



## Effect of mode of addition of inducer on enhanced biosynthesis of Penicillin V acylase by *Fusarium oxysporum* FUS-04

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### ABSTRACT

A total of thirty fungal strains expressing penicillin V acylase activity belonging to genus *Aspergillus* sp., *Fusarium* sp. *Mucor* sp., *Penicillium* sp. and *Rhizopus* sp. were screened for the production of enzyme under solid state fermentation in conical flasks. Among all fungal strains, *Fusariumoxysporum* FUS-04 was found to be the most potential strain.  $\beta$ -lactamase test was also performed to get  $\beta$ -lactamase negative strain. Different fermentation media were evaluated for enzyme production. The medium M2 supplemented with wheat bran gave two fold higher enzyme production than M3, M4 and M5. It was observed that the amount of penicillin V acylase produced was highest at substrate to moisture ratio 1:2. Glutamic acid was found to be a best inducer. Concentration and time of addition of glutamic acid was found to be critical for penicillin V acylase production. It was found that production of penicillin V acylase was maximized by mode of addition of inducer. The medium optimization by the Plackett-Burman and central composite designs resulted in six times increase in PVA production.

**Keywords:**  $\beta$ -lactam antibiotics, SSF, Penicillin V acylase,  $\beta$ -lactamase negative.

### INTRODUCTION

Penicillin V Acylase (Cephalosporin acylase) is a crucial enzyme for the development of new enzymatic pathways leading to the production of semi-synthetic cephalosporins. 7-amino desacetoxycephalosporanic acid (7-ADCA) is an important intermediate in these pathways. Large quantities of this compound are needed, but the traditional production routes are time-consuming, expensive and/or polluting. Filamentous fungi are commonly used in SSF, due to their relatively high tolerance to low water activities, their high potential to excrete hydrolytic enzymes and their morphology [7]. Considerable work has been done on bacterial acylases. However, research report on fungal acylases is very limited. Pakistan being an agricultural country has potential resources of biomass, such as wheat bran, molasses, corn steep liquor, cheese whey, distillery wastes and defatted oil seed cakes for their exploitation in the manufacture of cell biomass, enzymes or microbial metabolites. Maintenance of optimum concentration as well as the addition time of inducer is very critical for maximizing the enzyme activity [5]. It has been reported that phenylacetic acid and its salts induces the production of penicillin G acylase in *E.Coli* and other bacteria. A very little data has been published on the type and mode of addition of inducer for the biosynthesis of penicillin V acylase.

Optimization of any bioprocess can be conducted either by changing one factor at a time or by varying several factors at the same time and examining their effects and interactions using statistical analysis. PB designs are very

useful for screening process variables from a large number of factors, and require fewer runs than other factorial designs [10]. The influence of six variables on enzyme production was investigated using the methodology of PB design. So keeping in view the importance of industrial production of  $\beta$ -lactam antibiotics, the present research work focused on the biosynthesis of penicillin V acylase for the hydrolysis of penicillin V to 7-amino desacetoxyccephalosporanic acid using fungal acylases with new enzyme technology which leads to significant cost reductions. Hence, this work objective is to enhance the biosynthesis of penicillin V acylase by applying factorial design to determine the optimum media components, using *Fusariumoxysporum* FUS-04 and to increase the enzyme production by mode of addition of inducer.

## EXPERIMENTAL SECTION

### Microorganism:

Fungal strain, *Fusariumoxysporum* FUS-04 used in the present study was obtained after screening of thirty different fungal strains isolated from local habitats (soil, water, air, spoiled fruits and vegetables, moldy bread etc. The culture was stored in culture tubes containing mineral salt agar media (g/L; 1.0  $\text{KH}_2\text{PO}_4$ , 0.5 KCl, 0.5  $(\text{NH}_4)_2\text{SO}_4$ , 0.2  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.1  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.5 Lasparginine, 0.5 yeast extract with 1.8% agar) as described by Eggins and Pugh at  $4 \pm 1^\circ\text{C}$  [2].

### Fermentation Technique:

Enzyme production was carried out in 250 mL cotton wool plugged conical flasks. Six grams wheat bran (dry weight equivalent) was wetted with 25 ml minimal solution setting the moisture content to 83%. The composition of minimal solution was as follows (in % w/v): Lactose 5.0; Glucose 0.5;  $\text{NaNO}_3$  0.5;  $\text{KH}_2\text{PO}_4$  0.1;  $\text{Na}_2\text{SO}_4$  0.1;  $\text{CaCO}_3$  1. Media was inoculated with 1.0 % seven days old spore inoculum (conidial inoculum with  $3.0 \times 10^6$  conidia/ml). The flasks were incubated at  $30^\circ\text{C}$  for 72 h. The crude enzyme extract was prepared by blending of fermented media in distilled water for 3-5 min and filtered it by Whatman filter paper no. 1 (pore size=0.44cm). The clear filtrate was assayed for enzyme activity.

### Optimization of Cultural Conditions for PVA Production:

The effect of aeration on the production of penicillin V acylase was studied by changing the depth of fermentation medium (3.0, 6.0, 12.0, 15.0 and 18.0 g) in 250 ml conical flasks. The optimum moisture level was measured by varying substrate to moisture ratio (1:1, 1:2, 1:3, 1:4 and 1:5). Effect of POAA, PAA, glutamic acid, citric acid, acetic acid, lactic acid, formic acid, uric acid and oxalic acid was studied as an inducer. The concentration of selected inducer was varied from 0.05 % to 0.35 %. The effect of time of addition inducer was also determined at different time intervals (2, 4, 6, 8 and 10 h). All fermentation media were incubated at  $30^\circ\text{C}$  for 72 h.

### Factorial Design for Optimization of Medium:

The selection of the medium components was carried out based on the Plackett-Burman and central composite designs. Penicillin V acylase produced was measured in each batch, followed by statistical analysis in order to interpret the significant medium components.

### Enzyme Assay:

Penicillin V acylase activity was determined by PDAB-method [4]. The absorbance of enzyme was measured at 415nm under UV-Vis spectrophotometer (U-2800 HITACHI, Germany). One unit of enzyme activity was defined as the amount of enzyme required to produce 1  $\mu\text{mole}$  of 7-ADCA at  $40^\circ\text{C}$  per min.

## RESULTS AND DISCUSSION

### Effect of Aeration:

The effect of aeration was studied by varying depth of medium in 250 ml conical flasks. The results showed that an increase in depth of medium (lower aeration) contributed to increase in enzyme production (Figure 1). However, this was hold up to 12 g (15.78 U/ml/min) and beyond that further increase lowered the enzyme production. In SSF the aeration is needed for oxygenation,  $\text{CO}_2$  removal, heat dissipation (regulating the temperature of the medium), distribution of water vapor (regulating humidity), and distribution of volatile compounds produced during metabolism [1].

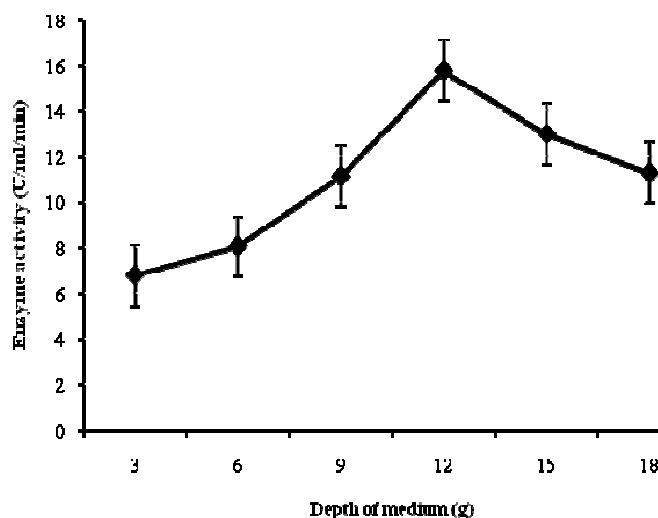


Figure 1: Effect of aeration on production of PVA by *Fusariumoxysporum* FUS-04 Fermentation conditions: Inoculum size 1.0 %, temperature 30 °C, pH 6.5, inducer concentration 0.05 % and time course 72 h

#### Effect of Moisture Content:

In SSF the moisture level should be adjusted at an optimum level. Our results revealed that enzyme production in the medium with increasing initial moisture was increased. Enzyme production was maximum when the substrate to moisture ratio was 1:2 (Figure 2). This can be due to faster growth of the organisms at higher moisture content and subsequent early initiation of enzyme production [9]. Low moisture content is known to decrease the metabolic and enzymatic activity probably due to reduced solubility of nutrients from solid substrate, low substrate swelling and higher water tension [3].

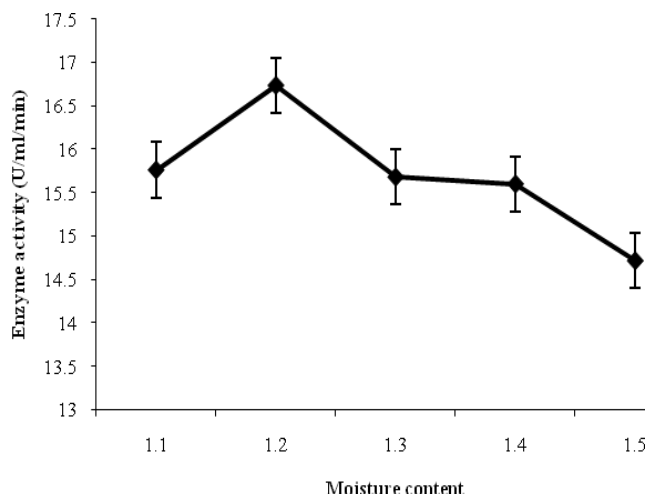


Figure 2: Effect of moisture content on production of PVA by *Fusariumoxysporum* FUS-04 Fermentation conditions: Inoculum size 1.0 %, temperature 30 °C, pH 6.5, inducer concentration 0.05 % and time course 72 h

#### Effect of Inducer:

On the basis of findings of Savidge effect of different inducers was studied on the production of PVA [11]. Among all inducers, enzyme with glutamic acid showed maximum enzyme activity (33.96 U/ml/min) (Figure 3). Effect of concentration of glutamic acid and the time of addition of glutamic acid on the biosynthesis of PVA was also investigated (Figure 4 & 5). It was clearly demonstrated that inducer concentration higher than 0.1 % was toxic to cell growth. Strong inhibitory effect was also observed on the growth and enzyme formation when it was added

during early growth phase [3]. Such results are also reported by Siewinski *et al.* for PGA productivity [6].

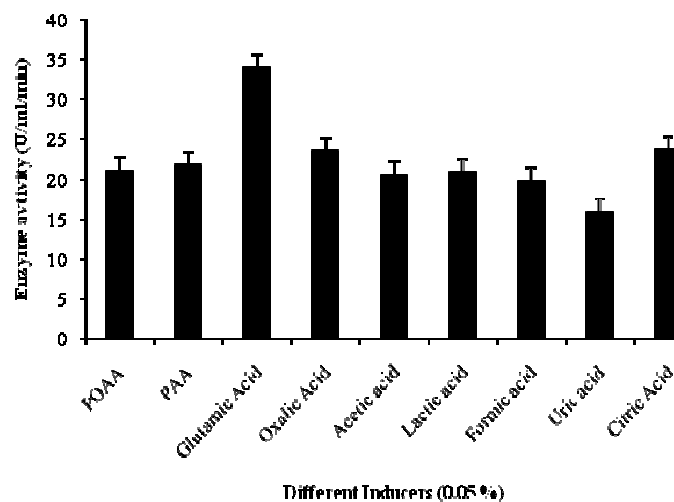


Figure 3: Effect of different inducers on production of PVA by *Fusarium oxysporum* FUS-04 Fermentation conditions: Inoculum size 1.0 %, temperature 30 °C, pH 6.5, inducer concentration 0.05 % and time course 72 h

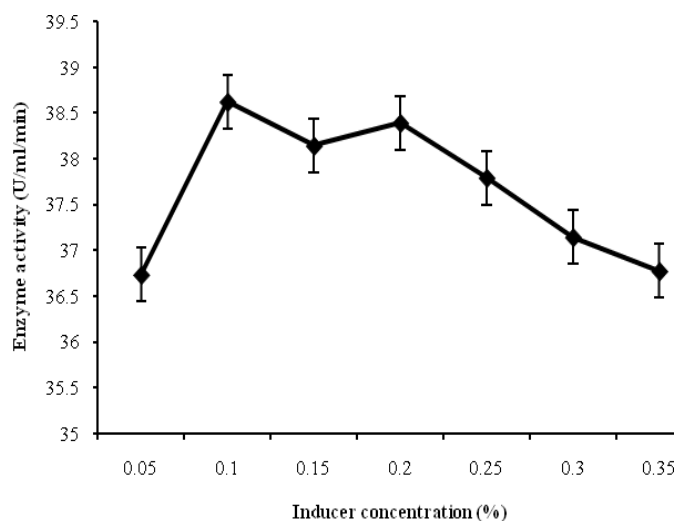


Figure 4: Effect of inducer (glutamic acid) concentration on production of PVA by *Fusarium oxysporum* FUS-04 Fermentation conditions: Inoculum size 1.0 %, temperature 30 °C, pH 6.5 and time course 72 h

#### Optimization of Medium Components:

The amount of enzyme produced varies according to the composition of the medium and its constituents. Plackett-Burman statistical experimental design was applied to optimize the fermentation medium components (Table 1). On analysis of regression coefficient of six medium components, glucose, lactose,  $\text{KH}_2\text{PO}_4$  and  $\text{CaCO}_3$  showed positive effect for enzyme activity, whereas  $\text{NaNO}_3$  and  $\text{Na}_2\text{SO}_4$  showed negative effect in the tested range of concentration (Table 2). The significant factors identified by Plackett-Burman design were considered for the next stage of the medium optimization. Central composite design indicated the exact quantity of each constituent required for the medium and its effects on the production of PVA (Table 3). Analysis of variance was performed in order to validate regression model (Table 4).

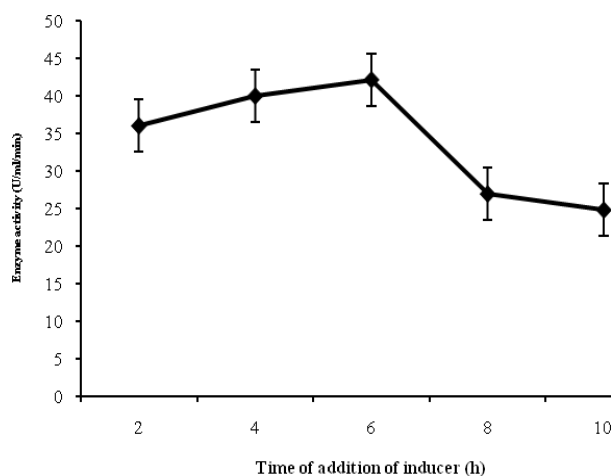


Figure 5: Effect of time of addition of inducer (glutamic acid) on production of PVA by *Fusariumoxysporum* FUS-04 Fermentation conditions: Inoculum size 1.0 %, temperature 30 °C, pH 6.5, inducer concentration 0.1 % and time course 72 h

Table 1: Plackett-Burman design for six wheat bran supplements assigned different levels of factors and experimental runs

Trial No.	Medium Components (% w/v)						Enzyme Activity (U/ml/min)
	Lactose	Glucose	NaNO <sub>3</sub>	KH <sub>2</sub> PO <sub>4</sub>	Na <sub>2</sub> SO <sub>4</sub>	CaCO <sub>3</sub>	
1	5.0	0.5	0.5	0.1	0.1	1.0	45.75
2	---	0.5	0.5	0.1	0.1	1.0	46.14
3	5.0	---	0.5	0.1	0.1	1.0	46.66
4	5.0	0.5	---	0.1	0.1	1.0	45.61
5	5.0	0.5	0.5	---	0.1	1.0	45.70
6	5.0	0.5	0.5	0.1	---	1.0	45.82
7	5.0	0.5	0.5	0.1	0.1	---	46.66
8	5.0	---	---	0.1	0.1	1.0	46.78
9	---	---	0.5	0.1	0.1	1.0	45.42
10	5.0	0.5	---	0.1	---	1.0	47.43

Fermentation conditions: Inoculum size 1.0 %, temperature 30 °C, pH 6.5, inducer concentration 0.1 % and time course 72 h

Table 2: Regression analysis for the Plackett-Burman design

Coefficient of determination,  $r^2 = 0.647$

Sr. No.	Variable	Coefficient	t-value	p-value
1	Lactose	0.0793	0.75	0.473
2	Glucose	0.4105	0.44	0.674
3	NaNO <sub>3</sub>	-1.4562	-1.82	0.106
4	KH <sub>2</sub> PO <sub>4</sub>	4.411	0.62	0.552
5	Na <sub>2</sub> SO <sub>4</sub>	-6.600	-1.34	0.217
6	CaCO <sub>3</sub>	6.6256	0.90	0.393
7	Intercept	44.882	20.61	0.000

Table 3: Central composite design for wheat bran supplements assigned different levels of factors and experimental runs

Trial No.	Medium Components (% w/v)				Enzyme Activity (U/ml/min)
	Lactose	Glucose	KH <sub>2</sub> PO <sub>4</sub>	CaCO <sub>3</sub>	
1	5.00	0.50	0.10	1.00	43.46
2	4.00	0.50	0.10	1.00	43.29
3	5.00	0.40	0.10	1.00	43.19
4	5.00	0.50	0.05	1.00	40.29
5	5.00	0.50	0.10	0.50	40.12
6	3.00	0.50	0.04	1.00	41.41
7	2.00	0.40	0.10	0.40	39.37
8	5.00	0.50	0.03	1.00	52.96
9	5.00	0.30	0.02	0.30	40.72
10	5.00	0.20	0.10	0.20	37.35

Fermentation conditions: Inoculum size 1.0 %, temperature 30 °C, pH 6.5, inducer concentration 0.1 % and time course 72 h

**Table 4: Regression analysis for the central composite design**  
Coefficient of determination,  $r^2 = 0.678$

Sr. No.	Variable	Coefficient	t-value	p-value
1	Lactose	-2.3477	-3.07	0.015
2	Glucose	-1.010	-0.12	0.904
3	KH <sub>2</sub> PO <sub>4</sub>	-33.12	-1.00	0.349
4	CaCO <sub>3</sub>	6.531	2.45	0.040
5	Intercept	46.143	8.23	0.000

### CONCLUSION

*Fusariumoxysporum* FUS-04 was found to be the best producer of penicillin V acylase among all other isolates. The medium M2 gave twofold higher enzyme production than M1, M3, M4 and M5. Concentration and time of addition of glutamic acid was found to be critical for penicillin V acylase production. Media optimization by Plackett-Burman and central composite designs enhanced the enzyme activity six times.

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