Enhanced Production of Catalase by Penicillium Chrysogenum in Benchtop Bioreactor

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Abstract— Batch cultures of P. chrysogenum were grown in a 7.5-L benchtop bioreactor under the optimum culture conditions previously established at the flask level and new parameters like agitation (300 rpm) and aeration (1vvm). Time courses of growth, enzyme production, protein content, substrate consumption and pH were followed for 7 days. Maximum intracellular catalase activity (44491 U/l) was obtained after 7 days of growth. pH control (pH 6) enhanced the production of catalase by more than 300 % while increasing agitation to 400 rpm destroyed the growth and reduced catalase production by more than 70 %. Just low concentrations (> 0.03 U/ml) of the produced enzyme were found to be sufficient to completely degrade the residual H2O2 in the bleaching liquor of textile industry after 10 min.

Keywords- Catalase, Penicillium Chrysogenum, Fermentation, Optimization, Production, Bioreactor

1 Introduction

Catalase [hydrogen peroxide oxidoreductase: (EC 1.11.1.6)] is a common enzyme found in nearly all living organisms which are exposed to oxygen, where it functions to catalyze the decomposition of hydrogen peroxide (a powerful and potentially harmful oxidizing agent) to water and free oxygen. Traditional commercial sources of catalase are mammalian liver and *Aspergillus niger* **[1], [2]**. This ubiquitous enzyme has important diverse applications in many pharmaceutical, medical and industrial fields **[3], [4], [5], [6], [7],[8], [9], [10] and [11]**. The main uses of catalase include the elimination of H_2O_2 used in chemical bleaching before dyeing in textile process, large-scale municipal water treatments and cold pasteurization of milk **[12]**.

Although fungal catalases offer stability advantages, the corresponding mammalian enzymes such as beef liver catalase appear to have higher catalytic activity **[13], [14] and [15]**. However, since enzyme stability is an important factor in the biotechnological utilization of enzymes, there is a considerable interest in the use of fungal catalases, especially for applications involving neutralization of high concentrations of hydrogen peroxide.

From our previous studies *Penicillium chrysogenum* was selected out of molds belonging to 10 species as the most effective producer of catalase and the optimum conditions of its production including carbon and nitrogen sources, pH and temperature were established at flask level. The enzyme was stable for 3 h at 50°C and over a range of pH from 6 to 8 and showed higher stability towards different concentrations of H_2O_2 than the commercial one. The present study aims at scaling up of catalase production by *Penicillium chrysogenum* to the fermentor level, enhancement of enzyme production using pH control and optimum agitation in the benchtop bioreactor and evaluation of the produced enzyme.

2 Materials and Methods

1- Micro-organism, media.

Penicillium chrysogenum RCMB 001015(2) was maintained on potato dextrose agar and grown on Czapek-Dox medium consisting of: 10g/l sucrose, 3g/l $NaNO_3$, 1g/l KH_2PO_4, 0.5 g/l KCl, 0.5g/l $MgSO_4.7H_2O$ and 0.01g/l $FeSO_4.7H_2O$.

2- Growth conditions

The batch fermentations were carried out in a 7.5 L BioFlo 310 benchtop Fermentor/ Bioreactor (New Brunswick scientific Co., Inc.) filled with 4.5 L Czapek-Dox medium and sterilized at 121°C for 30 min. the fermentor was equipped with a top stirrer bearing two six-blade Rushtone- type turbines. A pre-incubated broth (24-h-old, 500 ml) of *P.chrysogenum* grown in a shaken flask in the same medium was used as a seed for fermentor inoculation. The standard conditions of fermentation were as follows: agitation rate, 300 rpm; aeration rate, 1v/v/m; temperature, 28°C; occasionally, silicon antifoam (Sigma) was added to break the foam. The pH was controlled using 1M NaOH and HCl and samples were taken every 24h.

3- Preparation of crude homogenate

Mycelia were firstly collected by filtration, washed with distilled water and then homogenized using mortar and pestle in 50 mM potassium phosphate buffer, pH 7.0. The homogenates were centrifuged at 30000 xg for 30 min and the supernatants were saved at –20°C for further analyses.

4- Analytical procedures.

Extracellular and intracellular (measured in cell-free extracts) catalase activity was measured spectrophotometrically by observing the decrease in light absorption at 240 nm during decomposition of H_2O_2 by the enzyme **[16].** One unit (U) of catalase activity was defined as the amount of enzyme catalysing the decomposition of one micromole of hydrogen peroxide per minute at 25°C. The protein content of the medium and cell free extract was determined by the method of **[17]** using Coomassie Brilliant Blue G-250 (CBB G-250) and bovine serum albumin (BSA) as a standard. Residual sugar was measured by the method of **[18]**. Reidual H_2O_2 concentration was determined in the bleaching liquor, before and after addition of the catalase enzyme for 10 min, by titration with a standard 0.1 N $KMnO_4$ solution in acidic medium.

RESULTS AND DISCUSSION

Batch fermentation for catalase production by *P.chrysogenum*:

For each fermentation, the precise physiological conditions must be established for maximal product formation **[19].** The optimum conditions including carbon and nitrogen sources, pH and temperature previously established in the flask level (1% sucrose, 0.3% $NaNO_3$, initial pH 5, temperature 28 °C) were used in our batch fermentations.

The batch was operated without pH control and with agitation of 300 rpm and aeration of 1 vvm. Fresh weight of mycelia, residual sugar in culture medium, catalase activity and protein content in both culture medium and cell free extract were followed for 7 days (**Table 1**). Maximum catalase activity (44491 U/l) was obtained at the end of the fermentation process, by the seventh day, after the biomass growth was considerably high enough (11.9 g/l). This was in agreement with **Kacem-Chaouche [20]** who reported that the best value of total catalase activity was obtained by *Aspirgillus phoenicis* after the production of biomass.

Table (1): Growth and catalase production by *P. chrysogenum* in bench top bioreactor without pH control and 300 rpm.

Time (days)	Fresh weight (g/l)	Activity(U/l)		Protein (mg/l)		Remaining sugar (g/l)	pH
		Intra	Extra	Intra	Extra		
1	1.41	6491	3440	8.19	4.55	5.8	6.03
2	3.11	19141	5161	12.5	7.18	3.61	3.75
3	5.85	25860	6881	12.3	9.36	2.82	3.9
4	8.57	29165	6881	15.2	6.65	1.51	4.69
5	11.1	31644	6881	14.5	9.74	0.790	6.91
6	11.5	35890	12031	16.1	11.9	0.320	7.48
7	11.9	44491	12061	16.7	12.7	0.235	7.62

The pH profile **(Fig 1)** showed a decrease in pH toward the acidic side fom pH 6.03 to pH 3.9 by the second day and then begun to increase continually till reach pH 7.14 by the seventh day. In the study of **Petruccioli [21]** where glucose was used as a carbon source, the pH drop was attributed to the production of gluconic acid by the action of glucose oxidase. In the present study, the use of sucrose and not glucose as a carbon source beside the drop in pH during the increase in growth (exponential phase) suggested that the drop in pH was due to the organic acids produced by the normal cellular metabolism and TCA cycle rather than production of gluconic acid.

The drop in pH was accompanied by a drastic drop in specific rates of growth, catalase production and carbon substrate consumption (**Table 2**) which may reflect the pH-sensitivity of the culture. Since the maximum specific rates of growth, sugar consumption and enzyme production were achieved in the first day at pH 6, it was expected that keeping the pH of the fermentation culture at pH 6 may enhance the growth and catalase production. Moreover, in other investigations, pH control at pH 6 was employed for the production of catalase by *Penicillium variabile*, *A. niger* and *Aspergillus phoenicis* in stirred tank fermentors **[20], [21] and [22]**.

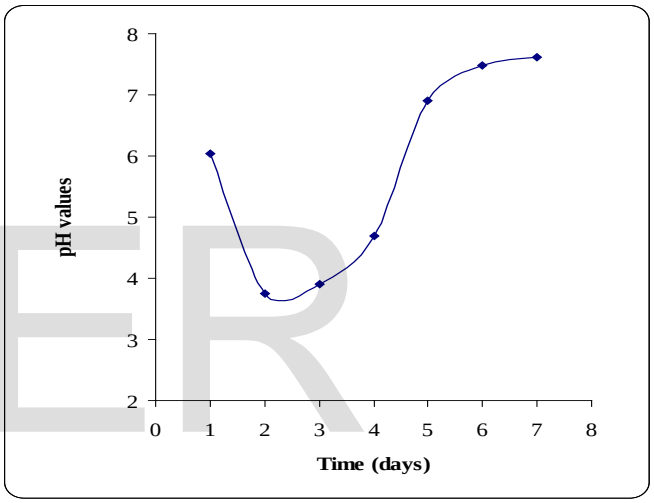


Fig. (1): pH profile of batch culture of *P. chrysogenum* in benchtop bioreactor without pH control and 300 rpm.

Table (2): The specific rates of growth, substrate consumption and enzyme production of batch culture of *P. chrysogenum* in bench top bioreactor without pH control and 300 rpm.

Time (days)	Specific growth rate (day^{-1})	Specific substrate consumption rate (day^{-1})	Specific enzyme production rate (day^{-1})
1	1.46	-1.56	13873
2	0.74	-0.46	2785
3	0.45	-0.21	771
4	0.29	-0.11	289
5	0.17	-0.05	275
6	0.05	-0.02	502
7	-0.09	0.00	986

Effect of pH control on catalase production by *P. chryso-*

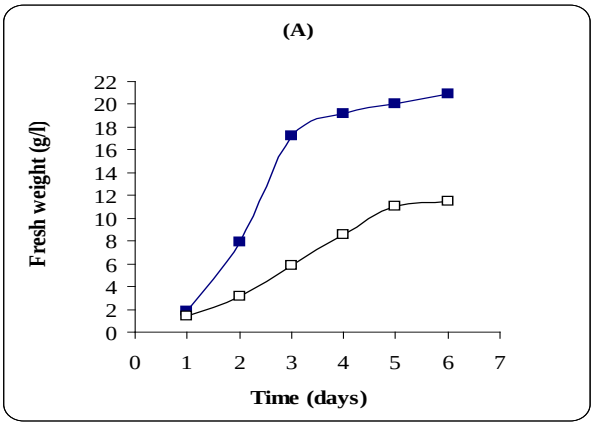
***genum* in benchtop bioreactor:**

The effect of controlling pH at the optimum value (pH 6) on catalase production by *P.chrysogenum* was examined (**Table 3**). The comparison between uncontrolled and controlled pH batches (**Fig 2**) showed that pH control enhanced the growth by about 2 folds and catalase activity by over 3 folds. The shorter exponential phase and the earlier stationary phase of the growth in the pH controlled batch also indicated that pH control enhanced the growth cycle of the microorganism. It was also noticed that pH control reduced the time course of the batch by 24h since the carbon substrate was almost removed by the sixth day instead of the seventh day. One interpretation of these results is that the biological activities of the culture like growth and enzyme production are sensitive to the pH changes.

Kurakov [2] reported the pH-dependence of the synthesis of extracellular catalase by *Penicillium pinophilum* 210K and *P. chrysogenum* K. The maximum activity was found in the medium with pH 7 and any shift towards acidity or alkalinity resulted in much lower catalase activities. The pH-sensitivity of the production of other enzymes like glucose oxidase, α-amylase and xylanase by *Penicillium fellutanum*, *Aspergillus oryzae* and *Aspergillus awamori*, respectively was also previously reported in the literatures **[23], [24] and [25]**.

Table (3): Growth and catalase production by *P. chrysogenum* in bench top bioreactor with pH control and 300 rpm.

Time (days)	Fresh weight (g/l)	Activity(U/l)		Protein (mg/l)		Remaining sugar (g/l)
		Intra	Extra	Intra	Extra	
1	1.85	6111	3440	11.4	9.97	6.07
2	7.92	19542	3440	32.2	12.6	4.47
3	17.3	51818	5160	39.1	14.2	2.84
4	19.2	56449	6881	37.4	14.4	1.7
5	20.0	79511	6881	32.7	13.1	0.74
6	20.9	112222	6881	30.3	10.0	0.1



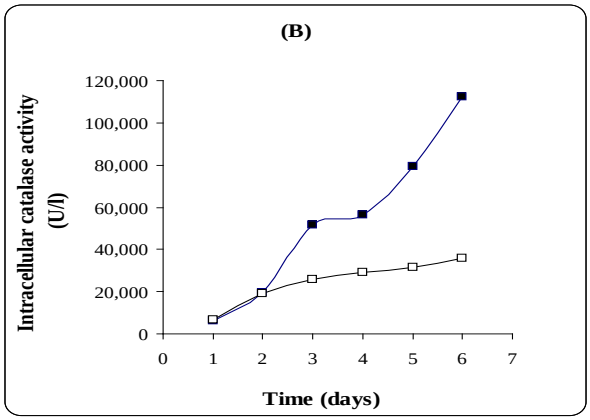


Fig. (2): Time course of growth (A) and catalase production (B) in controlled (■) and uncontrolled (□) pH batches of *P. chrysogenum* in bench top bioreactor at 300rpm.

Effect of increasing agitation on catalase production by *P. chrysogenum* in benchtop bioreactor:

The effect of increasing agitation from 300 to 400 rpm on catalase production by *P.chrysogenum* was examined (**Table 4**). The fermentor was operated with pH control at pH 6 and agitation of 400 rpm. The time course of growth and catalase production (**Fig 3**) showed that increasing agitation had a harmful effect on the growth of the fungus and catalase production. Much lower biomass (from 20.9 to 12.3 g/l) and catalase activity (from 112222 to 27855 U/l) were obtained. Moreover higher excretion (>125%) of the catalase into the medium (extracellular catalase) was observed. In addition, the cells grew in the form of less hairy pellets in the beginning of the batch instead of usual moderate round smooth pellets and hollow pellets plus fragmented pieces were obtained at the end of the batch instead of usual large compact pellets. These results may be attributed to the shear forces created by the agitation that affect the fungus in several ways, e.g., damage to cell structure, morphological changes, as well as variations in growth rate and product formation.

Table (4): Growth and catalase production by *P. chrysogenum* in bench top bioreactor with pH control and 400 rpm.

Time (days)	Fresh weight (g/l)	Activity(U/l)		Protein (mg/l)		Remaining sugar (g/l)
		Intra	Extra	Intra	Extra	
1	3.08	4875	1376	7.50	11.70	5.04
2	4.50	8639	4128	5.87	9.10	3.76
3	7.30	16030	5504	9.07	14.20	2.09
4	9.54	26153	4128	13.8	13.8	1.12
5	11.22	28959	4128	15.4	13.5	0.46
6	12.34	27855	2752	21.2	12.8	0.11

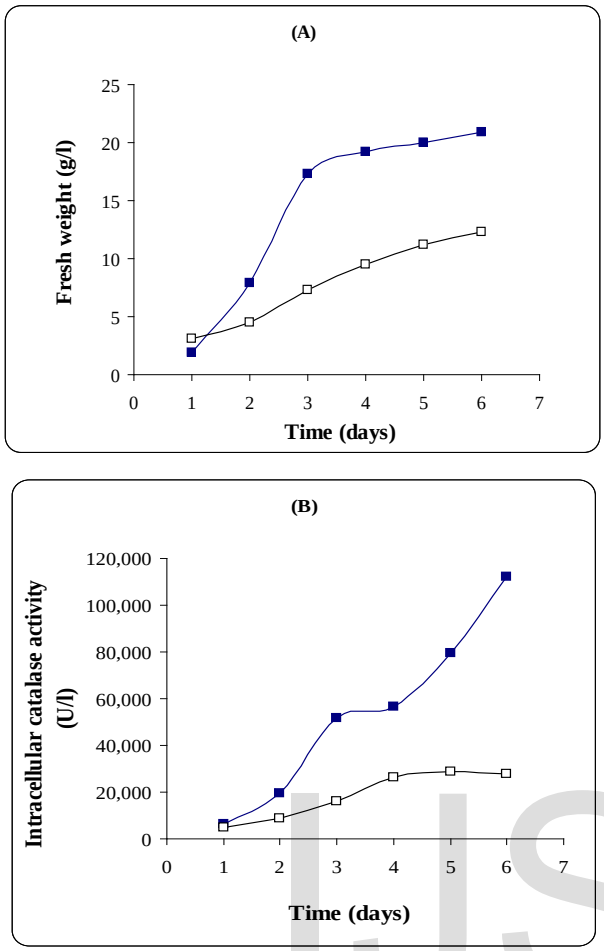


Fig. (3): Time course of growth (A) and catalase production (B) in 300 (■) and 400 (□) rpm batches of *P. chrysogenum* in bench top bioreactor with pH control.

The harmful effect of agitation speed (over 300 rpm) on the cell growth of *A. niger* was previously investigated by **Fiedurek and Gromada [22].** The cells grew almost exclusively in the form of pellets at agitation ranging from 100 to 300 rpm. Increasing agitation from 400 to 700 rpm induced transformation of fungal biomass to less hairy pellets. All biomass was mechanically disintegrated at 900 rpm exhibiting short tiny threads. On the other hand, **Petruccioli [21]** studied the influence of agitation ranged from 300 to 900 rpm on catalase production by *P. variabile*. A dramatic increase in catalase activity was obtained with increasing agitation from 300 to 400 rpm while further increase in agitation from 400 to 900 rpm caused a reduction in catalase activity reaching its minimum at 900 rpm. Therefore, for each culture, optimum conditions of agitation will exist that will partly depend on the resistance of the hyphae to mechanical forces and also on their physiological state **[26] and [27].**

Evaluation of the produced *P. chrysogenum* catalase in textile processing:

The produced *P. chrysogenum* catalase was prepared in different concentrations (0.01, 0.03, 0.05, 0.1 U/ml) and evaluated for its ability to eliminate the residual H_2O_2 in the bleaching liquor originating from textile processing. In separate flasks, 1ml of each concentration of *P. chrysogenum* catalase was added to 100 ml of bleaching liquor neutralized to pH 7 and mixed well. H_2O_2 concentration was determined, before and after addition of the catalase enzyme for 10 min, by titration with a standard 0.1 N $KMnO_4$ solution in acidic medium. The results **(Table 5)** showed that low concentrations of the enzyme (> 0.03 U/ml) were sufficient to completely degrade the residual H_2O_2**.**

One has to note that bleaching with peroxide carries out at temperatures above 60 °C and pH values above 9. Commonly, optimum activity of catalase is achieved at moderate temperatures (20–50°C) and neutral pH, therefore, the commercial use of catalase requires adjustments of pH and temperature for bleaching effluent treatment. Various additives were used by **Costa [28]** for improvement of the stability of native catalase obtained from *Bacillus sp* against these aggressive conditions. Amines, polyethylene glycol and glycerol shifted the maximum pH of activity of the native catalase toward more alkaline region, while glycerol was the only additive to improve the temperature profile of the enzyme.

Table (5): Removal of residual H_2O_2 from bleaching liquor using *P. chrysogenum* catalase.

Enzyme concentration (U/l)	Residual H_2O_2 (%)
0.00	100
0.01	14
0.03	0.1
0.05	-
0.1	-

CONCLUSION

The present study dealt with the scaling up of catalase production by *P.chrysogenum* to the fermentor level. pH control was found to be extremely important to enhance the production of catalase by *P. chrysogenum* in the bench top bioreactor (300 % enhancement). Three hundred rpm was found to be the optimum agitation rate for good growth and best catalase production in bench top bioreactor; above which, production will be reduced. The produced *P. chrysogenum* catalase showed good potentialities for elimination of hydrogen peroxide in the bleaching liquor of textile industry. These findings will justify further investigations on the larger scale and application.

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