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Upgrading of Exoglucanase Production By *Trichoderma reesei* NRC 210

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Abstract:- Cellulases find extensive applications in food, fermentation, textile industries and the hydrolysis of cellulosic wastes to useable carbon source for most of the microbes in food, fuel and chemical production. *Trichoderma reesei* is an efficient producer of exoglucanase enzyme. Physiological factors affecting the production of enzyme were studied in shake flask to obtain the optimum parameters for enzyme production. Batch fermentations were performed in 7 liter fermentor using an initial cellulose concentration corresponding to 10g/l. Results obtained indicate that adjusting the fermentation process at a pH range of 4-5 and agitation speed to 350 rpm resulted to an increase in enzyme activity about 15 folds and 1.8 fold respectively. In addition Monod growth kinetics and Leudeking Piret product formation kinetics were studied using *Trichoderma reesei* with best medium under optimized conditions of inoculum concentration, agitator speed, temperature and pH value.

Keywords:- Trichoderma reesei, Cellulase, Exoglucanase, Fermentation, Fed batch, Modeling.

I. INTRODUCTION

Cellulase is an important and essential kind of enzyme for carrying out the depolymerization of cellulose into fermentable sugars. As a major resource for renewable energy and raw materials, it is widely used in the bioconversion of renewable cellulosic biomass. Glucose, from appropriate hydrolysis of this cellulosic biomass under the treatment of advanced biotechnology can be used in different applications such as production of fuel ethanol, single cell protein, feed stock, industrially important chemicals **[1,2,3]**. Microbial conversion of cellulosic/ lignocellulosic biomass into useful products is a complex process involving combined action of three enzymes namely endoglucanase (EC 3.2.1.4), exoglucanase EC (3.2.1.91) and β-glucosidase (EC 3.2.1.21) **[4]**. Exoglucanases are of two types; 1, 4-β-D-glucan cellobiohydrolase (EC 3.2.1.91) which removes cellobiose units and 1, 4-β-D-glucan glucohydolase (EC 3.2.1.74) which removes glucose units both acting from the non-reducing ends of oligosaccharides produced by the action of endoglucanase **[5]**. Cellobiohydrolase, often called an exoglucanase, is the major component of the fungal cellulase system accounting for 40-70% of the total cellulase proteins and can hydrolyse highly crystalline cellulose **[6]**. Cellobiohydrolases remove mono- and dimers from the end of the glucose chain. Most commercial cellulases are mesophilic enzymes produced by the filamentous fungi *Trichoderma reesei* and *Aspergillus niger* with *Trichoderma* as the most leading strain **[7]**. This reflects well the fact that filamentous fungi are naturally excellent protein secretors and can produce industrial enzymes in feasible amounts **[8]**.

In the last decades, the high production cost and low yields of cellulases are the major problems for industrial application. Therefore, investigations on the ability of microbial strains to utilize inexpensive substrate **[9]** and improvement of enzyme productivity **[10,11]** have been implemented. Concerning the optimization of biotechnological processes **modeling** has become a prerequisite for the development of optimization and control methods. The usefulness of **simulation** models in connection with experimental planning is also evident. Thus, modeling and simulation are valuable tools in basic biological research by directing investigators towards a search for quantifiable results. Furthermore, for the practical implementation of control, estimation of non-measurable state variables is necessary as well.

The aim of this study is to produce an inexpensive and highly active cellulase lies in a combination of critical factors such as, improved enzyme quality, enhanced enzyme productivity and yield, prolonged enzyme lifetime, and minimal cost of media and substrate.

II. MATERIALS AND METHODS

Microorganism: *Trichoderma reesei* NRC 210 was obtained from the Culture Collection of Microbial Chemistry Department, Division of Genetic Engineering and Biotechnology, National research centre. Egypt.

Media:

a. Maintenance medium for stock fungal cultures

Organism was cultivated and kept on slants of Potato Dextrose agar **[12]**.

b. Inoculum's medium

Organisms are grown on Potato Dextrose broth medium for 2 days and then inoculated into the screening medium.

c. Screening medium for exoglucanase production

Modified Mandel's liquid medium was used which has the following composition (g/l): Carbon source (Carboxymethylcellulose), 5.0; Urea, 0.3.; $(NH_4)_2SO_4$, 1.4; Proteose peptone "Difco", 1; KH_2PO_4, 2; $CaCl_2$, 0.3; $MgSO_4.7H_2O$, 0.3; $FeSO_4.7H_2O$, 5mg; $MnSO_4.7H_2O$, 1.6 mg; $ZnSO_4.7H_2O$, 1.4 mg; $CoCl_2$, 2 and Tween 20, 2.5 ml. Initial pH value was adjusted to 5.6. The medium was sterilized by autoclaving at 1.5 atmosphere and 121°C for 20 min. **[13]**.

Batch Fermentation: Enzyme production experiments were performed in a 7.5L BioFlo 310 benchtop Fermentor/ Bioreactor (New Brunswick scientific Co., Inc.) with a working volume 5L. To 2.7L growth medium, containing cellulose as a carbon source with a concentration 10g/L, about 300ml of the inoculum prepared on cellulose was added which corresponds to 10% of the total worked volume (3L). The initial pH was adjusted in the range 5.5-6.0. Exoglucanase production was followed for 7 days; samples were regularly withdrawn and analyzed for exoglucanase activity. The pH was controlled above or below 5 by 1N HCl or NaOH using peristaltic pumps. Foam was controlled by the addition of silicon antifoam (Fluka). The fermentor was aerated at a flow rate of 1.5L/min, corresponding to 0.5 (v/v)/min. Oxygen level in the fermentor was monitored during the experiments.

Analytical determination:

Exoglucanase (FPase) activity was determined by the method described by **Elshafei *et al.*, (1990) [14]** while Protein determined by the lowry method **[15]**. Total reducing sugars was determined by DNS method described by **Ghose (1984) [16]**.

Kinetic (Mathematical) procedures:

MODEL FOR GROWTH KINETICS

- **Monod model**

Monod model relates the specific growth rate and an essential substrate concentration and was described by equation **[17]**:

$$\mu = \frac{\mu_{max} S}{k_s + s}$$

Where S is the nutrient (substrate, S) concentration and K_s is the half saturation constant for that nutrient, i.e. the nutrient concentration at which one-half the maximum specific growth rate is achieved.

MODEL FOR PRODUCT FORMATION KINETICS

- **Leudeking-Piret model**

The kinetics of cellulase protein production was described by Leudeking-Piret model **[18]** which states that the product formation rate varies linearly with both the instantaneous cell mass concentration (x) and growth rate $\left(\frac{dX}{dt}\right)$ as:

$$r_p = \frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta X$$

$\frac{dx}{dt}$ Biomass accumulation in the culture medium ($g_b\ L^{-1}\ h^{-1}$)

$\frac{dp}{dt}$ Exoglucanase accumulation in the culture medium ($U\ L^{-1}\ h^{-1}$)

α Growth-associated coefficient for enzyme production (U g_b^{-1})
β Growth-independent coefficient for enzyme production (U g_b^{-1} h^{-1})
X Biomass concentration (g_b L^{-1})

III. RESULTS AND DISCUSSION

From previous study in the shake flask experiments, we found that the medium containing cellulose at a concentration of 1% with a pH value 5.6 and incubation period for 7 days at 25°C were the best conditions for exoglucanase production. Batch type is the most common operational mode of cellulase production because it is the easiest to start up and control. So a set of fermentation batches were established to optimize the production of enzyme in semi-industrial scale.

Batch fermentation for exoglucanase production by *T. reesei* NRC 210 (under conditions of Uncontrolled pH, Stirrer-speed: 350 rpm, Temperature: 25°C and Aeration level: 0.5 (vvm)

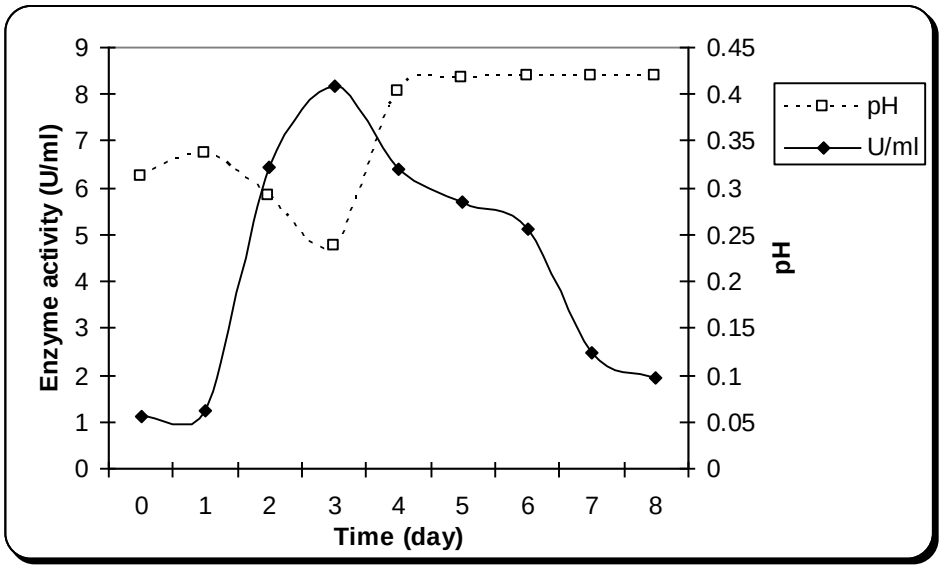

Figure (1)

From these results shown in figure (1) which represents the time course relationship between **pH** values along with exoglucanase activity in (U/ml), we found that maximum enzyme activity was obtained at a pH range 4-5 then the enzyme activity decreases with the increase of pH values, from these results, we obtained the optimum range of pH of exoglucanase production at fermentor level at pH 4-5 which was previously reported by **[19]**.

Comparison between uncontrolled and controlled pH batch fermentations for production of exoglucanase enzyme from *T. reesei* NRC 210

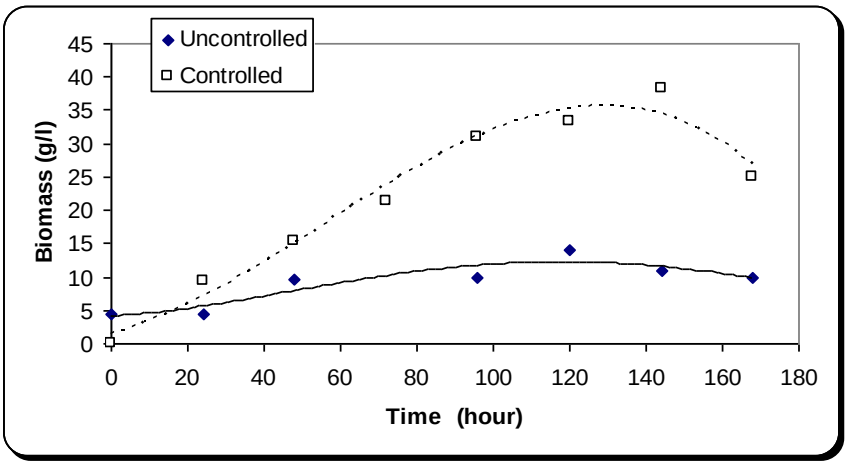

Figure (2)

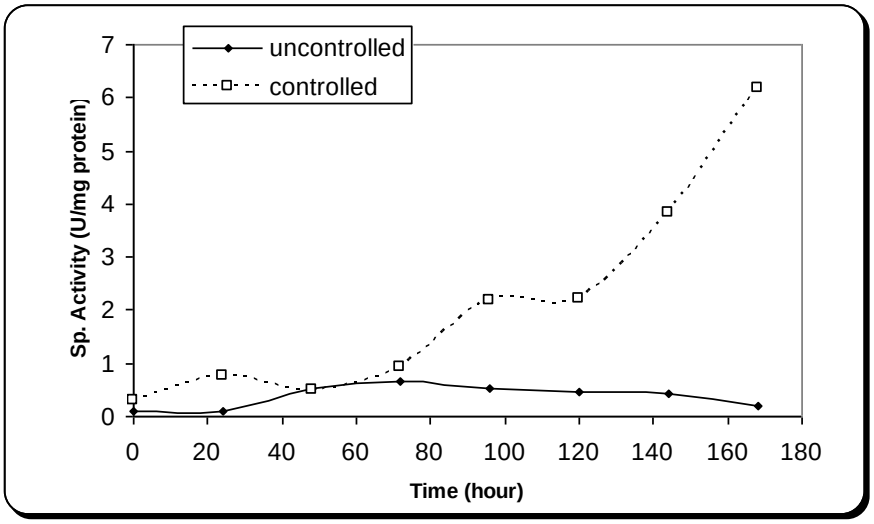

Figure (3)

From these results shown in figure (2) which represents the time course relationships of biomass in (g/l) of the two batches, in both patterns growth begin to increase after the first 24 hrs in the batch to give a maximum biomass weight after 120 hrs for uncontrolled pH batch and 144 hrs for controlled pH one, we conclude that by controlling the pH value of the culture medium, we could improve the fungal growth about **63%** with 24hrs excess time. While figure (3) represents the pattern of specific enzyme activity (U/mg protein) for the two batches, we conclude that by controlling the pH of the culture medium, we could improve the exoglucanase activity 15 fold.

Comparison between batch fermentations for production of exoglucanase enzyme from *T. reesei* NRC 210 with 250 and 350 rpm agitation speed.

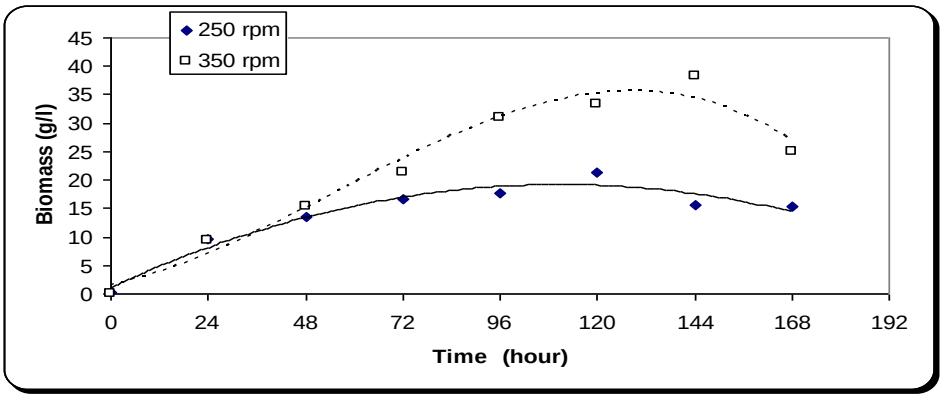

Figure (4)

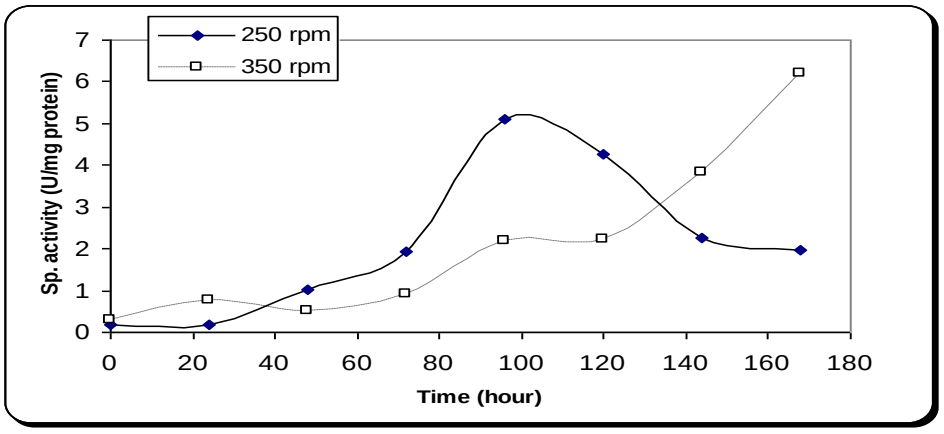

Figure (5)

From the results shown in figure (4) which represents the time course relationship of the biomass in (g/l) for the two batches, we can conclude that by increasing the agitation speed we could improve the fungal productivity with about 80% by further 24 hrs excess times. While figure (5) represents the pattern specific enzyme activity in (U/mg protein) for the two batches, we estimated that we could improve the exoglucanase activity by increasing the agitation speed to 350 rpm to about **79%** fold by 72 hrs excess time. This conclusion was reported previously by **Reczey [20].**

Application of Kinetic Models on the Fermentation Process

The experimental results of the pH controlled process with 350 rpm agitation speed and cellulose as sole carbon source were used to apply a kinetic model for this process. For this purpose the biomass growth rate, enzyme production rate, specific growth rate and specific enzyme production rate were calculated.

The enzyme production followed **Luedeking-Piret equation** (By constructing a relationship between specific growth rate and specific enzyme production rate) as shown in the following equation and represented in figure (6).

$$dP/dt = \alpha * dX/dt + \beta * X$$

where **α** = 0.2136, **β** = 0.0087 (h^{-1}),
dP/dt: Production rate, **dX/dt**: Growth rate, **α & β** : Constants**, X**: Biomass conc.

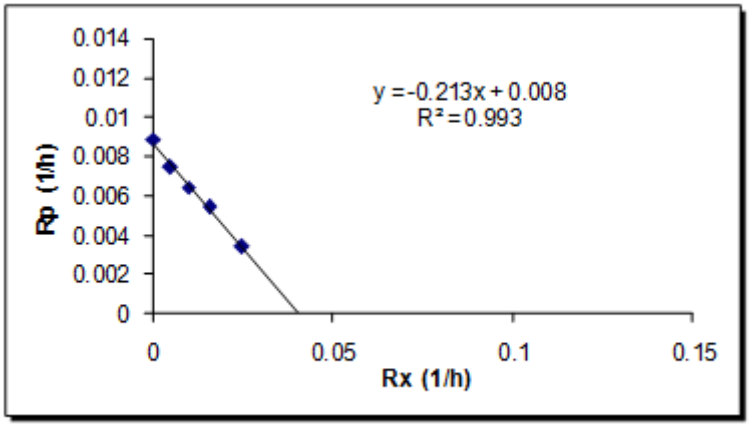

Figure (6)

Due to a major difference between the values of α and β values with high β value, it recommends that the production of exogluconase enzyme is mainly growth associated type, and this confirmed our experimental data. This conclusion was in complete agreement with that reported by **Muthuvelayudham & Viruthagiri (2006)** with approximately the same values of α and β.

The substrate consumption followed **Monod kinetic equation** and using the values of maximum specific growth rate calculated experimentally and saturation constant from literature, the substrate concentration was calculated during the fermentation time and represented in figure (7). The substrate decreased gradually from 10 g/l cellulose at zero time to be consumed completely after 5 days.

$$\mu = (\mu_{max} * S) / (K_s + S)$$

Where **μ**: Specific growth rate = (dX/dt)/X**, μ_{max} & K_s** : Constants**, S**: Substrate conc. (cellulose)

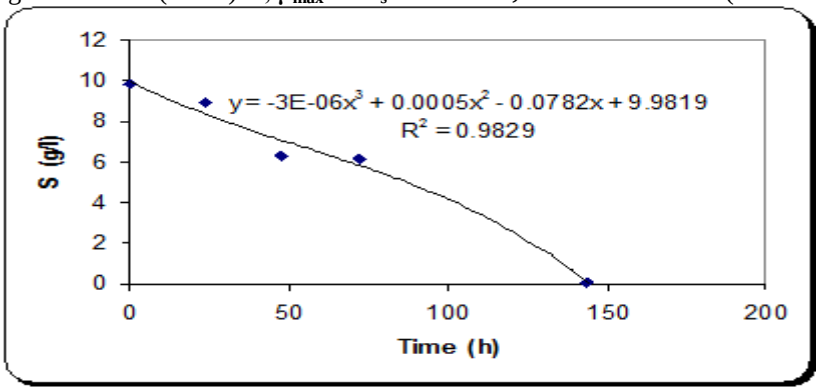

Figure (7)

IV. CONCLUSION

Factors affecting the optimization of exoglucanase production from *Trichoderma reesei* NRC210 were studied using cellulose as a carbon source.

By controlling the pH value between (4-5) and agitation speed to 350 rpm, the enzyme activity was increased by about 15 and 1.8 folds respectively.

By applying the kinetic models Leudeking & Piret model for exoglucanase production and Monod model for fungal growth to the fermentation process, a best fit was achieved with $\alpha = 0.2316$ and $\beta = 0.0087$ (h^{-1}) which led to the conclusion that the enzyme production is growth associated.

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