Optimization of cellulase production by *Trichoderma reesei* in solid-state fermentation

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Mohamed A. Farid* and Hoda M.A. Shata**

*Natural and Microbial Products Department and **Microbial Chemistry Department, National Research Centre, Dokki, Cairo, Egypt

*Corresponding author. E-mail address: nrcfarid@yahoo.com

ABSTRACT

A solid state fermentation (SSF) in a laboratory scale was used for cellulase production by Trichoderma reesei NRRL 6156 using wheat straw and wheat bran (3:1 w:w) as substrate. The solid substrates (initial water content 54.5 %), supplemented with a SSF mineral medium before optimization showed carboxymethyl cellulase (CMC-ase) and filter paper-cellulase (FP-ase) activities of 129 and 8.8 U/g respectively. After a three-step medium optimization CMC-ase activity had significantly increased to ca. 2690 U/g culture, while FP-ase increased to 16 U/g culture. The optimized SSF medium allowed 17.5 and 1.2 fold respective increases in CMC-ase and FP-ase activities. The maximum extracellular cellulase production was obtained after 10 days. Adding 20g/l of (NH4)2SO4 led to a proportional increase in cellulase activity. Combined supplementation with (NH4)2SO4, urea wheat bran and wheat straw have a stimulating effect on cellulase production. Furthermore, the concentration of KH2PO4 showed a strong effect on enzyme production, suggesting the importance of buffering of the SSF medium.

Key words: *Cellulase, solid-state fermentation, Trichoderma reesei, medium optimization*

INTRODUCTION

Cellulase production is the most important step in the economical production of single cell protein, ethanol and other chemicals from renewable cellulosic materials. To date, cellulase production has been widely studied in submerged culture processes. Various methods have been reported for cellulase production by solid-state fermentation (SSF) due to its lower capital investment and lower operating expansion (Couri *et al*., 2000; Yang *et al*., 2001). Another approach to reduce the cost of cellulase production is the use of lingo-cellulosic materials as substrates rather than expensive pure cellulose. Numerous agricultural wastes such as wheat straw, rice straw, baggasse, corn stover, etc. were used in cellulase production (Panagiotou *et al*., 2003; Liming and Xueliang 2004). Also, the effects of fermentation conditions including moisture content, initial pH, temperature, composition of mixed substrate and mixed cultures were studied to improve cellulase production (Yang *et al*., 2003; Hanif *et al*., 2004).

This paper reports the results of research done on medium optimization for cellulase production by *Trichoderma reesei* NRRL 6156

using wheat straw and wheat bran as cheap substrates.

MATERIALS AND METHODS

Microorganism and inoculum

Trichoderma reesei strain NRRL 6156 was obtained from Agricultural Research Service (ARS) Culture Collection (NRRL), Peoria, Illinois, USA. Stock cultures were maintained on potato dextrose agar (Fluka) slants. For inocula fungal preparation, spores were washed from 10-day agar slant cultures with 10 ml sterile distilled water.

Substrates

Air-dried and milled wheat straw (WS) and wheat bran (WB) were obtained from the Agricultural Research Center, Giza, Egypt. Wheat straw has the following composition: cellulose, 43.5%, hemicellulose, 19.3%, lignin, 13%, protein, 1.6%, and ash 10.6%. Wheat bran charactarised by: cellulose, 21.2%, hemicellulose, 26.3%, starch, 8.6%, sugar, 4.5%, protein, 15.3%, and ash 6.3%.

Solid-state fermentation (SSF)

The SSF was carried out in Petri dishes (Ø 9 cm), either was loaded with 4 g dry weight substrate containing wheat straw and wheat bran in a ratio of 3: 1 (w: w). After sterilization (121°C/ 30 min) and cooling to the room temperature, 2 ml of spore suspension (~10^7 spores/ml) were aseptically added to each Petri dish containing 4 g of the substrate and mixed. The initial water content of the substrates after sterilization and addition of nutrients and inocula was 44 – 80 % (w/w). The inoculated Petri dishes were incubated for 10 days at 30°C in a humidity chamber of 95 % RH. The mineral SSF medium composed of (g/100 ml): $(NH_4)_2SO_4$, 2; urea, 0.5; KH_2PO_4, 0.5; $MgSO_4$.$7H_2O$, 0.5; $CaCl_2$, 0.45; $CoCl_2$, 0.05 and lactose 0.2, the pH was 4.5.

Extraction of cellulase system

The SSF culture from each Petri dish was mixed soaked in distilled water (5 ml/g culture) and shaked (200 rpm) for 3 hr on a rotatory shaker. Extracts were centrifuged for 20 min at 6000 rpm at 4^0C. The obtained supernatants were used for enzyme preparation.

Endoglucanase (CMC-ase) activity was determined according to Mandels *et al.* (1976) using 1 % (w/v) carboxymethyl cellulose (Fluka). The amount of released reducing sugars was determined as glucose as stated by Nelson (1944) and Somogi (1952). The enzyme activity was expressed in international units (IU) defined as the amount of enzyme required to produce one µmol glucose per min.

Filter paper-cellulase (FP-ase) activity was measured as described by Mandels *et al.* (1976) at pH 4.6 using phosphate-citrate buffer (Jones and Hayward, 1973) at 50°C and expressed as IU equivalent to one µmol glucose produced per min from Whatman filter paper No. 1.

Soluble proteins in the culture filtrates were estimated by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Medium optimization for cellulase production

Optimization was carried out using the fractional factorial experimental design described by DeMeo *et al.* (1985).The experimental design reported in Table (1) was applied to evaluate the influence of different media components including $(NH_4)_2SO_4$, urea, KH_2PO_4, $MgSO_4$.$7H_2O$, $CaCl_2$, $CoCl_2$ and lactose. The rows represent eight different experiments and each column represents a different component. For each component, high (H) and low (L) concentrations were tested.

For evaluation of the effect of each component, the coefficients Cp_1 (CMC-ase

activity), Cp_2 (FP-ase activity) and Cs (specific activity) were calculated as follows: if P is the cellulase activity and S is the specific activity of the enzyme, the coefficients Cp and Cs relating to each of the seven given components are given by:

$$Cpj = \frac{1}{8} \times \{\sum_{i=1}^{8} Aj \times Pi\}$$

$$Csj = \frac{1}{8} \times \{\sum_{i=1}^{8} Aj \times Si\}$$

Here, Aj means either high (H) or low (L) level in experimental run i. If a calculated coefficient has a positive value, it means that the particular component has a positive effect at its high level and visa versa.

RESULTS AND DISCUSSION

Effect of initial water content

The water content of solid substrates is one of the key factors in cellulase production by SSF (Panagiotou *et al.*, 2003). Therefore, SSF experiments with different initial water contents were carried out. The optimal initial water content in the solid substrate appears to be 54.5 %. Under these conditions, cellulase activities of 129 U/g culture of CMC-ase and 8.8 U/g culture of FP-ase were obtained (Table 2). An increase in the initial water content of the fermented substrate has a negative effect on the production of cellulolytic enzymes.

Actually, moisture level in SSF depends on the substrate nature, microorganism and type of end-product (Ramesh and Lonsane, 1990). On the other hand, Tao *et al.* (1997) mentioned that a decrease in the water content of SSF medium or an increase in the quantity of salts result in high osmotic potential. This will have an adverse effect on growth and enzyme production, since nutrient concentration is inversely related to the quantity of water present in the medium. However, Raghavarao *et al.* (2003) reported that many microorganisms are capable of growing on solid substrate, but only filamentous fungi can grow to a significant extent in the absence of free water. The authors also mentioned that bacteria and yeasts could grow on solid substrates at 40-70 % moisture level. Favels-Torres *et al.* (1998) reported that SSF systems usually perform under conditions of low free water content where the overall packing density of the wet solid materials in the fermentation vessels is between 0.2–0.5 g/cm^3. They suggested that SSF system should contain more than 50 % of interparticle space, which otherwise occupied by air in aerobic processes. The authors added that the microorganisms always are in contact with liquid (culture medium) and gas (air) phases that strongly reduce the osmotic pressure provoked by salts and sugar concentrations. Analogously, Hesseltine (1972) mentioned that there is optimum moisture content for metabolic activities, which related to the dissolved oxygen concentration.

Table (1): The DeMeo's design of experimental runs used for cellulase (CMC- ase and PF-ase) production by T. reesei NRRL 6156.

Expt. run	Constituents						
	A	B	C	D	E	F	G
1	L	L	L	H	H	H	L
2	H	L	L	L	L	H	H
3	L	H	L	L	H	L	H
4	H	H	L	H	L	L	L
5	L	L	H	H	L	L	H
6	H	L	H	L	H	L	L
7	L	H	H	L	L	L	L
8	H	H	H	H	H	H	H

Letters A-G represent the components used as follows: (A) urea, (B) $(NH_4)_2SO_4$, (C) KH_2PO_4, (D) Co Cl_2, (E) Ca Cl_2, (F) $MgSO_4$, (G) lactose.

H denotes a high-level value; L denotes a low-level value.

Table (2): Effect of initial water content on cellulase (CMC-ase and FP-ase) production by T. reesei NRRL 6156 after 10 days of SSF.

Initial water content (%)	Cellulase activity				pH
	CMC-ase activity		FP-ase activity		
	U/g culture	U/mg protein	U/g culture	U/mg protein	
44.0	15.01	0.62	5.62	0.23	4.5
54.5	128.89	3.07	8.8	0.21	4.5
61.5	111.43	2.65	8.9	0.21	4.4
66.6	71.73	2.29	6.3	0.20	4.4
68.6	39.00	1.61	3.6	0.14	4.4
72.0	42.29	1.68	3.2	0.13	5.0
78.2	27.58	1.29	1.7	0.08	4.3
80.3	24.85	0.95	1.4	0.05	5.0

Medium optimization for cellulase production

Optimization of the SSF mineral medium was performed with the fractional factorial experimental design (DeMeo *et al.*, 1985) in three steps for cellulase production by *T. reesei* strain NRRL 6156. The first step was used to determine which components have a significant effect on cellulase (CMC-ase and FP-ase) production while the second and the third to adjust their concentrations. The influence of $(NH_4)_2$ SO_4, KH_2PO_4, $MgSO_4.7H_2O$, $CaCl_2$, $CoCl_2$, urea and lactose when added to the solid substrate consisting of wheat straw and wheat bran (3:1 w: w) was studied. All optimization experiments were carried out at initial water content of 55 %.

First optimization step

The design of media optimization (Table 1) and the concentration of each component for the first optimization step is given in Table (3). The corresponding SSF results (CMC-ase, FP-ase activities and their specific activities) are summarized in Table (4a). The CMC-ase level in the different runs varied from 37.9 to 606.8 U/g culture (Ø 259.7 U/g culture). Similarly, wide variations (0.95 to 14.5 U/mg culture) in its specific activity were recorded. On the other hand, FP-ase level and its specific activity were relatively low (4 to 10.8 U/g culture). The calculated coefficients are given in Table (4b). Increasing in the level of urea, ammonium sulphate, calcium chloride cobalt chloride, lactose or magnesium sulphate had a

negative effect on CMC-ase production. While, potassium dihydrogen phosphate has strongly influenced the level of CMC-ase and its specific activity. The changes in the amount of urea, calcium chloride and magnesium sulphate negatively affected FP-ase level. Ammonium sulphate, potassium dihydrogen phosphate and cobalt chloride showed a positive effect on FP-ase and its specific activity. The CMC-ase and FP-ase levels and their specific activities were relatively low at pH more than 5.

Table (3): Different components levels used in medium optimization steps for CMC-ase and FP-ase production by T. reesei NRRL 6165 in SSF.

Optimization step	Components	Low level g/100 ml	High level g/100 ml
First step	A) Urea	0.5	1.5
	B) $(NH_4)_2 SO_4$	2.0	6.0
	C) $KH_2 PO_4$	0.5	1.5
	D) $Co Cl_2$	0.05	0.15
	E) $Ca Cl_2$	0.45	1.35
	F) $Mg SO_4$	0.5	1.5
	G) Lactose	0.1	0.3
Second step	A) Urea	0.05	0.5
	B) $(NH_4)_2 SO_4$	0.2	2.0
	C) $KH_2 PO_4$	1.0	2.0
	D) $Co Cl_2$	0.01	0.05
	E) $Ca Cl_2$	0.1	0.5
	F) $Mg SO_4$	0.01	0.05
	G) Lactose	0.02	0.1
Third step	A) Urea	0.01	0.05
	B) $(NH_4)_2 SO_4$	0.2	2.0
	C) $KH_2 PO_4$	0.5	1.5
	D) $Co Cl_2$	0.001	0.01
	E) $Ca Cl_2$	0.01	0.1
	F) $Mg SO_4$	0.001	0.001
	G) Lactose	0.02	0.1

Second optimization step

Based on the results of the first optimization step, the concentration of urea, ammonium sulphate, magnesium sulphate, calcium chloride, cobalt chloride and lactose were more reduced whereas, the level of KH_2PO_4 was increased. The concentrations of each component in the second step are shown in Table (3). Results in Table (5a) show that the CMC-ase level produced varied between 98 and 933 U/g culture (Ø, 389.9 U/g culture). The specific activity of the enzyme ranged from 4.1 to 22.7 U/mg. On the other hand, the FP-ase level ranged from 1.3 to 8.7 U/g culture. The maximum level of FP-ase in the second optimization step was significantly lower than that of the first one and before optimization. The calculated coefficients (Table 5b) showed that ammonium sulphate, calcium chloride, cobalt chloride and lactose exhibited positive effects on CMC-ase production. Consequently, their concentrations can remain at their low levels. On the other hand, increasing the level of potassium dihydrogen phosphate to 2 g/l did negatively affect the CMC-ase production and its specific activity. Similarly, urea and magnesium sulphate at their levels in the second step showed also negative effects on CMC-ase production. These results suggested

that the concentration of both should be decreased to lower levels or omitted from the fermentation medium.

Table (4a): First optimization step for CMC-ase and FP-ase production and their specific activities after 10 days of SSF.

Run i	CMC-ase activity			FP-ase activity			pH
	U/ml extract	U/g culture	U/mg protein	U/ml extract	U/g culture	U/mg protein	
Control	30.6	153.0	4.8	1.12	5.6	0.16	4.6
1	22.66	113.32	1.97	1.02	4.1	0.003	4.6
2	7.59	37.95	0.95	0.00	0.0	0.0	5.3
3	21.25	106.23	2.40	1.32	6.6	0.17	4.4
4	31.96	159.23	3.02	1.00	5.0	0.11	5.0
5	102.59	512.93	11.40	1.52	7.6	0.17	4.6
6	85.59	427.93	10.37	0.85	4.25	0.10	4.5
7	121.35	606.76	14.48	2.17	10.87	0.26	4.4
8	22.65	113.25	2.86	1.50	7.51	0.19	4.5
Average	51.95	259.77	5.93	5.93	5.74	0.13	4.6

Table (4b): Coefficients of CMC-ase and FP-ase production and their specific activities for each component resulting from first optimization step.

Coefficient	A	B	C	D	E	F	G
Cp_1 CMCase	600.29	106.07	1243.55	279.55	-556.73	-1549.45	-537.47
Cs_1 CMC-ase	-13.05	193.00	30.77	-9.95	-12.25	-26.89	-12.13
Cp_2 FP-ase	-12.41	14.03	14.53	2.49	-1.01	-22.71	-2.51
Cs_2 FP-ase	-0.20	0.457	0.437	-0.06	-0.08	-9.62	0.06

Table (5a): Second optimization step for CMC-ase and FP-ase production and their specific activities after 10 days of SSF.

Run i	CMC-ase activity			FP-ase activity			pH
	U/ml extract	U/g culture	U/mg protein	U/ml extract	U/g culture	U/mg protein	
Control	30.6	153.0	4.8	1.12	5.6	0.16	4.6
1	49.00	245.27	6.54	0.91	4.55	0.13	5.0
2	69.91	349.53	8.04	1.17	5.83	0.16	5.1
3	196.61	933.03	22.76	1.74	8.70	0.23	4.6
4	134.58	672.92	17.80	1.12	5.60	0.21	4.4
5	68.29	341.47	13.24	1.18	5.90	0.25	5.0
6	19.26	98.26	4.10	1.08	5.40	0.25	4.9
7	19.67	98.36	4.11	0.26	1.30	0.05	4.8
8	76.00	380.68	16.03	1.20	5.98	0.23	4.7
Average	77.92	389.94	11.58	1.08	5.40	0.18	4.8

Table (5b): Coefficients of CMC-ase and FP-ase production and their specific activities for each component resulting from second optimization step.

Coefficient	A	B	C	D	E	F	G
Cp_1 CMCase	116.74	1050.46	-1282.66	161.16	194.96	-1168.56	889.9
Cs_1 CMC-ase	-2.48	27.8	-18.08	12.12	5.4	-32.64	27.0
Cp_2 FP-ase	2.33	0.07	-6.07	0.83	6.03	-10.57	9,53
Cs_2 FP-ase	0.19	-0.07	0.05	0.13	0.17	-0.47	0.23

Third optimization step

Based on the results of the first and second optimization steps, the basal composition of the SSF medium was readjusted (Table3). The concentration of magnesium sulphate was fixed at 0.001 g/l, while urea, cobalt chloride and calcium chloride were reduced to a level below their levels in the second step. The level of lactose was set at its levels in the second step. While, the dosage of potassium dihydrogen phosphate was set at its level in the first step. Under these conditions, Tables (6a and 6b) show that the level of CMC-ase varied between 442 and 2697 U/g culture (Ø 1127 U/g culture). The mean value of CMC-ase after the third optimization step increased to 4.3, 2.9 and 7.3 fold of the first step, second step and before medium optimization, respectively. The specific activity of CMC-ase also increased from 20 to 102 U/mg. These results suggested that the concentration of every component was optimized in the third optimization step except that of $MgSO_4$. As previousely mentioned, $MgSO_4$ could be omitted from the constituents of the SSF medium.The final pH values in the third optimization step were falling in the range of cellulase stability. Whereas, maximum level of CMC-ase was observed at pH value of 4.6 – 4.7.

Table (6a): Third optimization step for CMC-ase and FP-ase production and their specific activities after 10 days of SSF.

Run i	CMC-ase activity			FP-ase activity			pH
	U/ml extract	U/g culture	U/mg protein	U/ml extract	U/g culture	U/mg protein	
Control	30.6	153.0	4.8	1.12	5.6	0.16	4.6
1	114.93	574.67	23.65	1.50	7.52	0.31	4.90
2	88.43	442.17	20.86	1.50	7.50	0.35	4.90
3	146.14	730.72	24.56	3.20	16.06	0.54	4.60
4	156.122	780.61	28.73	2.28	11.44	0.42	4.70
5	201.72	1008.58	41.20	0.60	2.98	0.12	4.90
6	130.42	652.09	28.50	0.30	1.5	0.05	4.90
7	426.20	2131.90	70.55	0.99	4.95	0.16	4.70
8	539.40	2697.08	102.62	1.61	8.03	3.3	4.60
Average	225.42	1127.23	42.58	1.50	7.49	0.66	4.78

Table (6b): Coefficients of CMC-ase and FP-ase production and their specific activities for each component resulting from the third optimization step.

Coefficient	A	B	C	D	E	F	G
Cp1 CMCase	126.08	3662.8	3961.48	1104.06	291.30	-1589.98	739.3
Cs1 CMC-ase	20.75	112.25	145.073	51.73	17.99	-46.41	37.86
Cp2 FP-ase	-3.04	20.98	-25.06	-0.04	6.24	-13.88	9.16
Cs2 FP-ase	2.99	3.57	2.01	3.05	3.15	2.67	3.37

In conclusion, a new SSF medium could be formulated for high CMC-ase and FP-ase production (g/l) as follows: urea, 0.1; $(NH_4)_2SO_4$, 20; KH_2PO_4, 15, $CoCl_2$, 0.1; $CaCl_2$, 1; and lactose, 1. Wheat straw and wheat bran were used in proportion of 3:1 w:w. Consequently, a comparative study of cellulase (CMC-ase and FP-ase) production by *T. reesei* NRRL 6156 in SSF was carried out using the mineral salts medium (before and after optimization) and solid substrate (Fig. 1). For both cases, maximum extracellular cellulase production was obtained after 10 days of SSF. The optimized SSF medium allowed 17.5 and 1.2 fold increase in CMC-ase and FP-ase respectively.

In general, addition of 20 g/l $(NH_4)_2SO_4$ led to a proportional increase in cellulase activity. At 60 g/l $(NH_4)_2SO_4$, the enzyme activity decreased probably due to high initial salt concentration/ osmotic stress in the SSF medium. However, enzyme formation in the medium generally requires the exhaustion of nutrients such as nitrogen. The relatively low enzyme yield at low level (2 g/l) of $(NH_4)_2SO_4$ could be explained by that most of the nitrogen source being used for mycelial build-up. Gupta *et al.* (1972) reported that a combination of different nitrogen sources such as urea, ammonium sulphate and peptone gave better production of cellulase than when a single nitrogen source was used. Our findings indicates that a combined supplementation with $(NH_4)_2SO_4$ (20g/l), urea (0.1 g/l) and wheat bran with wheat straw have a stimulating effect on cellulase production (Fig. 1).

In this respect, Hanif *et al.* (2004) reported that wheat bran and cellulose were the most effective promoters of cellobiodydrolase and filter paper cellulase (FP-ase) activities produced by *Aspergillus niger*. The authors mentioned that *A. niger* required limiting concentration of carbon, nitrogen and phosphorous for production of these enzymes. Chandhuri and Sahai (1993) recommended using lactose as an inducing component for production of cellulose enzymes by *T. reesei*. Also, the previousely addition of lactose (1 g/l) showed a positive effect on cellulase activities. Finally, the concentration of KH_2PO_4 exhibited a strong effect on enzyme production, suggesting the importance of buffering the medium during fermentation.

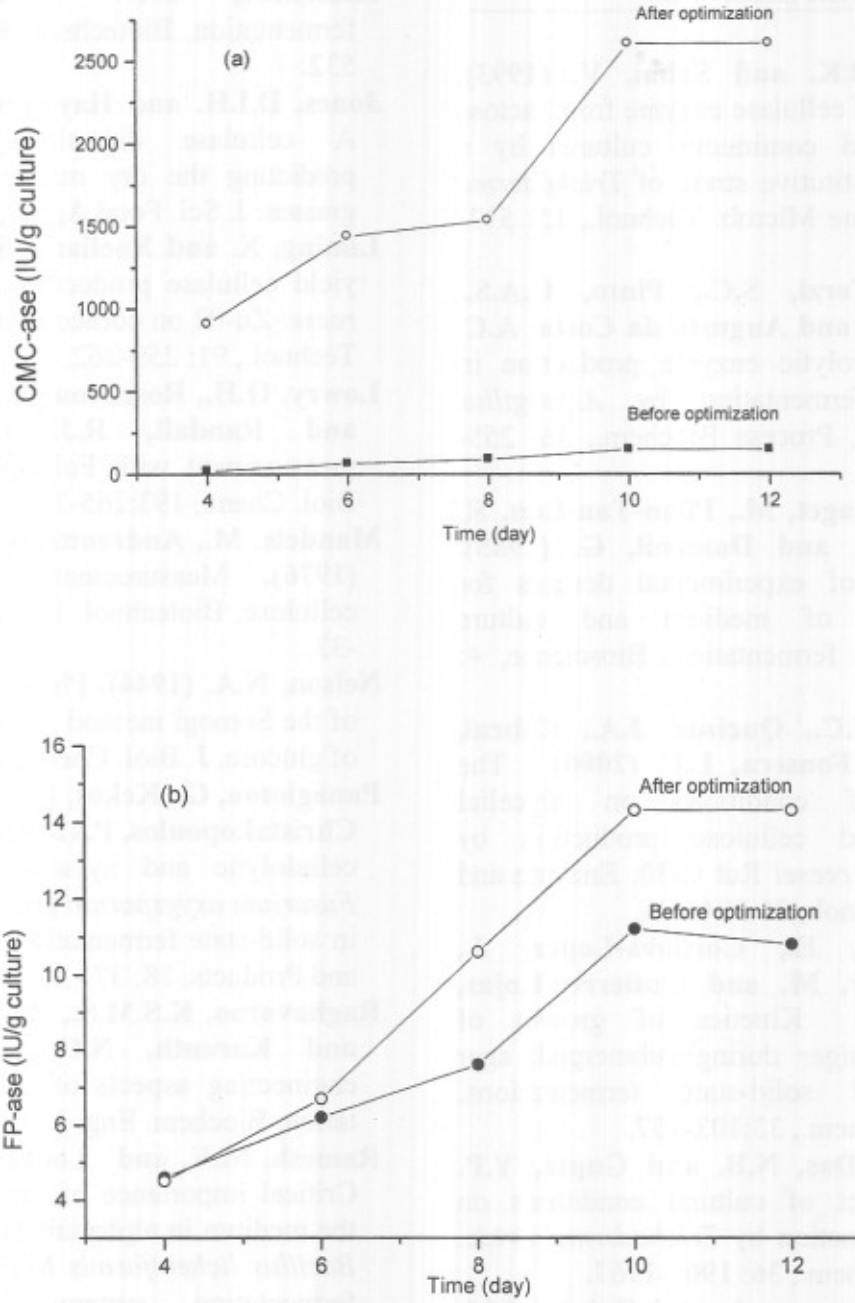

Fig. (1): CMC-ase and FP-ase production by T. reesei NRRL 6156 in SSF before and after optimization.

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الملخص العربي

الإنتاج الأمثل لإنزيم السيليولاز بواسطة طر تراى كودرما ريزى فى المنابت التخمرية الصلبة

***محمد عبد الفتاح فريد و** هدى محمد عبد الحليم شطا**

**قسم المنتجات الطبيعية والميكروبية – المركز القومى للبحوث – الدقى – الجيزة – مصر

**قسم كيمياء الكائنات الدقيقة – المركز القومى للبحوث – الدقى – الجيزة – مصر

فى هذا البحث تم استخدام طريقة التخمرات الصلبة على المستوى المعلمى لإنتاج انزيم السيليولاز بواسطة فطر التراى كودرماريزى NRRL 6156 باستخدام قش القمح وردة القمح (1:3 وزن/وزن) كمادة غذائية وباستخدام المواد الصلبة بنسبة رطوبة 54.5% ومزودة ببيئة من المواد المعدنية تم إنتاج حوالى 129 وحدة نشاط انزيمى من CMC-ase و 8.8 وحدة نشاط إنزيمى من FP-ase لكل جرام. تم إجراء ثلاث خطوات لتعظيم الإنتاج باستخدام نموذج المضروب الجزئى حيث أظهرت النتائج زيادة معقولة وصلت إلى 2690 وحدة نشاط من CMC-ase و 16 وحدة نشاط إنزيمى من FP-ase لكل جرام من المادة الصلبة المتخمرة. حيث تضاعف الإنتاج إلى ما يقرب من 17.5 و 1.2 مرة لكلا الإنزيمين على التوالى. وقد وصل الإنتاج الأقصى للانزيمات المحللة للسيليولاز بعد فترة تحضين 10 أيام. وأوضحت النتائج أن إضافة 20جم/لتر من سلفات الامونيوم أدى إلى زيادة نسبية لكمية الإنزيم كما بينت النتائج أن إضافة سلفات الأمونيوم واليوريا وردة القمح وقش القمح معا لها تأثير محفز لإنتاج إنزيم السيليولاز علاوة على ذلك وجد أن إضافة فوسفات البوتاسيوم ثنائى الهيدروجين له تأثير قوى على إنتاج الإنزيم حيث يعمل كمنظم لبيئة التغذية.