	1.503796891×10^{-4}	$5.1650280008\times10^{-4}$	$-3.6612311097\times10^{-4}$	$6.668824891901\times10^{-4}$
F	$-2.843834076\times10^{-6}$	$1.09762358369\times10^{-5}$	$-1.3820069912\times10^{-5}$	$8.132401760862\times10^{-6}$
G	-4.139675061312	8.53896723036981	-12.678642291682	4.39929216905743
H	17.164260970579	42.4118638761597	-25.247602905583	59.5761248467394
I	-38.19495817791	116.513008971325	-154.70796714923	78.3180507934138
J	44.17599099998	155.282126726103	-111.106135726123	199.458117726084
K	-20.29864273787	77.0714999001104	-97.3701426379845	56.7728571622363

Table 4.7. 99% Confidence Intervals, GYMP inocula, 20 g L^{-1} CMC concentration.

Variable	Value	99% (+/-)	Lower Limit	Upper Limit
A	-0.195967998705784	0.212926714707484	-0.408894713413268	0.0169587160017
B	0.097589834945159	0.1751453258648	$-7.75554909196\times10^{-2}$	0.272735160809959
C	-0.059366937822175	$7.72762659269\times10^{-2}$	-0.13664320374913	$1.79093281047\times10^{-2}$
D	$1.00976794424\times10^{-2}$	$1.21347769432\times10^{-2}$	$-2.03709750076\times10^{-3}$	$2.22324563856\times10^{-2}$
E	$-6.7993704006\times10^{-4}$	$8.08411223855\times10^{-4}$	$-1.48834826392\times10^{-3}$	$1.28474183786\times10^{-4}$
F	$1.60913142649\times10^{-5}$	$1.94658505382\times10^{-5}$	$-3.37453627327\times10^{-6}$	$3.55571648032\times10^{-5}$

G	9.50261695585926	8.44837895380627	1.05423800205299	17.9509959096655
H	-74.5261128987237	74.0104789650578	-148.536591863781	-0.515633933665924
I	248.787204221237	293.048631318461	-44.2614270972242	541.835835539698
J	-365.15263433979	535.560256765751	-900.712891105541	170.407622425961
K	187.009298188757	364.67266935097	-177.663371162213	551.681967539727

Table 4.8. 99% Confidence Intervals, GYMP inocula, 20 g L^{-1} CMC concentration.

Variable	Value	99% (+/-)	Lower Limit	Upper Limit
A	$-3.8326930553\times10^{-3}$	0.12650689311311	-0.130339586168148	0.122674200058073
B	$8.1279232575\times10^{-3}$	0.121851621351852	-0.113723698094257	0.129979544609447
C	$-3.00704464063\times10^{-3}$	$4.89885034898\times10^{-2}$	$-5.19955481304\times10^{-2}$	$4.5981458849\times10^{-2}$
D	$6.047679012666\times10^{-4}$	$6.83757664127\times10^{-3}$	$-6.23280874001\times10^{-3}$	$7.4423445425\times10^{-3}$
E	$-3.61771702459\times10^{-5}$	$4.11100177757\times10^{-4}$	$-4.47277348003\times10^{-4}$	$3.7492300751\times10^{-4}$
F	$6.521991517601\times10^{-7}$	$9.01818609418\times10^{-6}$	$-8.36598694242\times10^{-6}$	$9.6703852459\times10^{-6}$
G	-0.150564632032693	3.31587091202226	-3.46643554405495	3.16530627998956
H	1.86103251910994	21.2083512617351	-19.3473187426252	23.0693837808451
I	-2.61317968142973	66.003442567459	-68.6166222488887	63.3902628860292
J	-2.42795095443168	95.5188248503896	-97.9467758048213	93.0908738959579
K	3.83606876158159	50.1261769517205	-46.2901081901389	53.9622457133021

Nonlinear modelling study was also perform to determine a goodness-of-fit for media with PDA inocula. Hence, 240 different mathematical models were solved and automatically sorted according to the goodness-of-fit criteria (section 2.2.3).

The proposed model for media with PDA inocula is defined as a function of two operating variables [Enzyme activities = *f* (viscosity, time)]:

$$y = a + b\ln(x_1) + \frac{c}{x_2} + d\ln(x_1)^2 + \frac{e}{x_2^2} + f\ln\frac{(x_1)}{x_2} + g\ln(x_1)^3 + \frac{h}{x_2^3} + i\ln\frac{(x_1)}{x_2^2} + j\ln\frac{(x_1)^2}{x_2} \quad \text{Eq. (4.2)}$$

where y is enzyme activity rate (U ml^{-1}), x_1 is the fermentation time (12 hours interval) and x_2 is viscosity (cP). Fit information for each concentration is summarized in Table 4.9.

Table 4.9. Summary of regression results for the best fit modelling study for media with PDA inocula.

Concentration ($g\ L^{-1}$)	SEE	SR	AR	RSS	R^2	R_a^2
5	5.55×10^{-3}	1.396×10^{-12}	8.213×10^{-14}	2.162×10^{-4}	0.989	0.977
10	0.00695	-4.33×10^{-13}	-2.54×10^{-14}	3.389×10^{-4}	0.986	0.970
15	7.54×10^{-3}	-3.66×10^{-12}	-2.15×10^{-13}	3.982×10^{-4}	0.987	0.970
20	8.164×10^{-3}	-1.36×10^{-11}	-8.01×10^{-13}	4.665×10^{-4}	0.99	0.978
25	1.355×10^{-2}	-1.02×10^{-10}	-6.05×10^{-13}	1.286×10^{-3}	0.981	0.957

SEE, standard error of the estimate; SR, sum of residual; AR, average residual; RSS, residual sum of squares; R^2, coefficient of multiple determinations; R_a^2, adjust coefficient of multiple determination.

According to the results, the selected mathematical function (Eq. 4.2) proposed the highest correlation coefficient, R^2 as the best-fit equation for the prediction of enzyme activities. The results are shown in Figure 4.14 (a) to (e).

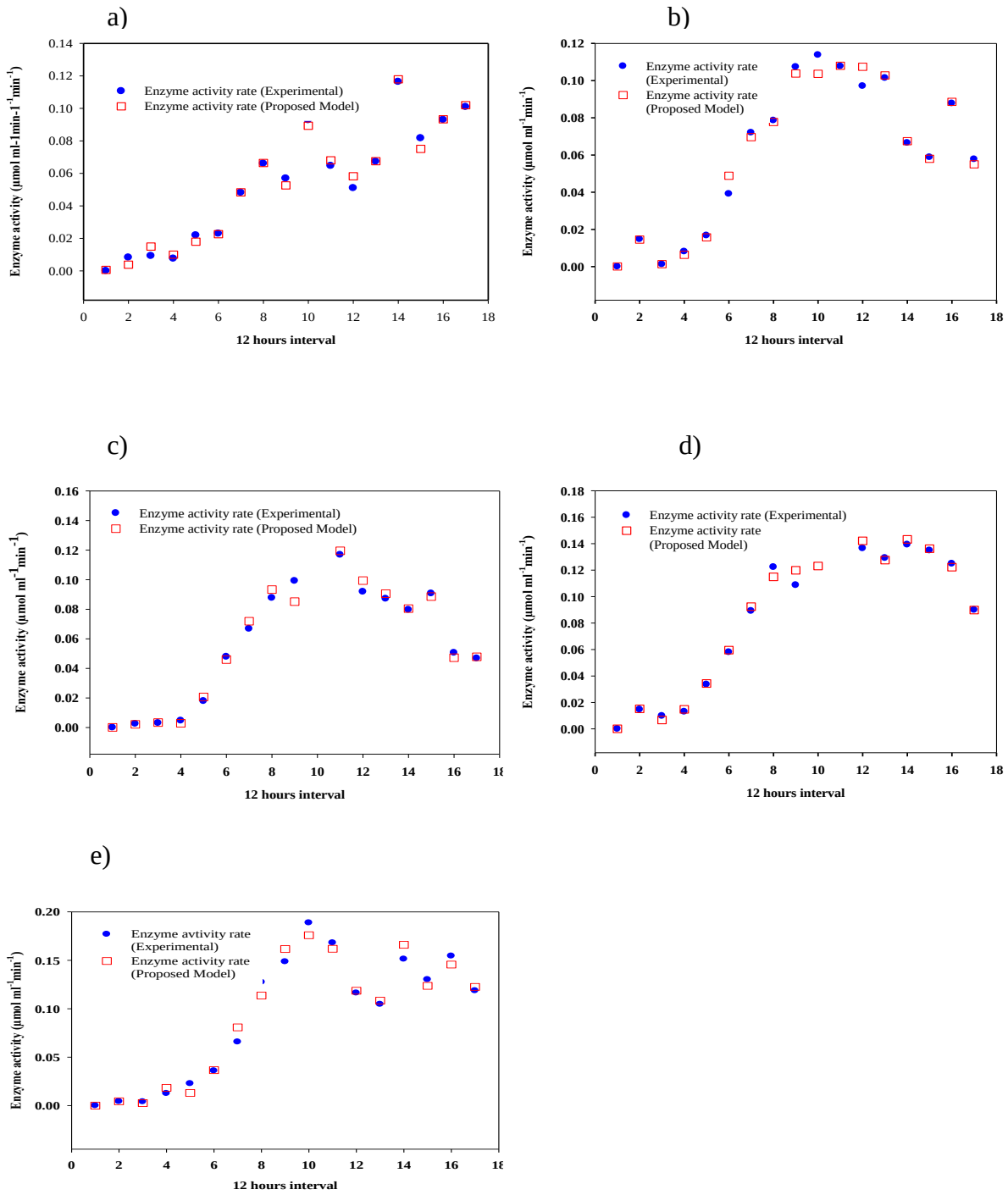


Figure 4.145. Agreement between the proposed model outputs and experimental data **PDA** inocula, a) **5** g L^{-1}, b) **10** g L^{-1}, c) **15** g L^{-1}, d) **20** g L^{-1}, d) **25** g L^{-1} CMC concentration.

All media with different CMC concentrations were analysed using this model while the coefficients of each medium concentration was tested in four percentages with 99% confidence intervals are shown below and other percentages (68% to 95%) are shown in Appendix A.

Table 4.10. 99% Confidence Intervals, PDA inocula, 5 g L^{-1} CMC concentration.

Variable	Value	99% (+/-)	Lower Limit	Upper Limit
A	-0.54906152000312	0.31244007292304	-0.86150159292617	-0.236621447080078
B	0.22086601023693	0.15599836614016	$6.48676440967\times10^{-2}$	0.376864376377093
C	3.78111904405442	2.07933973842923	1.70177930562519	5.86045878248365
D	-0.18531876521216	0.11905159981934	-0.304370365031511	$-6.62671653928\times10^{-2}$
E	-1.465306389666	1.55567568549579	-3.02098207516179	$9.036929582979\times10^{-2}$
F	-1.6372219411651	1.45747848977592	-3.09470043094102	-0.179743451389183
G	0.114139824839289	$5.6526349782\times10^{-2}$	$5.76134750569\times10^{-2}$	0.170666174621583
H	-0.77136373199334	0.90333640656888	-1.67470013856222	0.131972674575539
I	1.44902405144951	1.04596194894505	0.40306210250446	2.49498600039456
J	-0.26124165566106	0.470506029025842	-0.73174768468691	0.209264373364774

Table 4.11. 99% Confidence Intervals, PDA inocula, 10 g L^{-1} CMC concentration.

Variable	Value	99% (+/-)	Lower Limit	Upper Limit
A	0.21544393179808	0.2468112803057	$-3.1367348507\times10^{-2}$	0.462255212103857
B	-0.2176491981205	0.23968910044522	-0.45733829856564	$2.2039902324\times10^{-2}$
C	-1.8814477000619	2.34897971961511	-4.2304274196771	0.467532019553121
D	$-4.5312651803\times10^{-2}$	0.16702996693193	-0.2123426187353	0.121717315128943
E	-3.2707630804928	6.77668554919299	-10.047448629688	3.50592246870017
F	3.0966887295543	3.02619887996065	$7.04898495935\times10^{-2}$	6.12288760951495

G	$4.07259702339\times10^{-2}$	0.15428371985725	-0.11355774923266	0.19500969009126
H	-0.209215701955868	11.5559691836655	-11.7651848856214	11.3467534817097
I	1.25233673765332	9.1839327104543	-7.93159597280098	10.4362694481076
J	-0.89169561262431	2.33519195577461	-3.22688756839892	1.44349634315029

Table 4.12. 99% Confidence Intervals, PDA inocula, 15 g L^{-1} CMC concentration.

Variable	Value	99% (+/-)	Lower Limit	Upper Limit
A	0.104444294478189	$5.7919763243\times10^{-2}$	$4.65245312350\times10^{-2}$	0.162364057721283
B	0.20007996974059	0.180262813682078	$1.98171560585\times10^{-2}$	0.380342783422668
C	-4.92054689045709	2.48887478227588	-7.40942167273297	-2.43167210818121
D	-0.4085331770416	0.40447160065932	-0.813004777700921	$-4.06157638228\times10^{-3}$
E	-5.0220714644538	10.9638556907457	-15.9859271551995	5.94178422629193
F	6.46545591740028	5.00823300118752	1.45722291621276	11.4736889185878
G	0.162586030951437	0.19359504095357	$-3.1009010002\times10^{-2}$	0.356181071905008
H	-4.1873113622921	11.4279538298697	-15.6152651921618	7.24064246757758
I	4.67071371322835	10.316081886474	-5.64536817324567	14.9867955997024
J	-2.31564263320795	2.69026794395732	-5.00591057716527	0.374625310749366

Table 4.13. 99% Confidence Intervals, PDA inocula, 20 g L^{-1} CMC concentration.

Variable	Value	99% (+/-)	Lower Limit	Upper Limit
A	$4.47092607846\times10^{-2}$	$5.6085259529\times10^{-2}$	$-1.13759987449\times10^{-2}$	0.10079452031406
B	$6.97660397852\times10^{-2}$	0.13185534542864	$-6.20893056433\times10^{-2}$	0.20162138521389
C	-2.12745200082632	2.44708675426851	-4.57453875509483	0.31963475344219
D	-0.19319260663071	0.211041108432452	-0.40423371506316	$1.7848501801\times10^{-2}$
E	-2.84393125386273	2.68187538443505	-5.52580663829778	-0.1620558694276
F	3.27412984398445	2.47495153620821	0.79917830777624	5.74908138019266

G	$9.14598255001\times10^{-2}$	0.08948264725579	$1.97717824438\times10^{-3}$	0.18094247275596
H	-1.87456833838967	2.58829796259235	-4.46286630098202	0.71372962420268
I	2.4735492786153	2.69633399494993	-0.222784716334628	5.16988327356523
J	-1.25940426227771	0.954299499912816	-2.21370376219053	-0.30510476236489

Table 4.14. 99% Confidence Intervals, PDA inocula, 25 g L^{-1} CMC concentration.

Variable	Value	99% (+/-)	Lower Limit	Upper Limit
A	0.40078956465779	0.26424554274397	0.13654402191382	0.6650351074766
B	-0.2909959839997	0.36946579148888	-0.66046177548868	$7.8469807489\times10^{-2}$
C	-31.279701171853	20.3857312067849	-51.665432378638	-10.893969965068
D	$8.1399424138\times10^{-2}$	0.64409526568416	-0.56269584154594	0.72549468982238
E	-43.55589168488	29.3588070441439	-72.914698729023	-14.197084640736
F	34.8798266890423	23.1319489102495	11.7478777787928	58.0117755992918
G	$-3.573453765\times10^{-2}$	0.286401270594538	-0.32213580824682	0.25066673294224
H	-14.5904101668015	15.4696123247922	-30.060022491593	0.87920215799068
I	23.0683324406242	19.0346566435267	4.03367579709748	42.1029890841509
J	-9.31350337223644	7.03013416427409	-16.34363753651	-2.2833692079623

The other percentages of coefficients were also tested and presented in Appendix A.

Viscometric analysis can be applied to evaluate endocellulase activity because the hydrolysis of internal bonds within polymer molecules alters the viscosity of a solution (Almin and Eriksson 1967 a,b). The decrease in viscosity resulting from endocellulase activity is primarily due to the fragmentation of CMC by cleavage of glucosidic linkages remote from the glucoside chain end of the substrate.

Hence, the data presented in the modelling part showed that viscometric method can be used for rapid assaying of endoglucanase activity. The method developed in this study has sensitivity comparable to that spectrophotometric assay method for endoglucanase. The advantage of the viscometric method is that relatively small assay volume is required 15 ml of supernatant of CMC media and shorter time (5 minutes) for each enzyme activity assay as compared to spectrophotometric method.

On the other hand, in spectrophotometric method approximately 30 mL of media was used and it takes approximately 2 hours for each enzyme activity assay. Ishihara *et al*. (2005) has reported for 10 minutes for each assay of enzyme activity using viscometric analysis that in this study the time is less than what it was reported.

The modelling study also showed that for different inoculation media (GYMP and PDA), two distinct mathematical functions were obtained for the same sets of CMC concentrations range and cultivation time. This indicates that the fungal culture's history may significantly influence the subsequent behaviour of the enzyme activities profile with time and CMC concentration.

Chapter 5

Conclusions

The successful use of cellulosic resources as renewable carbon sources relies on the improvement of economically feasible processes for cellulase enzyme production and enzymatic hydrolysis of cellulosic materials to some lower molecular weight products such as hexose and glucose.

Many microorganisms have been classified as cellulolytic but there is scarcely any report on the ability of *Pycnoporus sanguineus* CY788 capable of synthesizing cellulase enzyme. In the present study, specific media formulation was used to investigate the efficiency of cellulase enzyme production by *P. sanguineus* which may satisfy reasonable manufacturing costs and enzyme productivity level.

The results of different media composition in the shake flasks cultivation exhibited the economical and technical advantages of enzyme production process using mixtures of CMC and Tween 80.

In the modelling part, the correlation between endoglucanase activities level and CMC media viscosity were studied and two different mathematical functions were proposed for two different inoculation media, GYMP and PDA (Fig. 5.1). The fact that two distinct mathematical functions were used to describe the fungal fermentation with the same CMC concentration range tested and cultivation time underlined the importance of culture's history.

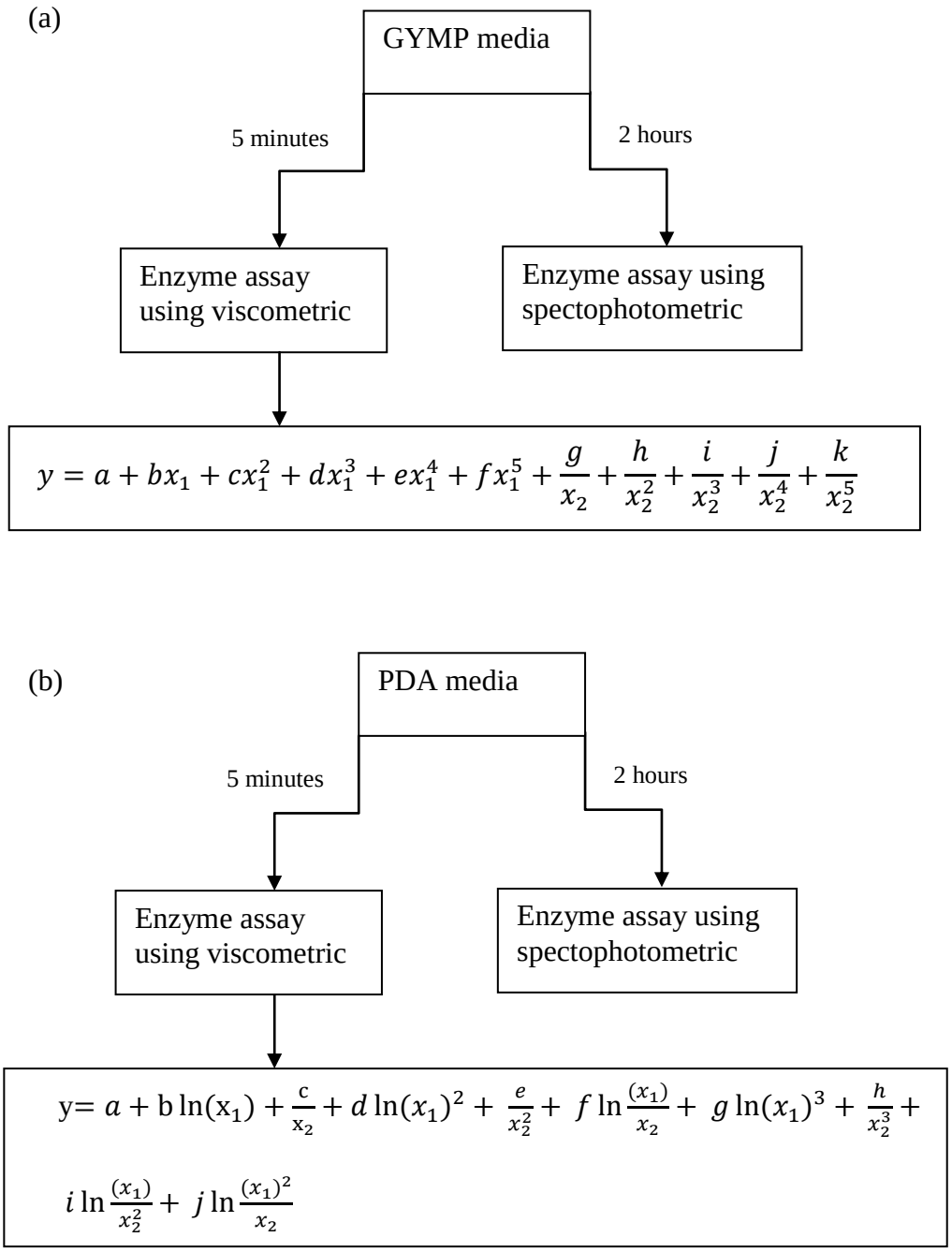


Figure 5.1.Enzyme assay using viscometric and spectrophotometric methods in (a) GYMP media and (b) PDA media.

The data presented in this study showed that viscometric method can be used for rapid assaying of endoglucanase activity. The method developed in this study has sensitivity comparable to that spectrophotometric assay method for endoglucanase. The advantage of the viscometric method is the relatively small assay volume (15 ml of CMC media) and shorter time (5 minutes) for each enzyme activity assay as compared to spectrophotometric method (30 ml, 2 hours).

sAdvance investigations will be needed in order to understand how the resulting enzyme activity quantitatively related to the culture's origin and history.